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Bioactivity properties of fermented beverage made from various fruits after *in vitro* gastrointestinal digestion*

Çeşitli meyvelerden yapılan fermente içeceğin *in vitro* gastrointestinal sindirim sonrası biyoaktivite özellikleri

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ABSTRACT

Objective: This study aimed to develop a functional fermented beverage using whole forms of nine different fruits and to evaluate its bioactive properties under simulated gastrointestinal digestion.

Material and Methods: A mixture of pomegranate, sour cherry, red and green apples, orange, lemon, pear, strawberry, nectarine, and raisins was fermented with kefir culture under controlled conditions. The beverage was evaluated for physicochemical properties, bioactive compound content, and biological activities, including antioxidant, antidiabetic, and antihypertensive effects.

Results: After fermentation, the final product had a pH of 3.85, a soluble solids content of 5.4°Brix, and malic acid content of 0.73 g/100 mL. The beverage was analyzed for its content of total phenolics, total flavonoids, anthocyanins, and ascorbic acid, yielding results of 620 mg CE/L, 230 mg CE/L, 38 mg/L, and 14 mg/L, respectively. After undergoing *in vitro* digestion, the DPPH and ABTS radical scavenging activities were found to be 480 and 570 µmol TEAC/mL, respectively, indicating that the antioxidant potential was preserved. The ACE inhibition reached 32.1% following digestion, suggesting moderate antihypertensive activity. Moreover, the inhibitions of α-amylase and α-glucosidase indicated moderate antidiabetic activity.

Conclusion: The data obtained indicate that fermentation of whole fruits with kefir culture can produce a bioactive-rich beverage that remains functionally active after digestion. This beverage, without heat treatment, added sugar, or juice extraction, has been evaluated as a functional alternative to traditional fruit-based beverages.

Keywords: ACE inhibition, antioxidant activity, bioactive compounds, fermentation, *in vitro* digestion, α-Amylase inhibition, α-Glucosidase inhibition

ÖZ

Amaç: Bu çalışmada, dokuz farklı meyve türünü bütün şeklinde kullanarak fonksiyonel fermente bir içecek geliştirilmesi ve simüle edilmiş gastrointestinal sindirim koşullarında biyoaktif özelliklerinin değerlendirilmesi amaçlanmıştır.

Materyal ve Yöntem: Nar, vişne, kırmızı ve yeşil elma, portakal, limon, armut, çilek, nektarin ve kuru üzümden oluşan meyve karışımı, kontrollü koşullar altında kefir kültürüyle fermente edildi. İçecek, fizikokimyasal özellikleri, biyoaktif bileşik içeriği ve antioksidan, antidiyabetik ve antihipertansif etkiler de dahil olmak üzere biyolojik aktiviteleri açısından değerlendirildi.

Araştırma Bulguları: Fermantasyondan sonra, son ürünün pH'ı 3,85, çözünür kuru madde içeriği 5,4°Brix ve malik asit içeriği 0,73 g/100 mL olarak saptandı. İçeceğin toplam fenolik madde, toplam flavonoid, antosiyanin ve askorbik asit içerikleri sırasıyla 620 mg CE/L, 230 mg CE/L, 38 mg/L ve 14 mg/L olarak saptandı. *İn vitro* sindirim sonrası, DPPH ve ABTS radikal temizleme aktiviteleri sırasıyla 480 ve 570 µmol TEAC/mL olarak bulundu. ACE inhibisyonu, sindirimden sonra %32,1'e ulaşarak orta düzeyde antihipertansif aktivite gösterirken, α-amilaz ve α-glukosidaz inhibisyon değerleri orta düzeyde antidiyabetik aktivite olarak değerlendirildi.

Sonuç: Elde edilen veriler bütün halindeki meyvelerin kefir kültürüyle fermantasyonu ile, sindirimden sonra da fonksiyonel olarak aktif kalan, biyoaktif açıdan zengin bir içecek üretilebileceğini göstermektedir. Isıl işlem, ilave şeker veya meyve suyu ekstraksiyonu olmadan bu içecek geleneksel meyve bazlı içeceklere alternatif fonksiyonel bir içecek olarak değerlendirilmiştir.

