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Research Article

Assessment of the bioactivity, preservation potential and sensory acceptance of a propolis extract applied in a functional fruit-herbal beverage

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Abstract

Functional foods and beverages can be defined as processed products that provide health benefits beyond the basic nutritional needs and reduce the risk of diseases. The design of functional products with improved shelf-life is a new challenge for the food industry. Therefore, this research aimed to: design a functional fruit-herbal beverage; compare the preservation effect of two preservatives (propolis extract and potassium sorbate) added in the beverages; evaluate the sensory characteristics of the newly designed beverages; observe the physico-chemical and microbiological changes during storage at 4℃ for 42 d. The results demonstrated that the addition of propolis extract (0.02%) in the beverage, increased the total polyphenols, total flavonoids and antioxidant activity, compared to the control beverage and the beverage containing potassium sorbate (0.05%). The results from microbiological analyses showed that potassium sorbate and propolis extract effectively inhibited the microbial growth in the treated beverages. However, the propolis extract in a lower concentration (0.02%) was as effective as potassium sorbate in a higher concentration (0.05%) during the entire storage period. Consequently, in addition to being a functional ingredient and a food enhancer, propolis is a prospective natural preservative, which can improve the shelf-life of the food products and beverages.

Keywords
propolis, functional beverages, natural preservatives, biopreservation

Abbreviations
ANOVA – one-way analysis of variance; CGA – chloramphenicol glucose agar; DPPH – 2,2-diphenyl-1-picrylhydrazyl; FHB – fruit-herbal beverages; FRAP – ferric-reducing antioxidant power assay; PCA – plate count agar; QE – quercetin; SEM – standard error of means; TE – Trolox equivalent; TFC – total flavonoid content; TPC – total phenolic content; TPlC – total plate count; TPTZ – 2,4,6-Tris(2-pyridyl)-s-triazine;

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Introduction

In recent years, the quest for a healthy lifestyle has become an enduring trend leading to the development of a new branch of the food industry involving functional foods and beverages. Functional foods and beverages are those that exert positive effects on one or more body functions beyond the basic nutritional needs, thereby having a beneficial effect on the human health. In both functional foods and functional beverages, one or more ingredients have been added, modified or removed in order to enhance a certain physiological function, or to reduce some adverse health effects (Granato et al. 2010).

As a source of essential nutrients, milk has long been considered the most suitable natural matrix for the manufacture of functional food products. However, recent studies reveal that some milk components (lactose, proteins, vitamin D, calcium, cholesterol, saturated fatty acids, hormones and contaminants such as pesticides) may have a harmful impact on the organism (Corbo et al. 2014; Willett and Ludwig 2020). In this regard, fruit juices are an alternative for production of functional beverages for consumers who are lactose-intolerant or allergic to dairy products. Besides the high amounts of valuable nutrients (carbohydrates, phenolic compounds, vitamins and minerals), health-promoting properties and the low costs of production, fruit juices represent an excellent medium for the incorporation of additional ingredients in various combinations as well as a good environment for the growth of different probiotic strains (Žuntar et al. 2020). One of the technological approaches to increase the health benefits of the functional beverages is the addition of plant extracts, or creating so called fruit-herbal beverages. Plant extracts are rich sources of biologically active compounds that possess antioxidant, antimicrobial, immuno-modulatory, cardioprotective and antidiabetic activities, thus improving functional value of the product. Plant extracts also exert a protective effect on anthocyanin content and color stability of some functional beverages (Teneva et al. 2022) as well as enhance their sensory properties making them more attractive for the consumers (Owczarek et al. 2004; Skapska et al. 2020).

Apple (*Malus domestica*) is a tree from the family *Rosaceae* cultivated worldwide, whose fruits are important raw material for processing. The apple fruit contains many valuable nutrients – carbohydrates, dietary fibers, polyphenols, minerals (Na, K, Ca, P, Mg), vitamins (B9, C, E, pro-vitamin A carotenes), pectin, lutein and organic acids (Kowalska et al. 2018). Apple phytochemicals have been shown to possess strong antioxidant activity, anticarcinogenic, antidiabetic, cardioprotective, anti-asthmatic, anti-obesity and other health benefit effects (Boyer and Liu 2004).

Ginger (*Zingiber officinale* Roscoe), which belongs to the family *Zingiberaceae* is a popular spice used in the culinary and a healing plant in the medicine. Ginger root is a rich source of phenolic compounds (gingerols and shogaols), terpenes, polysaccharides, lipids, organic acids and dietary fibers that contribute to its antioxidant, antimicrobial, antidiabetic, anti-inflammatory, anticarcinogenic, neuroprotective, cardioprotective, respiratory protective, anti-obesity and antiemetic activities (Mao et al. 2019).

