CHARACTERIZATION OF HAZELNUT MILK FERMENTED BY *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS*

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ABSTRACT

Raw hazelnut milk were used to develop new plant-based fermented product using commercial starter mix containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains in order to address the current demand for health promoting and functional plant-based dairy-like products. The pH, titratable acidity (TA), serum separation (SS), protein content, fat content and ash content of fermented product were found to be 4.95, 1.25 g lactic acid/ 100 mL, 28.00%, 2.60%, 7.03% and 0.66%, respectively. Fermented hazelnut milk demonstrated non-Newtonian pseudoplastic flow behavior and it's structure was more predominantly elastic than viscous. Sensory analysis showed that some attributes of fermented hazelnut milk such as color, aroma, consistency, typical yoghurt odor and overall acceptability were less appreciated than Ayran (commercial cow milk yogurt drink) whereas some other attributes were found as satisfying.

Keywords: Hazelnut, fermentation, functional foods, foods of plant origin, Ayran

LACTOBACILLUS DELBRUECKII SUBSP. *BULGARICUS* VE *STREPTOCOCCUS THERMOPHILUS* İLE FERMENTE EDİLMİŞ FINDIK SÜTÜNÜN KARAKTERİZASYONU

ÖZ

Ham fındıktan *Lactobacillus delbrueckii* subsp. *bulgaricus* ve *Streptococcus thermophilus* ticari suşları kullanılarak sağlığı teşvik edici, fonksiyonel özellikte fermente bir ürün elde edilmiştir. İlk aşamada, ultrasonikasyon uygulaması ile fındık sütünün homojenizasyonu sağlanmıştır. Elde edilen fermente ürününün pH, titre edilebilir asitlik, serum ayrılma kapasitesi, protein içeriği, yağ içeriği ve kül içeriği sırasıyla 4.95, 1.25 g laktik asit/100mL, %28.00, %2.60, %7.03 ve %0.66 olarak bulunmuştur. Reolojik analizlerden elde edilen veriler fermente fındık sütünün Newtonian olmayan psödoplastik akış davranışı sergilediğini ve yapısının viskozdan daha elastik olduğunu göstermiştir. Duyusal analiz, renk, aroma, tipik yoğurt kokusu ve genel kabul edilebilirlik gibi fermente fındık sütünün bazı niteliklerinin Ayran'dan daha az takdir edildiğini, ancak diğer bazı özelliklerin tatmin edici olduğu sonucuna varılmıştır.

Anahtar kelimeler: Fındık, fermentasyon, fonksiyonel gıdalar, bitki esaslı gıdalar, Ayran

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INTRODUCTION

The consumer demand for alternatives to cow's milk increased due to the rise of health problems such as lactose intolerance and cow's milk allergy. In addition, avoiding dairy products has been a lifestyle including vegetarian and vegan diets and based on health concerns including antibiotic residues and cholesterol and based on diet preferences or based on ethical considerations (Jeske et al. 2017). The total sales of plant-based milk substitute in EU market has been reached $1.9 billion in 2015 (Jeske et al. 2017). Hazelnut milk (HM) which is prepared by grounding the hazelnut in water, homogenizing and filtering could supply almost the same potential health benefits as whole hazelnuts (Bernat et al. 2014, 2015; Jeske et al. 2017). HM may be used in the production of fermented products as substitute to bovine milk products to contribute to the health benefits of the products and possibly to be alternative to animal milk products in lacto-intolerant and vegetarian consumers (Bernat et al. 2014).

Hazelnuts are extensively used by food industry to produce products including ice-cream and dairy, baking, confectionery, candy and chocolate products. They are mostly used in various products to give taste and aroma (Simsek and Aykut 2007). Hazelnuts play a crucial role in human health and nutrition due to their composition of protein, carbohydrates, lipids, vitamins, dietary fibres, phenolic compounds, tocopherols, minerals, phytosterols and squalene, and it is a potential energy source (600-650 kcal in 100 grams) (Alasalvar et al. 2003; Köksal et al. 2006). HM contains monounsaturated fatty acids (i.e. oleic acid) and phytosterols (i.e. β-sitosterol) and antioxidant compounds (Lainas et al. 2016). Given the combination of its health-promoting properties and nutritional composition, hazelnut could be the raw material to manufacture a new generation of plant based fermented milk products.