Anahtar sözcükler: ACE inhibisyonu, antioksidan aktivite, biyoaktif bileşikler, fermantasyon, *in vitro* sindirim, α-Amilaz inhibisyonu, α-Glukosidaz inhibisyonu

INTRODUCTION

One of the recommendations outlined in the dietary guidelines is to choose foods and beverages that provide the lowest possible calorie content while delivering the highest possible nutrient density per serving. This approach helps maintain a healthy body weight, ensures optimal intake of essential nutrients, and reduces the risk of chronic diseases, thereby promoting a healthier lifestyle (Karasawa & Mohan, 2018; FAO, 2020). However, in today's fast-paced world, following these nutritional recommendations can be challenging due to various limitations. Spending most of the day away from home significantly restricts meal and beverage choices. One frequently unmet recommendation, as highlighted in the dietary guidelines, is to consume at least three servings of fruit each day. In the approach to optimal nutrition, the recommendation to increase the consumption of fruits and vegetables is based not only on their richness in essential nutrients but also on their abundance of various bioactive compounds. Major bioactive compounds include polyphenols, carotenoids, glucosinolates, and dietary fiber, among others (Yalcin & Çapar, 2017). Key mechanisms influenced by these bioactive compounds include: Antioxidant activity, where they scavenge free radicals, protect cells against oxidative stress, delay the aging, and reduce the risk of chronic diseases; ACE (angiotensin-converting enzyme) inhibition, promoting vascular relaxation, lowering blood pressure, and thus decreasing the risk of hypertension and cardiovascular diseases; Antidiabetic activity, achieved by partially inhibiting the activities of carbohydrate digestive enzymes (α-amylase and α-glucosidase), thereby controlling postprandial blood glucose levels. Notably, fruits rich in phenolics, such as grape, pomegranate, and apple, have demonstrated strong inhibitory effects on antidiabetic activity (Zhao et al., 2022; USDA, 2023).

The intake of bioactive compounds from natural food sources is considered more effective than from dietary supplements. Supplements often contain a single compound in high doses, which when ingested may lead to undesirable side effects. In contrast, bioactive compounds from natural foods are better tolerated and absorbed by the human body. These compounds can act synergistically, providing enhanced health benefits when consumed together. Moreover, the “food matrix” — the complex physical and chemical environment within foods — plays an important role in facilitating the bio accessibility of these compounds by interacting with other nutrients. This interrelationship also underscores the importance of using simulated digestion models to assess the stability and bio accessibility of bioactive compounds under physiological conditions (Mishra et al., 2024). Fermentation is a process that allows the effective release of bioactive compounds from the food matrix and is also used to increase consumer consumption of fruits and vegetables (Ozcelik et al., 2021; Saud et al., 2024; Sun et al., 2024). Ozcelik et al. (2021) evaluated the total phenolic compound contents and antioxidant activities after fermenting cranberry, hawthorn, red plum, rosehip and pomegranate fruit juices with water kefir grains at 25°C for 48 h and reported that the water kefir could be used in the production of fruit juices and the antioxidant activity of the produced beverages increased. Similarly, it has been reported that fermentation of chokeberry juice with milk kefir grains results in improved flavor, increased physicochemical properties, total phenol and total antioxidant activities (DPPH, FRAP, ABTS). Researchers have reported that fermentation with milk kefir grains is a promising technique that improves the nutritional properties and overall acceptability of fruit juices (Sun et al., 2024).

A traditional beverage known as “Tükenmez” has been part of Anatolian cuisine for many years. This drink is prepared by fermenting a mixture of various fruits—such as medlar, apple, pear, and quince—together with sugar and chickpeas in glass jars. After a period of fermentation, the beverage is consumed, and each time it is poured, the container is refilled with fresh water, allowing it to be continuously consumed over an extended period. This feature is the origin of its name, which means “inexhaustible” or “never-ending” in Turkish. Current evidence indicates that it has been traditionally prepared and consumed in various regions of Anatolia. This study was inspired by this ancient traditional beverage. Accordingly, the aim was to develop a functional beverage that could offer a solution to the limited consumption of fruits, and to investigate its potential health effects.

Considering the difficulty of consuming nine fruits, either together or separately, in a daily diet, the study aimed to produce a fermented fruit beverage and evaluate its positive health effects by determining their bioactive properties. To this end, nine different fruits (two varieties of apples, orange, lemon, pear, strawberry, sour cherry, nectarine, and pomegranate) were fermented using different kefir concentrations and fermentation times. The product with the highest antioxidant activity and sensory properties was selected and analyses of pH, soluble solids, malic acid, total sugar, total phenolic, total flavonoid, vitamin C, and anthocyanin contents were carried out. The fermented beverage was then subjected to *in vitro* digestion and bioactivity analyses (antioxidant activity, antidiabetic activity, antihypertensive activity) were performed.

MATERIALS and METHODS

Materials

The fruits (two varieties of apples, orange, pear, lemon, strawberry, sour cherry, nectarine, pomegranate), raisins, cinnamon sticks and vanilla were purchased from local markets. Kefir culture was provided by Davisco (KEFIR DC1 1000 I). All chemicals and enzymes (Pancreatin P1750; pepsin P7012, bile acids B8631, pefabloc 76307, α amylase A6255, α-glucosidase G5003) utilized for the analyses were purchased from Sigma-Aldrich.