Peppermint (*Mentha piperita* L.) is one of the most widely consumed single ingredient herbal teas. Tea obtained from the peppermint leaves and the peppermint essential oil are used in the traditional medicine. The phenolic compounds of peppermint include rosmarinic acid and several flavonoids (eriocitrin, luteolin and hesperidin). The main volatile components of the essential oil are menthol and menthone. It has been found that peppermint exhibits significant antibacterial, antiviral, antioxidant, anti-allergenic, antitumor, immunomodulatory, anaesthetic, gastroprotective and neuroprotective effects (McKay and Blumberg 2006).

Propolis (bee glue) is a complex biological mixture produced by European honey bees (*Apis mellifera* L.) after collecting exudates from flowers and leaf buds of various plant species. Propolis plays an important role as a building and defensive material in the bee hive, but also demonstrate high antioxidant, antimicrobial, antiparasitic, immuno-modulatory, anti-inflammatory, antiviral, anti-carcinogenic, hepatoprotective, anti-ulcerogenic, anti-allergic, antidiabetic, astringent and anaesthetic activities. Owing its rich composition and health benefits, propolis is widely applied as a remedy, a

promising food biopreservative and a nutritional value enhancer (Tumbarski et al. 2022).

In the food industry, special attention is paid to the product shelf-life and the ways for the product quality retention. Having in mind that fruit products are usually prepared using raw materials, the high water content and high amounts of nutrients of fruits, fruit beverages are extremely susceptible to microbial spoilage, which is a prerequisite for their short shelf-life. The conventional methods for preservation by a thermal impact as pasteurization can cause a significant decrease in some bioactive components such as anthocyanins and vitamins, and change in the color intensity (Teneva et al. 2022; Vilas-Boas et al. 2022). On the other hand, chemical or synthetic preservatives (benzoates, sorbates, nitrites and nitrates of sodium or potassium, sulphites, glutamates, etc.) have proven harmful effects on the organism as they are considered to be cause of residual toxicity, carcinogenicity and teratogenicity (Vara et al. 2019). Therefore, the application of safe natural antimicrobial substances such as propolis can have not only functional and nutritional enhancing effects, but also can act as biopreservatives improving the shelf-life of the product (Vasilaki et al. 2019; Tumbarski et al. 2022).

Therefore, the present research aimed to: a) design a functional fruit-herbal beverage based on concentrated apple juice with the addition of fresh ginger juice, peppermint decoction and two types of preservatives – propolis extract and potassium sorbate; b) evaluate the sensory characteristics of the newly designed functional fruit-herbal beverages; c) compare the preservation effect of the propolis extract with those of the chemical preservative potassium sorbate in the beverages; d) observe the physicochemical and microbiological changes in the product during storage for 42 d.

Materials and Methods

Materials

Concentrated apple juice. Natural, non-pasteurized, concentrated apple juice (pH 3.55, 70° Brix, refraction index 1.466) without addition of sugar and preservatives was purchased from Krichimfrukt Ltd., Krichim, Bulgaria.

Fresh ginger juice. Ginger roots were purchased from the local fruit market, Plovdiv, Bulgaria. The roots were transported to the laboratory, washed with tap water, peeled and finely chopped by a blender (Bosch, Germany). Next, the ground mass was left at refrigerated conditions (4°C) for 3 - 4 h, then squeezed through a gauze, and filtered through a filter paper in order to obtain a fresh ginger juice.

Peppermint decoction. Dried and chopped peppermint leaves from Rhodope Mountains were purchased from Monsi-52 Ltd., Bulgaria. In order to obtain decoction, 10 g of dried peppermint leaves were placed in 250 ml of boiling water, then the pot was closed and left at room temperature to cool. The decoction was strained and filtered through a filter paper before use.

Propolis extract. Fresh propolis obtained in 2022 from a beekeeper located in the village of Burya, Gabrovo district, Bulgaria (43°02'N 25°19'E) was delivered to the laboratory by a courier. To obtain 10% propolis extract, a mass of 4 g of the sample was finely chopped, placed in a plastic tube and macerated with 40 ml of 70% ethanol (Sigma-Aldrich, Merck, Germany). Next, the sample was vigorously shaken on vortex V-1 (Biosan, Latvia) for 15 - 20 s and kept at room temperature for 72 h in darkness. During the extraction, the sample was periodically vortexed. The obtained ethanolic extract was filtered through a filter paper and stored at 4°C for further analyses (Tumbarski et al. 2022).