In this paper, it was aimed to produce a dairy-like fermented product for the ones sensitive to bovine products. For this purpose, HM was produced and fermented by commercial yogurt starter culture *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. In addition to analysis of microstructure and chemical composition, rheological behavior and sersory attributes were investigated. Comparison of the data obtained to those of Ayran was also given.

MATERIALS AND METHODS

All chemicals and solvents (analytical grade) were purchased from Merck (Darmstad, Germany). FAME (fatty acid methyl esters) mix was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of hazelnut milk

Hazelnut raw kernels were obtained from a local market in İstanbul, Turkey. The methods found in the literature (Al-nabulsi et al. 2014; Bernat et al. 2014) were modified and used for production of HM. For this purpose, unroasted hazelnut kernels were grounded and soaked in distilled water and homogenized for 2 minutes by a laboratory homogenizer (WiseTis HG-15A, witeg Labortechnik GmbH, Wertheim, Germany) after filtered through two folded cloth filter. Then, the mix was further homogenized by an ultrasound homogenizer (Hielscher UP400S, Teltow, Germany) at 100 W and 20 kHz for 10 minutes to achieve homogeneous dispersion of oil droplets (Ertugay et al. 2004).

Colloidal stability of HM

Colloidal stability of ultrasound-homogenized and non-homogenized (control) HM samples was determined through the phase separation analysis during 7 days of storage time at 4 °C . For this purpose, about 15 g of HM was poured into 16 mm diameter of glass tubes and the degree of the separated phases was visually evaluated after ultrasonication. Additionally, the colloidal structure of the HM before and after ultrasound homogenization was evaluated using a light camera (Wu et al. 2000)

Fermentation process

The activated starter culture mix (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) in liquid form was obtained from a milk processing company (Atesoglu Milk Products

Co., Istanbul, Turkey). The activated commercial culture mix containing *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* cells were enumerated on M17 agar medium (pH 6.9) and on acidified MRS agar medium (pH 5.4), respectively (Ghoddusi and Robinson 1996; Ashraf and Shah 2011). For this purpose, 25 ml starter mix was poured into 225 ml sterile peptone diluents (0.1g/L). Appropriate dilutions were made by adding 1 mL of aliquot to 9 mL of sterile peptone diluent. Pour plate technique was carried out for enumeration of the cells. *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were incubated 37°C for 48 h. Plates containing 20 to 300 colonies were enumerated, and the counts were expressed as CFU/mL. Additionally, total number of cells was determined using a Thoma cell chamber (Glaswarenfabrik Karl Hecht GmbH & Co KG, Germany) for rapid enumeration prior to inoculation (Pleissner et al. 2016). Counting the colonies on respective mediums showed that the commercial inoculum contained around 8 log CFU/mL *L. delbrueckii* subsp. *bulgaricus* and around 9 log CFU/mL *S. thermophilus*.

As suggested previously, HM samples were enriched with 1.5% (w/w) glucose solution prior to the fermentation process in order to achieve the growth of the mixed culture used, which also resulted in acidification (Bernat et al. 2014). The liquid starter mix was inoculated into HM at a rate of 1% (v/v). The inoculated HM samples were incubated at 45 °C and the fermentation was stopped at pH 4.6. The fermented hazelnut milk (FHM) samples were immediately cooled and stored at 4 °C for 12 hours prior to analyses.

Chemical properties and proximate analyses
Analyses of moisture, total nitrogen (protein) and fat content in HM and FHM samples were conducted based on the methods as outlined, AOAC 16.006, AOAC 958.48, and AOAC 945.16, respectively (Horwitz, 2000). The ash content was determined by incineration in an electric muffle furnace at 550 °C.

The pH values were measured at 25 °C using a calibrated pH meter (Hanna HI2211, Germany). The AOAC standard method AOAC 947.05 (AOAC 2000) was used to determine the titratable acidity in samples (Yasmin et al. 2015). 25 mL sample was titrated against 0.1 mol/L NaOH solution until pH 8.3 remain stable for 10 s using phenolphthalein as indicator and the results were expressed as grams of lactic acid equivalent per 100 ml of the sample.