Propagation of kefir starter

A commercially available freeze-dried starter culture (Kefir DC1, Danisco, Poland), was used in this study. The culture contains a complex microflora similar to traditional kefir grains, including lactic acid bacteria (LAB, *Lactococcus lactis*, *Leuconostoc* spp., *Lactobacillus* spp., *Streptococcus thermophilus)* and kefir yeasts. The description of the culture states that it is non-GMO, certified according to ISO 9001, 14001, 22000 and PN-N-18001 standards, and it is recommended to incubate at 26-30°C. For activation, 1 g of the culture was suspended in 200 mL of sterile water and incubated at room temperature (approx. 22–25°C) for 3 days before inoculation to fruits (Anonymous, 2015).

Preparation of fermented fruit juice

In the production of fermented fruit juice, three different pre-formulations were tested to optimize the complementary effects of bioactive compounds obtained from various fruits. In these formulations, different inoculum kefir concentrations (2.5, 5.0, and 6.25 mg/mL) and different fermentation times (65 h, 5 days, and 7 days) (Paredes et al., 2021) were used. Fruits were immersed in drinking water containing 2% hypochlorite, rinsed, and then coarsely chopped together with the peels and seeds. After adding cinnamon bark (5 g), vanilla (1/4 teaspoon), and raisins (100 g) to enhance aroma and flavor, the mixture was transferred to 9 L glass jars and supplemented with sterilized drinking water (Figure 1). It was then hermetically sealed and incubated at 23°C in the dark. After the incubation period, the fermented juices were filtered using a wire kitchen strainer with a pore width of 1.5 mm and subjected to preliminary DPPH and ABTS antioxidant assays, as well as sensory evaluation. The sample showing the strongest antioxidant effect and the highest sensory acceptance score was selected for further analysis.

Figure 1. The state of the fruits prepared for fermentation.
Şekil 1. *Meyvelerin fermantasyona hazırlanmış durumu*.

Sensory evaluation

Refrigerated (14°C) beverage with a volume of 100 mL was presented to 27 untrained panelists from students and staff of the Food Engineering Department, Ege University, Türkiye, with different ages (18-65), healthy males and females. The panelists were asked to rate their evaluation of flavor, taste, and overall acceptability in a hedonic scale (0: dislike to 9: like extremely). Literature indicates that a mean score of 6 or higher on a 9-point hedonic scale typically signifies acceptable consumer liking for one sample (Altug & Elmaci, 2011).

Chemical measurements

Analyses of pH, malic acid and total sugar (Lane Eynon) contents were determined following the methodologies outlined by AOAC (2019). The soluble matter content was determined using a digital Abbe refractometer.

Determination of ascorbic acid (AA)

The vitamin C (ascorbic acid) content of the beverage was determined using the 2,6-dichlorophenolindophenol (DCPIP) titration method, as described by AOAC (2019). Briefly, an aliquot of appropriately diluted sample was titrated with a standardized DCPIP solution until a persistent light pink color was observed, indicating the endpoint. The ascorbic acid content was expressed as milligrams of ascorbic acid per liter of sample (mg AA/L).

Determination of total anthocyanin

The total anthocyanin content of the fermented fruit juice was determined using the pH differential method, as described by Ercan & El (2021). Briefly, the sample was diluted separately with two buffer solutions: potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5). The absorbance of the diluted samples was measured at 520 nm and 700 nm using a UV–visible spectrophotometer (Thermo Scientific Varioskan Flash, Finland).

The total anthocyanin content (TAC) was calculated using the pH differential method, according to Equation (1):

$$TAC_{(M3OG)}\ (mg/L) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l} \qquad (1)$$

where $A = [(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}]$;
MW is the molecular weight of the reference anthocyanin (cyanidin-3-glucoside, 449.2 g·mol^{-1});
DF is the dilution factor;
ε is the molar extinction coefficient (26,900 L·mol^{-1}·cm^{-1} for malvidin-3-O-glucoside);
and l is the path length (cm).

Determination of total phenolic compounds (TPC)

The total phenolic content (TPC) of the fermented fruit juice was carried out using the Folin–Ciocalteu colorimetric method, as described by Singleton and Rossi (1965). In brief, 0.5 mL of appropriately diluted sample was mixed with 2.5 mL of 10% (v/v) Folin–Ciocalteu reagent. After 5 minutes of incubation at room temperature in the dark, 2.0 mL of 7.5% (w/v) Na_2CO_3 (0.7 M) solution was added. The reaction mixture was further incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 765 nm using a UV microplate reader (Thermo Scientific Varioskan Flash, Finland). The results were expressed as µg of catechin equivalent (CE).