Potassium sorbate. Granulated potassium sorbate (E 202) was purchased from Sigma-Aldrich, Merck, Germany.

Culture media

Plate count agar (PCA). This medium was used for determination of the total plate count of mesophilic aerobic and facultative anaerobic microorganisms. A quantity of 23.5 g of the PCA agar medium base (containing 5 g casein peptone, 2.5 g yeast extract, 1 g dextrose and 15 g agar) was dissolved in 1 L of deionized water, pH 7.0 ± 0.2. The medium was autoclaved at 121°C for 15 min.

Chloramphenicol glucose agar (CGA). CGA is a selective medium for the enumeration of yeasts and fungi. A quantity of 40 g of the CGA agar medium base (containing 20 g dextrose, 5 g yeast extract, 0.1 g chloramphenicol and 15 g agar) was dissolved in

1 L of deionized water, pH 6.6 ± 0.2. The medium was autoclaved at 121℃ for 15 min.

Methods

Experimental procedure. The three experimental groups of functional fruit-herbal beverages (FHB 1, FHB 2 and FHB 3) were prepared by mixing the ingredients (Table 1) in plastic bottles. The functional beverages were stored under refrigeration conditions (4℃) for a period of 42 d. During the storage, physicochemical and microbiological analyses according to the Bulgarian State Standards were performed at 7-day intervals.

Table 1. Ingredients of the functional fruit-herbal beverages

Ingredient	Functional beverages		
	FHB 1 (C*)	FHB 2	FHB 3
Concentrated apple juice, g.L^{-1}	172	172	172
Peppermint decoction, ml.L^{-1}	100	100	100
Fresh ginger juice, ml.L^{-1}	10	10	10
Potassium sorbate, g.L^{-1}	-	0.5	-
EPE, ml.L^{-1}**	-	-	2
Dining water, L	ad 1	ad 1	ad 1

*C – Control; **EPE – Ethanolic propolis extract, 100 mg.ml^{-1}

Determination of pH. The pH was assessed using a pH-meter WTW pH 7110 (InoLab, Germany). The glass electrode was immersed directly into the beverages at room temperature until a constant value. The calibration was performed with standard buffer solutions (Tumbarski et al. 2022).

Determination of titratable acidity. The determination of titratable acidity was implemented according to the standard method (Tumbarski et al. 2022). The titratable acidity was assessed by titration of each sample with 0.1 N NaOH using phenolphthalein as an indicator until the appearance of a pale pink color persisting over 1 min. The results were expressed as g malic acid.L^{-1}.

Total phenolic content. The TPC was determined using the standard method. The reaction mixture was prepared with 1 ml of Folin-Ciocalteu reagent (Sigma-Aldrich, Merck, Germany), 0.8 ml of 7.5% sodium carbonate (Sigma-Aldrich, Munich, Merck) and 0.2 ml of the tested extract/beverage. After incubation at room temperature for 20 min (in darkness), the absorbance was measured by a spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, UK) at 765 nm against a blank (distilled water). The results were expressed as mg equivalent of gallic acid GAE.100 ml^{-1} extract/beverage (Ivanov et al. 2014).

Total flavonoid content. The TFC was evaluated according to the method described by Ivanov et al. (2014). An aliquot of 1 ml of the extract/beverage was added to 0.1 ml of 10% $Al(NO_3)_3$, 0.1 ml of 1 M CH_3COOK (Sigma-Aldrich, Merck, Munich, Germany) and 3.8 ml of distilled water. The samples were left at room temperature for 40 min, and then the absorbance was measured at 415 nm. Quercetin (QE) was used as a standard and the results are expressed as mg quercetin equivalents QE.100 ml^{-1} extract/beverage.

Antioxidant activity

DPPH radical scavenging assay. The reaction mixture was prepared with 2.85 ml of DPPH reagent (Sigma-Aldrich, Merck, Germany) and 0.15 ml of the tested extract/beverage. The samples were incubated at 37°C for 15 min. The reduction of absorbance was measured at 517 nm against a blank (methanol). The antioxidant activity was expressed as mM Trolox equivalents TE.100 ml^{-1} extract/beverage (Ivanov et al. 2014).