Determination of total flavonoid (TF)

The total flavonoid content (TFC) of the fermented fruit juice was determined using the aluminum chloride colorimetric method, as described by Chang et al. (2002) with slight modifications. Briefly, 0.5 mL of appropriately diluted sample was mixed with 2 mL of distilled water and 0.15 mL of 5% (w/v) sodium nitrite solution. After 5 minutes, 0.15 mL of 10% (w/v) aluminum chloride solution was added. At the 6th minute, 1 mL of 1 M sodium hydroxide was added, and the total volume was adjusted to 5 mL with distilled water. The mixture was thoroughly vortexed, and the absorbance was measured at 510 nm using a microplate reader (Thermo Scientific Varioskan Flash, Finland). The results were expressed as milligrams of catechin equivalent (CE).

***In vitro* digestion**

In vitro digestion of fermented fruit beverage was carried out according to the procedure described by Minekus et al. (2014). Briefly, the enzymatic activities of enzymes and the bile salt concentration in the porcine bile extract were determined. Oral digestion was simulated as 10 mL of the sample was mixed with 5 mL of simulated salivary fluid electrolyte stock solution for 2 min followed by gastric digestion with the addition of 8 mL simulated gastric fluid electrolyte stock solution (SGF) containing pepsin solution (20 000 U mL−1) for 2 h and finalized with intestinal digestion by addition of simulated intestinal fluid electrolyte stock solution (SIF) containing bile extract solution and pancreatin solution (800 UmL^{-1}, based on trypsin activity) under stirring for 2 h at 37°C. To terminate the enzyme activity for intestinal digestion, 0.05 mL of 0.1 M Pefabloc (enzyme inhibitor) was used. Following intestinal digestion, the sample was centrifuged at 8000 × *g* for 30 min at 4°C. Thereafter, the supernatant was used as the digested portion.

Determination of antioxidant activity

DPPH radical scavenging assay

The DPPH activity of the fermented fruit juice was determined by measuring its scavenging ability of DPPH (2,2-diphenyl-1-picrylhydrazyl), following the method described by Brand-Williams et al. (1995) with slight modifications. Briefly, 50 µL of appropriately diluted sample was mixed with 950 µL of 0.030 mg/mL methanol solution of freshly prepared DPPH solution (0.1 mM in methanol). The mixture was incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm using a microplate reader (Thermo Scientific Varioskan Flash, Finland). Antioxidant activity (AA) results were expressed as Trolox equivalents (µmol TEAC/mL sample)

ABTS radical scavenging assay

The ABTS radical inhibition activity assay was carried by the method of Re et al. (1999). $ABTS^{+}$• stock solution was used by reacting 7 mM ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) with 2.45 mM potassium persulfate. The mixture was left in the dark at room temperature for 12–16 hours. The $ABTS^{+}$• working solution was then diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. The sample (100 µL) was mixed with 900 µL of $ABTS^{+}$• working solution for 6 minutes of incubation at room temperature in the dark. The absorbance was recorded at 734 nm using a microplate reader (Thermo Scientific Varioskan Flash, Finland). Antioxidant activity (AA) results were expressed as Trolox equivalents (µmol TEAC/mL sample).

Determination of antidiabetic activity

α-Amylase inhibition assay

The antidiabetic potential of the fermented fruit juice was evaluated based on its inhibitory effect against α-amylase activity (El et al., 2015). The α-amylase solution was prepared at a final concentration of 2 mg/mL in a phosphate buffer (pH 6.9, 50 mM, with 6.85 mM NaCl). The fermented juice was diluted to different concentrations, and then 80 µL of the diluted sample was preincubated with 10 µL of porcine pancreatic α-amylase solution (1 U/mL in phosphate buffer, pH 6.9) at 37°C for 10 minutes. After this, 8 μL of potato starch solution (1% in phosphate buffer) was added to initiate the reaction. Then mixture was incubated at 37°C for 12 minutes. To stop the reaction, 50 μL of 10% HCl was used, and the mixture was heated in a boiling water bath for 5 minutes. To determine the starch content in the medium, 15 μL of iodine solution (0.0025 M I2/0.0065 M KI) and 50 μL of water were added, and the absorbance at 620 nm was measured using a microplate reader. A control (without sample) was prepared in the same way. The experiment was repeated using an inactive (denatured) enzyme that had been treated for 10 minutes in a boiling water bath at 100°C. Acarbose was used as a positive control. The inhibition effect of the beverage was expressed as IC_{50} (mg/mL).

α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity of the beverage was evaluated using the method described by El et al. (2015). The reaction was performed with p-nitrophenyl-α-D-glucopyranoside (PNPG, 30 mM) as the substrate and α-glucosidase enzyme (25 mg/mL), both dissolved in phosphate buffer (pH 6.9). Initially, the enzyme solution was mixed on ice for 15 minutes and then centrifuged at 10,000 × g for 30 minutes at 4°C to remove any insoluble fractions. The resulting supernatant served as the active enzyme solution. For the assay, 102 μL of sample solutions at various concentrations were added to separate wells of a microplate. Following this, 6 μL of the α-glucosidase enzyme solution was added to each well, and the plate was incubated at 37°C for 10 minutes. The reaction was initiated by adding 12 μL of PNPG solution to each well, followed by a second incubation at 37°C for 15 minutes. To stop the reaction, 60 μL of 1 M sodium carbonate (Na_2CO_3) solution was added. The amount of p-nitrophenol released was determined by measuring the absorbance at 400 nm using a microplate reader. A blank control was prepared by replacing the sample with phosphate buffer. Additionally, an enzyme inactivation control was conducted by pre-heating the enzyme at 100°C for 10 minutes. Acarbose was used as a positive control. The inhibition effect of the beverage was expressed as IC_{50} (mg/mL).

Determination of antihypertensive activity (ACE inhibition assay)

The ACE (angiotensin-converting enzyme) inhibitory activity of the fermented fruit juice was determined using the method described by Karakaya et al. (2016). The assay is based on the hydrolysis of the substrate hippuryl-histidyl-leucine (HHL) by ACE to release hippuric acid, which can be quantified spectrophotometrically. Briefly, 50 μL of the sample was preincubated with 50 μL of ACE enzyme solution (25 U/mL) at 37°C for 10 minutes. Then, 150 μL of 5 mM HHL solution prepared in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl was added to initiate the reaction. The mixture was incubated at 37°C for 30 minutes. The reaction was terminated by adding 250 μL of 1 N HCl. The hippuric acid formed was extracted with 1.5 mL of ethyl acetate, centrifuged, and the organic phase was evaporated at 95°C. The residue was dissolved in distilled water, and absorbance was measured at 228 nm using a UV–visible spectrophotometer (Thermo Scientific Varioskan Flash, Finland).

The ACE inhibitory activity (%) was calculated based on the reduction in absorbance relative to the control, indicating the ability of the sample to inhibit ACE-mediated substrate conversion.

$$\text{Inhibition (\%)} = (A_{\text{Control}} - A_{\text{Control blank}}) - (A_{\text{Sample}} - A_{\text{sample blank}}) / (A_{\text{Sample}} - A_{\text{sample blank}}) \times 100 \quad (2)$$

where
A_{control} is the absorbance of the reaction mixture containing ACE and the substrate (without sample), $A_{\text{control blank}}$ is the absorbance of the control mixture without ACE, A_{sample} is the absorbance of the mixture containing both ACE and the tested sample, and $A_{\text{sample blank}}$ is the absorbance of the mixture containing the sample but without ACE.

Statistical analysis

Statistical analyses were performed using Minitab software (version 21.3). The study was conducted using one sample with three independent experimental replicates, each analyzed in duplicate. Duplicate measurements were averaged prior to statistical analysis. Results are expressed as mean ± standard error of the mean (SEM) calculated from three independent experiments (n = 3). Differences among group means were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A significance level of $p \leq 0.05$ was considered statistically significant.

RESULTS and DISCUSSION

In the preliminary trials conducted to optimize the synergistic effects of bioactive compounds derived from different fruits, various fruit amounts, different culture inoculum concentrations (2.5, 5.0, and 6.25 mg/mL), and distinct fermentation durations (7 days, 5 days, and 65 hours) were applied. Among the three preliminary formulations tested, the one demonstrating the highest antioxidant activity (DPPH inhibition of 78.3% at 10 mg/mL) along with the highest mean sensory acceptance score (7.8 ± 0.5 on a 9-point hedonic scale) was selected for final fermented fruit beverage production. Based on sensory evaluation results, the fermented fruit drink can be described as a fresh, slightly carbonated, acidic beverage with a pleasant taste and moderate acid content.

The amounts of fruit used in the selected formulation are given in Table 1, and the fermentation conditions were 5 mg/mL kefir inoculum, 23°C, and 65 hours. The chemical and physical analysis results before *in vitro* digestion are shown in Tables 2 and 3, and the bioactivity analysis results of the fermented fruit juice after *in vitro* digestion are shown in Table 4.