Ferric-reducing antioxidant power (FRAP) assay. The FRAP reagent was freshly prepared with 300 mM acetate buffer with pH 3.6, 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine in 40 mM hydrochloric acid and 20 mM Iron (III) chloride hexahydrate (Sigma-Aldrich, Merck, Germany) in distilled water in a ratio of 10:1:1. The reaction mixture (3 ml of FRAP reagent and 0.1 ml of the extract/beverage) was incubated at 37℃ for 10 min in darkness. The absorbance was measured at 593 nm against a blank (distilled water). The antioxidant activity was expressed as mM TE.100 ml^{-1} extract/beverage (Ivanov et al. 2014).

Determination of color. The color of beverages was measured using a portable colorimeter FRU WR-10QC (China) by the CIELab method. The CIELab system consists of a lightness component (L*) and two chromatic components, as the a* value represents green (-a) to red (+a) and the b* value represents blue (-b) to yellow (+b) colors. The colorimeter was calibrated using a standard white plate (L* = 96.20, a* = 0.06, b* = -6.20) (Falcao et al. 2013). The values of these parameters were read and recorded automatically by touching the device to the bottles with the beverages. In the second stage of the analysis, the colorimeter was calibrated to the color of the control beverage (FHB 1) in order to determine the variations in the color components (ΔL, Δa, Δb) and the total color variation index (ΔE). To determine the chroma (C*), hue (H*) and browning index (BI), the following equations according to Abd El-Baset and Almoselhy (2023) were used:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (1)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$H^* = tan^{-1}[b^*/a^*] \quad (3)$$

$$BI = \frac{100}{0.17}\left(\frac{a^* + 1.75L^*}{5.645L^* + a^* - 0.012b^*} - 0.31\right) \quad (4)$$

Microbiological analyses

Total plate count (TPlC). The TPlC (mesophilic aerobic and facultative anaerobic microorganisms) was determined by colony-count technique on PCA at 30℃ according to the Bulgarian State Standard BSS EN ISO 4833-1:2013 (2013). The results were expressed as colony-forming units per ml (cfu.ml^{-1}).

Total number of yeasts and/or fungi. The enumeration of yeasts and/or fungi was determined by colony-count technique on CGA at 25℃ according to the Bulgarian State Standard BSS ISO 21527-1:2011 (2011). The results were expressed as cfu.ml^{-1}.

Sensory evaluation. To conduct the sensory analysis, twenty volunteer consumers over 18 years old were invited. Participants who had allergy to any of ingredients were not permitted to join the test. The analysis was performed by blind testing of the three functional beverages FHB 1, FHB 2 and FHB 3, offered in a small amount in plastic cups. The beverages were evaluated on the following organoleptic parameters: overall appearance, color, odor (aroma), flavor and viscosity, using the 9-points Hedonic scale (9 = liked extremely, 8 = liked very much, 7 = liked moderately, 6 = liked slightly, 5 = neither liked nor disliked, 4 = disliked slightly, 3 = disliked moderately, 2 = disliked very much, 1 = disliked extremely). The results from the sensory analysis were recorded on individual questionnaire cards and then a sensory profile of each beverage was made using MS Excel 2010 software.

Statistical analysis. The results from triplicate experiments were expressed as mean values ± standard error of the (means ± SEM). One-way analysis of variance (ANOVA) was performed using Statgraphics Centurion statistical program version XVI, 2009 (Stat Point Technologies, Inc., Warrenton, VA, USA). The mean differences were established by Fisher's least significant difference test for paired comparison with a significance level $P \leq 0.05$.

Results and Discussion

Total phenolic content, total flavonoid content and antioxidant activity of the ingredients of the functional beverages. As seen from the results in Table 2, the ethanolic propolis extract used as a biopreservative exhibited the highest TPC, TFC and antioxidant activity (determined by two independent methods DPPH and FRAP), compared to the other components of the functional beverage – concentrated apple juice, peppermint decoction and fresh ginger juice.

Physicochemical changes. The results presented in Table 3 demonstrate that the addition of propolis extract in concentration of 0.02% in the functional beverage FHB 3, led to an increase in the TPC, TFC and antioxidant activity, in comparison with the functional beverage FHB 1 (the control) and FHB 2 (with addition of 0.05% potassium sorbate). The values of TPC remained relatively constant in all experimental groups during the period of refrigerated storage at 4℃ for 42 d. However, the TFC and DPPH values significantly decreased in all beverages on the 42^{-nd} d of the storage. As regards the results from the FRAP method, the

Table 2. Total phenolic content, total flavonoid content and antioxidant activity of the ingredients of the functional fruit-herbal beverages

Ingredients	Total phenols, mg GAE.100 $ml^{-1}.g^{-1}$	Total flavonoids, mg QE.100 $ml^{-1}.g^{-1}$	Antioxidant activity	
			DPPH, mM TE.100 $ml^{-1}.g^{-1}$	FRAP, mM TE.100 $ml^{-1}.g^{-1}$
Concentrated apple juice*	109.20 ± 0.70	8.96 ± 0.11	688.02 ± 8.17	813.94 ± 15.00
Peppermint decoction*	34.49 ± 0.56	11.05 ± 0.08	331.97 ± 6.89	306.48 ± 2.01
Fresh ginger juice*	35.60 ± 0.45	1.67 ± 0.02	210.21 ± 2.32	254.84 ± 2.00
Ethanolic propolis extract**	233.00 ± 0.11	71.30 ± 0.10	1407.10 ± 2.37	950.60 ± 1.02

***** Values per ml; ****** - Values per g

beverages FHB 1 and FHB 2 retained similar antioxidant levels on the 42-nd day, while the antioxidant activity of the beverage FHB 3 significantly decreased compared to day 0. During the entire period of storage, the beverage with addition of propolis (FHB 3) showed the highest values of TPC, TFC and antioxidant activity, compared to the other experimental groups – FHB 1 and FHB 2. Consequently, the application of propolis can be considered a promising technological approach to improve the functional properties and enhance the nutritional value of food products and beverages. During the storage under refrigeration conditions for 42 d, a gradual increase in titratable acidity (expressed as g malic acid.L^{-1}) and a concomitant decrease in pH values in all experimental groups were observed (Table 4). At the end of the storage period (day 42), the titratable acidity reached values of 3.86, 3.82 and 3.83 for FHB 1, FHB 2 and FHB 3, while pH took values of 3.65, 3.75 and 3.66, respectively. These changes are normally associated with the microbial growth and sugar degradation in the samples during the storage period.

Table 3. Total phenolic content, total flavonoid content and antioxidant activity of the functional fruit-herbal beverages during storage at 4℃ for 42 d

Day	Functional beverage	Total phenols, mg GAE.100 ml^{-1}	Total flavonoids, mg QE.100 ml^{-1}	Antioxidant activity	
				DPPH, mM TE.100 ml^{-1}	FRAP, mM TE.100 ml^{-1}
0	**FHB 1 (C*)**	$25.14 \pm 0.21^{b,A}$	$4.89 \pm 0.01^{b,A}$	$166.16 \pm 3.63^{b,A}$	$142.25 \pm 1.98^{b,A}$
	FHB 2	$26.17 \pm 0.27^{b,A}$	$4.89 \pm 0.01^{b,A}$	$172.51 \pm 3.03^{b,A}$	$138.78 \pm 0.99^{b,A}$
	FHB 3	$29.38 \pm 0.99^{a,A}$	$8.33 \pm 0.06^{a,A}$	$187.05 \pm 3.04^{a,A}$	$160.32 \pm 0.42^{a,A}$
42	**FHB 1 (C)**	$24.45 \pm 0.17^{c,A}$	$4.60 \pm 0.01^{b,B}$	$149.26 \pm 2.50^{a,B}$	$138.04 \pm 0.49^{b,A}$
	FHB 2	$25.78 \pm 0.16^{b,A}$	$4.66 \pm 0.01^{b,B}$	$149.57 \pm 2.16^{a,B}$	$137.80 \pm 0.30^{b,A}$
	FHB 3	$29.30 \pm 0.17^{a,A}$	$7.75 \pm 0.06^{a,B}$	$152.70 \pm 1.43^{a,B}$	$151.65 \pm 0.49^{a,B}$

Values are means of three replicates ± SEM.

Values number in the same column followed by different superscripts are significantly different at $P \leq 0.05$.
*C – control

Table 4. Physicochemical changes in the functional fruit-herbal beverages during storage at 4°C for 42 d