Table 1. Fruits used in fermented fruit juice production
Çizelge 1. *Fermante meyve suyu üretiminde kullanılan meyveler*

Fruits	Binominal name	Amount (g)	Fruits (cont.)	Binominal name	Amount (g)
Pomegranate	*Punica granatum*	300	Strawberry	*Fragaria ananassa*	400
Pear	*Pyrus communis*	967	Sour cherry	*Prunus cerasus*	200
Red apple	*Malus domestica*	770	Nectarine	*Prunus persica*	570
Green apple	*Granny Smith*	438	Raisin (sultanas)	*Vitis vinifera*	100
Orange	*Citrus sinensis*	760	Vanilla (powder)	*Vanilla planifolia*	¼ teaspoon (2 g)
Lemon	*Citrus lemon*	405	Cinnamon (bark)	*Cinnamomum verum*	5

Physical and chemical evaluation

There are some studies in the literature on multi-fruit fermentation; however, these mainly focus on combinations of just 2 to 3 fruits, or involve a mixed beverage made from either single fruit or different fruit juices that have been fermented separately, or they use the juice of the fruits themselves. To date, we have not encountered any beverage that involves fermentation using all parts (seeds, fleshy parts, and peels) of nine different types of fruits with kefir culture. In this study, we used whole fruits, including peels and seeds, as fermentation substrates. After fermentation, we filtered the mixture to separate the juice from the solids. Therefore, we compared it with existing research on fermented fruit juices. Our results showed slightly lower soluble solids content (measured in °Brix) compared to similar juice fermentations because of limited diffusion of soluble sugars from the solid fruit tissues into the liquid phase (Table 2). The final pH (3.85) of the beverage was expected to range between 3.3 and 3.75, consistent with previous findings. Dikmetas et al. (2025) determined a final pH of approximately 3.5 in probiotic-fermented mixed fruit juice, while Lu et al. (2018) observed a pH of 4.41 in fruit juice fermented with *Lactobacillus rhamnosus*. Considering the high acidity of certain fruits used in this study (such as lemon, pomegranate, and sour cherry), a relatively low final pH was anticipated, enhancing the microbiological stability and sensory attributes of the beverage. The soluble dry matter content was estimated to be 5.4 °Brix post-fermentation. This is lower than values typically observed in juice-only fermentations reported by Dikmetas et al. (2025) after 48 hours of fermentation. This can be attributed to both microbial metabolism and the limited release of soluble solids from intact fruit matrices during fermentation. Titratable acidity, expressed as malic acid equivalents, was determined as 0.73 g/100 mL. The fermentation of whole fruits likely facilitated additional extraction of organic acids from peels and seeds, contributing to a slightly higher titratable acidity compared to juice-only fermentations. Similar observations have been documented during fermentation processes, where microbial acid production further elevates acidity levels (Dikmetas et al., 2025). Although chopped raisin is added to the fermentation medium at the beginning as a source of sugar for kefir yeast, most of them are consumed during the 65-hour fermentation period. During fermentation, this sugar is consumed by LAB and acids are formed, and light ethanol is formed by the kefir yeast. Kefir fermentation involves a symbiotic relationship between lactic acid bacteria (LAB) and yeasts, leading to the production of lactic acid, ethanol, and carbon dioxide. During fermentation, these microorganisms consume sugars, resulting in a decrease in sugar content over time (Paredes et al., 2021).

Table 2. Malic acid, pH, soluble dry matter and total sugar values fermented fruit beverage

Çizelge 2. *Fermente meyve içeceğinin malik asit, ph, çözünür kuru madde ve toplam şeker içerikleri*

	Malic acid g/100 mL	pH	^{0}Brix?	Total sugar g/L
Fermented fruit beverage	0.73 ± 0.03	3.85±0.06	5.4±0.50	4.62±1.02

Total phenolic compounds (TPC), total flavonoids (TF) and anthocyanin and ascorbic acid (AA) contents

Anthocyanins, phenolic compounds, and flavonoids present in fruits exhibit significant variations in their concentrations. These differences are influenced by a range of environmental factors such as soil composition, harvest time, and climate conditions. Additionally, food processing methods, temperature, oxygen exposure, pH, the presence of sugars and enzymes, as well as various physical and chemical conditions, can substantially affect the stability and concentration of these bioactive compounds. During mechanical, thermal, or chemical treatments such as juice production, the stability of these compounds may be positively or negatively impacted (Tiwari et al., 2009; Weber et al., 2019). Nevertheless, fermentation has been reported to enhance the anthocyanin content in fruit juices. Akarca & Baytal (2023) demonstrated that fermentation of juices derived from orange, grapefruit, and mandarin using lactic acid bacteria strains resulted in an increase in total phenolic content (TPC) and antioxidant activity, attributed to bacterial metabolism. Similarly, Dikmetaş et al. (2025) observed comparable enhancements when fermenting juices from kiwi, orange, dragon fruit, and apple. The highest TPC value (3257 mg GAE/g) was obtained in orange juice following inoculation with *Lactobacillus plantarum*. It is reported that enzymes produced by LAB can depolymerize complex phenolic structures or convert them into other derivatives. However, during fermentation, phenomena such as precipitation or oxidation—potentially due to alcohol production—can lead to a loss of phenolic content. Thus, TPC values may vary considerably depending on multiple factors, including fruit type, bacterial strains, and processing parameters (Wu et al., 2020). To our knowledge, no previous studies have evaluated the fate of these compounds after digestion in beverages made from fermenting combined whole fruits. In Mediterranean dietary patterns, the average daily intake of total polyphenols is estimated to be around 1905 mg. This is largely due to the consumption of polyphenol-rich foods, such as fruits, vegetables, olive oil, nuts, and red wine (Kapolou et al., 2021). Similarly, anthocyanins—a subclass of flavonoids daily intake ranges from 18.4 to 64.9 mg in Mediterranean populations, primarily through the consumption of red fruits and red wine (Gonçalves et al., 2021; Kapolou et al., 2021). The findings of our study suggest that consuming one serving (225 mL) of fermented fruit juice provides approximately 9–14% of the daily total polyphenol intake and 21–34% of the daily anthocyanin intake in a typical Mediterranean diet (Zamora-Ros et al., 2017; Kimble et al., 2019). Consistent consumption of these compounds has been linked to health benefits, including reduced oxidative stress and the prevention of chronic diseases.