Day	Functional beverage					
	FHB 1 (C*)		FHB 2		FHB 3	
	pH	TA**	pH	TA	pH	TA
0	$3.77 \pm 0.01^{a,B}$	$3.40 \pm 0.21^{b,A}$	$3.85 \pm 0.00^{a,A}$	$3.41 \pm 0.13^{b,A}$	$3.74 \pm 0.01^{a,B}$	$3.50 \pm 0.03^{a,A}$
7	$3.75 \pm 0.00^{a,B}$	$3.51 \pm 0.32^{ab,A}$	$3.84 \pm 0.01^{a,A}$	$3.42 \pm 0.03^{b,A}$	$3.73 \pm 0.00^{a,B}$	$3.54 \pm 0.27^{a,A}$
14	$3.68 \pm 0.00^{b,B}$	$3.51 \pm 0.11^{ab,A}$	$3.79 \pm 0.01^{b,A}$	$3.47 \pm 0.06^{b,A}$	$3.70 \pm 0.02^{b,B}$	$3.59 \pm 0.03^{a,A}$
21	$3.68 \pm 0.00^{b,C}$	$3.57 \pm 0.08^{ab,AB}$	$3.79 \pm 0.00^{b,A}$	$3.50 \pm 0.10^{ab,B}$	$3.70 \pm 0.01^{b,B}$	$3.68 \pm 0.07^{a,A}$
28	$3.68 \pm 0.01^{bc,C}$	$3.59 \pm 0.11^{ab,A}$	$3.79 \pm 0.01^{b,A}$	$3.50 \pm 0.10^{ab,A}$	$3.70 \pm 0.00^{b,B}$	$3.68 \pm 0.29^{a,A}$
35	$3.67 \pm 0.03^{bc,B}$	$3.68 \pm 0.08^{ab,A}$	$3.77 \pm 0.00^{bc,A}$	$3.63 \pm 0.30^{ab,A}$	$3.67 \pm 0.00^{c,B}$	$3.69 \pm 0.08^{a,A}$
42	$3.65 \pm 0.00^{c,B}$	$3.86 \pm 0.31^{a,A}$	$3.75 \pm 0.01^{c,A}$	$3.82 \pm 0.37^{a,A}$	$3.66 \pm 0.00^{c,B}$	$3.83 \pm 0.33^{a,A}$

Values are means of three replicates ±SEM.

Values number in the same column followed by different superscripts are significantly different at $P \leq 0.05$.
*C – control; **TA – titratable acidity, g malic acid.L^{-1}

Microbiological changes. During the first 7 d of the refrigerated storage, the number of mesophilic aerobic and facultative anaerobic microorganisms (the total plate count) remained at low limits, while yeasts in all experimental groups were not detected (Table 5). After the 14-th d of the storage, the total plate count and the number of yeasts in the control beverage (FHB 1) began to increase significantly (reaching values of 2.2×10^5 cfu.ml^{-1} and 7.6×10^4 cfu.ml^{-1} on the 21-st d, respectively), while the same parameters in FHB 2 (with 0.05% potassium sorbate) and FHB 3 (with 0.02% ethanolic propolis extract) retained values close to those in the beginning of the experiment. During the second half of the storage, total plate count and the number of yeasts in the control beverage (FHB 1) continue to increase, reaching the highest values of 7.6×10^8 cfu.ml^{-1} and 3.1×10^8 cfu.ml^{-1}, respectively at the end of the monitoring period (42-nd d). On the 28-th d a slight increase in the total plate count in the treated beverages FHB 2 and FHB 3 (1.0×10^2 cfu.ml^{-1}) was observed, while yeasts were not detected. After the 35-th d until the end of the storage period, the total plate count in the treated beverages FHB 2 and FHB 3 remained in the same limits, while the number of yeasts increased insignificantly (1.0×10^2 cfu.ml^{-1}). Fungi in all experimental groups were not found (Table 5).

Table 5. Microbiological changes in the functional fruit-herbal beverages during storage at 4°C for 42 d

Day	Functional beverage								
	FHB 1 (C*)			FHB 2			FHB 3		
	TPlC**, cfu.ml^{-1}	Yeasts, cfu.ml^{-1}	Fungi, cfu.ml^{-1}	TPlC, cfu.ml^{-1}	Yeasts, cfu.ml^{-1}	Fungi, cfu.ml^{-1}	TPlC, cfu.ml^{-1}	Yeasts, cfu.ml^{-1}	Fungi, cfu.ml^{-1}
0	35	< 1	< 1	20	< 1	< 1	15	< 1	< 1
7	70	< 1	< 1	60	< 1	< 1	20	< 1	< 1
14	1.7×10^3	4.0×10^2	< 1	60	< 1	< 1	30	< 1	< 1
21	2.2×10^5	7.6×10^4	< 1	80	< 1	< 1	50	< 1	< 1
28	2.3×10^7	2.7×10^7	< 1	1.0×10^2	< 1	< 1	1.0×10^2	< 1	< 1
35	6.0×10^7	6.0×10^7	< 1	1.0×10^2	1.0×10^2	< 1	1.5×10^2	1.0×10^2	< 1
42	7.6×10^8	3.1×10^8	< 1	1.2×10^2	1.0×10^2	< 1	4.0×10^2	1.0×10^2	< 1

*C – control; **TPlC – total plate count

The obtained results demonstrated that both preservatives – potassium sorbate and propolis extract, effectively inhibited the microbial growth in the treated functional beverages FHB 2 and FHB 3. However, the propolis extract applied in a lower concentration (0.02%) in the beverage FHB 3 was as effective as potassium sorbate applied in higher concentration (0.05%) in FHB 2. Therefore, in addition to being a functional ingredient and a food enhancer, propolis is a prospective natural preservative, which can improve the shelf-life of the food products and beverages.

Color changes. Color is the main visible sensory indicator, which is of great importance for the overall perception of the product by the consumers. As seen from the results in Table 6, no significant difference in the lightness component (L*) in the three experimental groups was observed.

Table 6. Color changes in the functional fruit-herbal beverages

Functional beverage	Color index									
	L*	**ΔL**	**a***	**Δa**	**b***	**Δb**	**ΔE**	**C***	**H***	**BI**
FHB1 (C*)	47.12 ± 0.13[a]	-	6.00 ± 0.05[a]	-	17.01 ± 0.13[b]	-	-	18.04 ± 0.11[b]	70.56 ± 0.29[b]	9.11 ± 0.06[b]
FHB 2	47.44 ± 0.51[a]	0.75	5.93 ± 0.09[a]	0.79	17.09 ± 0.33[b]	-0.74	1.31	18.09 ± 0.28[b]	70.85 ± 0.58[b]	8.95 ± 0.21[b]
FHB 3	46.66 ± 0.59[a]	1.79	5.11 ± 0.06[b]	0.45	18.55 ± 0.48[a]	-1.20	2.20	19.24 ± 0.46[a]	74.60 ± 0.45[a]	7.88 ± 0.13[a]

Values are means of three replicates ± SEM.
Values number in the same column followed by different superscripts are significantly different at $P \leq 0.05$.
*C – control

The parameters a* and b* of the beverage FHB 2 were not statistically different from those of the control (FHB 1). However, significant difference between the color components a* and b* of FHB 3 and the other two beverages (FHB 1 and FHB 2) as well as significant variations in the color parameters (ΔL, Δa and Δb) were found. The value of the parameter ΔE showed that the addition of potassium sorbate in concentration of 0.05% did not change the color of the beverage FHB 2 in comparison with the control, but the addition of 0.02% ethanolic propolis extract changed the color of the beverage FHB 3, compared to the other two groups (ΔE > 2.0), which reflected on the sensory profile and overall acceptance of this product. FHB 3 exhibited significantly higher values of C* and H*, but lower values of browning index (BI) in comparison with FHB 1 and FHB 2.

Sensory evaluation. The results from sensory analysis showed that FHB 1 (control) received the highest total score from the tasters in comparison with FHB 2 and FHB 3. The control kept the highest acceptance rate from the consumers for three from five organoleptic parameters – overall appearance (equal to FHB 2), odor/aroma and viscosity. FHB 2 (with 0.05% potassium sorbate) showed the highest score only for the color parameter, but similar to those of the control. FHB 3 showed the lowest values for all parameters, which showed that the application of 0.02 % ethanolic propolis extract adversely affected the sensory properties, in particular the color of the functional beverage (Fig. 2 and Fig. 3). Despite the insignificant differences between the experimental groups, they were arranged by the consumers as follows: FHB 1 (total score 8.65) > FHB 2 (total score 8.54) > FHB 3 (total score 8.29) from maximum 9 points (Fig. 3). Similar to our findings were reported by Vasilaki et al. (2019) who investigated a propolis green extract (30%) as potential substitute for potassium sorbate (0.03%) in a non-carbonated fruit beverage (orangeade). The authors stated that the beverage with addition of propolis extract showed higher antioxidant activity and total phenolic content in comparison with those containing potassium sorbate. Moreover, the application of the propolis extract as a natural preservative significantly improved the shelf-life of the product by effective inhibition of microbial growth at wide temperature limits for 120 d.

The effectiveness of propolis as a natural preservative in fruit juices was proven by Koc et al.