The stage of maturity, harvest timing, and climatic conditions significantly influence the levels of phenolic compounds and anthocyanins in fruits and vegetables. These variations typically depend on species and ripeness level, showing either increases or decreases in content. These factors interact with environmental conditions, collectively shaping the range of bioactive compound concentrations (Eseberri et al., 2022). Therefore, the changes that will arise from raw materials can be reduced by combining the fruits according to their seasons.

Table 3. Total phenolic compounds (TPC), total flavonoids (TF), anthocyanin and ascorbic acid (AA) contents

Çizelge 3. *Toplam fenolik madde (TPC), toplam flavonoidler (TF), antosiyanin ve askorbik asit (AA) içerikleri*

	TPC (mg CE/L)	TF (mg CE/L)	Anthocyanin (mg M3OG /L)	Ascorbic acid (mg/L)
Fermented fruit beverage	620 ± 30	230 ± 20	38 ± 5	14

Antioxidant, ACE inhibition and antidiabetic activities

Following the *in vitro* digestion process of beverage, its antioxidant activity, angiotensin-converting enzyme (ACE) inhibitory potential, and antidiabetic properties were assessed (see Table 4). The DPPH radical scavenging capacity was found to be 480 μmolTEAC/mL (65.3%) while the ABTS radical scavenging capacity was measured as 570 μmolTEAC/mL (72%). These results indicate that the fermented beverage maintained its stability throughout gastrointestinal simulation and exhibited high antioxidant activity. According to the literature, polyphenols undergo biotransformation during the fermentation of fruit-based products, which may enhance their free radical scavenging properties (Hur et al., 2014; Filannino et al., 2018). However, the preservation of polyphenolic compounds post-digestion is critical to sustaining their bioavailable antioxidant potential. These compounds are subject to degradation or transformation due to pH fluctuations, enzymatic hydrolysis, and microbial enzyme activity within the digestive environment (Tagliazucchi et al., 2009). Therefore, the retention of antioxidant activity following digestion is not solely dependent on initial phenolic richness but also the structural resilience of the compounds to digestive conditions.

Similarly, several studies have demonstrated that the antioxidant capacity of fermented plant-based products is maintained—or even enhanced—following simulated digestion. For instance, Filannino et al. (2018) determined that fermented pomegranate juice displayed minimal loss in antioxidant activity after *in vitro* digestion, and that certain aglycone forms (non-sugar entity) of phenolics became more bioactive during the process. This observation is consistent with the high DPPH and ABTS inhibition values recorded in our study. It has been suggested that phenolic matrices resulting from the co-fermentation of multiple fruit types may exhibit synergistic interactions during digestion, thereby contributing to the stabilization of overall antioxidant activity (Hur et al., 2014). This phenomenon may account for the effect observed in our study, in which a complex mixture of nine fruits was used. The finding that the fermented fruit filtrate retained high antioxidant activity even after *in vitro* digestion underscores its bioavailability potential and supports its application in the production of functional beverages. Further studies are warranted to identify the specific phenolic compounds that remain active post-digestion using advanced analytical techniques such as LC-MS/MS. The ACE inhibitory activity detected in our digested samples was 32.1%, indicating a noteworthy antihypertensive potential. The 32.1% ACE inhibition value was achieved under a simulated ingestion condition, above the "no effect" range (<25%) but below the pharmaceutical target (~80%). This positions it as "may contribute but is unlikely to normalize hypertension on its own". This can be considered a reasonable and safe amount to intake from the diet. The preservation of this activity after *in vitro* digestion suggests that the responsible compounds are resistant to gastrointestinal conditions. This effect may be attributed to bioactive peptides and phenolic compounds generated during fermentation, both of which are known to contribute to ACE inhibition

(Karakaya et al., 2016; Karagul & El, 2023). While our findings support the natural antihypertensive capacity of fermented fruit juice, further in vivo studies are necessary to confirm this effect under physiological conditions. The ACE inhibitory effects of fermented fruit products have been widely documented. For example, pomegranate and guava juices fermented with *Lactiplantibacillus plantarum* exhibited ACE inhibition ranging from 66.4% to 72.1% following *in vitro* digestion (Hashemi & Jafarpour, 2023), while the ACE inhibitory activity of pear and blackberry juice fermented with *Lactobacillus helveticus* was shown to increase with longer fermentation duration (Pucel et al., 2021). Moreover, the fermentation process is known to release bound polyphenolic compounds, which also play a role in the inhibition of carbohydrate-digesting enzymes (Hanhineva et al., 2010; Hashemi & Jafarpour, 2023). Similar to the findings in our study, fermented jujube juice has been reported to prevent α-amylase and α-glucosidase activities by 47.5% and 64.9%, respectively. Multiple studies have demonstrated that fermented plant-based beverages, due to their elevated polyphenol and flavonoid content, can effectively inhibit these enzymes (Hanhineva et al., 2010; Ercan & El, 2021).

Table 4. Bioactivity values of beverage before and after *in vitro* digestion

***Çizelge 4**. İçeceğin in vitro sindirim öncesi ve sonrası biyoaktivite değerleri*

	DPPH µmol TEAC/mL		ABTS µmol TEAC/mL		ACE (%)		α-Amylase IC_{50} (mg/mL)		α-Glucosidase IC_{50} (mg/mL)	
	Before	After	Before	After	Before	After	Before	After	Before	After
Fermented fruit beverage	376± 3^{a}	480 ± 4.1^{b}	525± 5^{c}	570± 6.5^{c}	31.8± 4.1^{d}	32.1±2.0^{d}	0.870±1^{e}	0.903±1.4^{f}	0.018 ± 2.1^{g}	0.020±1.6^{h}

Results are shown as mean ± SD (n = 3). Different superscript letters in the same row are significantly different ($p < 0.05$).

CONCLUSION

Many fruit-based beverages available in the market are presented in the form of fruit nectars rather than pure fruit juices. Only a limited number of fruits can be commercially offered as 100% fruit juice. Technologically, it is not possible to obtain and commercially process juice from every type of fruit. In general, in the fruit juice industry, fruits are processed during their harvest season to produce nectar or concentrate, which are later diluted and sold. Since nectar also contains added sugar, the label must state that the drink contains "added sugar". In recent years, dietary guidelines have recommended the consumption of “100% fruit juice” to prevent the intake of added sugars. In Türkiye, the Ministry of National Education has issued a circular concerning “Foods to Be Sold in School Canteens” aimed at reducing the risk of childhood obesity. According to this circular, fruit nectar and fruit juice concentrates are included in the list of foods and beverages not permitted for sale in educational institutions. In contrast, 100% fruit juices without added sugar are permitted. The fermented fruit beverage developed in this study can be described as a fresh, lightly carbonated, acidic beverage with a pleasant flavor and moderate acid content. In this beverage, while combining nine different fruits and flavor-enhancing ingredients such as vanilla and cinnamon, careful attention was given to incorporate fruits that are known to be rich in health-promoting bioactive compounds. Furthermore, the formulation was designed to maximize synergistic effects among the fruits, thereby increasing their potential bioactivity. Microbial characterization of this beverage and its probiotic or postbiotic properties should be revealed with further studies. Compared to conventional fruit beverages such as juices, concentrates, and nectars, this fermented beverage offers several distinguishing features:

- The whole fruit, rather than just the juice, is used.
- Multiple fruits are combined.
- Fermentation is carried out with a kefir starter culture known for its probiotic benefits.
- No additional table sugar is included.
- Seasonal flexibility allows for the formulation to be adapted according to the availability of fresh fruits throughout the year.

Overall, the fermented fruit beverage, due to its content of bioactive compounds and nutrients, offers a strong alternative to commercially available fruit nectars, fruit juices, and carbonated beverages. The beverage can be suitable for industrial production without additional investment and can be adapted by altering the fruit composition based on seasonal availability. The data of the study show that the physicochemical and bioactivity properties of the beverage obtained by fermentation of the whole fruits can offer a promising new approach for the development of functional beverages.

Data Availability

Data will be made available upon reasonable request.

Author Contributions

Conception and design of the study: SNE; analysis and interpretation of data: SNE, SK; statistical analysis: SNE; visualization: SNE, SK; writing manuscript: SNE

Conflict of Interest

There is no conflict of interest between the authors in this study.

Ethical Statement

We declare that there is no need for an ethics committee for this research.

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