(2007), who examined the antifungal effect of ethanolic extract of Turkish propolis in four non-pasteurized fruit juices (apple, orange, white grape, and mandarin) against six different yeast strains isolated from the same spoiled juices (*Candida famata*, *C. glabrata*, *C. kefyr*, *C. pelliculosa*, *C. parapsilosis* and *Pichia ohmeri*). The authors stated that the addition of propolis extract in concentrations between 0.01 mg.ml^{-1} and 0.375 mg.ml^{-1} had an inhibitory effect on all yeasts at 25°C, and exhibited greater antifungal effect than the conventional preservative sodium benzoate.



Figure 1. Overall appearance of the functional fruit-herbal beverages (from left to right: FHB 1 – control, FHB 2 and FHB 3)



Figure 2. Overall appearance of the functional fruit-herbal beverages with ice (from left to right: FHB 1 - control, FHB 2 and FHB 3)

According to research conducted by Silici and Karaman (2013), the application of a propolis extract in concentrations between 0.1 mg.ml^{-1} and 2 mg.ml^{-1} effectively inhibited the patulin-producing fungal strain *Penicillium expansum* in apple juice.

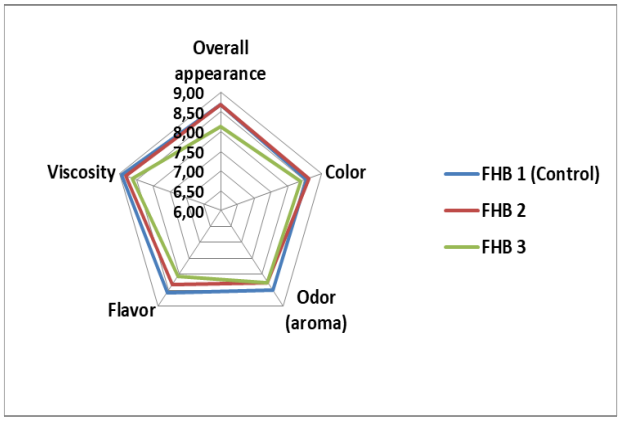


Figure 3. Sensory profile of the functional fruit-herbal beverages

The biopreservative potential of propolis extract (0.02 g.ml^{-1}) applied in orange juice was investigated also by Yang et al. (2017), who found that propolis possessed significant inhibition on the bacterial growth and L-ascorbic acid degradation. Orange juice pH value, titratable acidity, total phenolic content, color and antioxidant activity were effectively maintained by propolis application. The authors concluded that propolis prolonged the shelf-life of the orange juice up to 35 d and it can be used as a promising alternative to the chemical preservatives.

Lopes et al. (2022) developed a functional red fruit juice with addition of three different concentrations (3.1 mg.ml^{-1}, 4.6 mg.ml^{-1} and 6.1 mg.ml^{-1}) of aqueous extract of Brazilian green propolis. The results demonstrated that the addition of propolis extract increased the total phenolic and flavonoid contents, and improved the antioxidant capacity of the red juice as assessed by three different methods (DPPH, ABTS and FRAP). In addition to the functional benefits, the supplementation with propolis extract did not exert a negative impact on the sensory parameters of the red juice, as the beverage with the highest propolis extract concentration (6.1 mg.ml^{-1}) received the highest score from the consumers.

The search for safe and effective preservation methods that prevent the microbial spoilage while not altering the sensory and nutritional properties of foods, is still a major challenge in front of the food science and industry. Pasteurization is the main technological approach widely used to inactivate the microorganisms, thus improving the storage life of

the food products. However, the non-thermal and especially thermal pasteurization techniques used to inhibit the microbial growth and prolong the shelf-life may accelerate the quality degradation of fruit juices (Chang et al. 2021). On the other hand, the application of preservatives in foods must be consistent with two main conditions - they must not have harmful effects on the human health and should not degrade to toxic compounds after their consumption (Tzima et al. 2015). In this regard, propolis is a safe and promising natural preservation agent with significant antioxidant and antimicrobial potential, but its food applications should be very careful due to its water insolubility, low oral bioavailability, strong aroma and flavor, which can alter the sensory properties of the products. These limitations could be overcome by the application of some advanced techniques as microencapsulation, encapsulation, incorporation into edible films, coatings and packaging materials, which improve the propolis availability for certain food applications (El-Sakhawy et al. 2023).

Conclusions

Based on the results obtained, we can summarize that propolis is a natural product which can be successfully applied as an additive enhancing the functional properties of the foods and beverages, expressed in higher polyphenolic content, flavonoid content and antioxidant activity. Furthermore, propolis is a promising bio-preservative with significant antimicrobial potential that can be used to prolong the storage life of the products, and at the same time be an effective substitute for the chemical preservatives.

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