Fatty acid composition of FHM samples was determined by using GC (Agilent 6890 GC) equipped with HP-88 column (100 m x 0.25 mm ID x 0.2 μm) and FID. The methyl esters of the fatty acids (FAME) were prepared according to AOCS Official Method Ce 1h-05 (AOCS 2009). 0.1 gr of sample was mixed with 10 ml of n-Hexane and vortexed for 30 seconds with a vortex mixer. 100 microliters of 2 N potassium hydroxide (prepared in methyl alcohol) was added and mixed for 30 seconds using a vortex mixer. After centrifugation of whole mix, 1-2 ml of the liquid phase was poured into a vial and injected into GC-FID equipped with auto sampler (injection volume: 1 μL). Temperature and oven program of the device were as follows: Inlet temperature: 250 °C; Injection volume: 1 μl; Split Ratio: 1/50; Carrier gas: Helium; Pressure flow: 2 mL/min; Oven Temperature: 120 °C, 1 min, 10°C/min to 175 °C, 5 °C/min to 210 °C, 5 min 5°C/min 230 °C, 5 min; Detector temperature: 280 °C; Detector gases. Hydrogen: 40 mL/min, Air: 450 mL/min, Helium: 30 mL/min.

Serum separation
For the analysis of serum separation (SS) of FHM, the method as outlined by Lucey et al. (1999) was modified and used. Samples were placed in 50 mL graduated cylinders and stored at 4 °C. The volume of the separated serum at the top was read after storage for 10 days. The results were expressed as mL serum separated per 50 mL of the sample.

Rheological properties
Steady-state shear properties
The rheological behavior was characterized using a rheometer (Anton Paar, MCR 302, Austria) with a probe using a cone-plate configuration (cone diameter 50 mm, angle 4°, gap size 0.140 mm). The shear stress (σ) was measured as a function of

shear rate (Ɏ) from 1 to 100 s^{-1} at 25°C. 1.0 ml sample was placed between cone and plate and the measurement was started immediately. Each measurement was replicated three times on three different samples (each 1 ml) from the same sample with two repetitions. The Herschel–Bulkey model (Eq. 1) was fitted to the experimental points to determine the flow behavior index (n), consistency index (K) and yield stress (σ_0) using a non-linear procedure. Apparent viscosities were calculated at 50 s^{-1}, since the shear rates generated in mouth when food is being chewed and swallowed are between 10 and 100 s^{-1} (McDorman 2009).

$$\sigma = \sigma_0 + K(Ɏ)^n \qquad (1)$$

Viscoelastic properties

The amplitude sweep test was performed at 1 Hz in the strain range of 0.1–100% to determine the linear viscoelastic region (LVR). Frequency sweep test was performed at 1% strain (determined by amplitude sweep test) over a frequency range of 0.1–10 Hz at 25 °C. Each measurement was repeated three times with three replications. The viscoelastic parameters of G' (elastic or storage modulus) (Pa) and G'' (viscous or loss modulus) (Pa) were calculated using the following equations (Steffe 1996).

$$G' = K'(\omega)^{n'} \qquad (2)$$
$$G'' = K''(\omega)^{n''} \qquad (3)$$

where K, ω, n are the consistency index (Pa·s^n), oscillatory frequency and the flow behavior index (dimensionless) respectively,

Sensory properties

Sensory analysis (hedonic test) was conducted as described by (He and Hekmat 2015) with some modifications. Sensory evaluation was performed in open sitting in a room where the temperature was 25 °C. Twenty panelists (fourth year food engineering students) were randomly selected. The panelists were trained by giving them the explanation of the score sheet and method of scoring as well as the information about the definition of quality attributes selected for sensory evaluation (Routray and Mishra 2012). The sensory test included color, taste, mouthfeel, consistency, aroma, acidity, typical yogurt odor and overall acceptability. FHM was firstly served followed by the commercial full fat Ayran. They evaluated the samples using a 5-points hedonic scale. The scores were assigned from 1 (extremely dislike) to 5 (extremely like).

Statistical analysis

One way analysis of variance (ANOVA) was applied to determine whether any of the differences between the means are statistically significant. Tukey method was used to declare significant differences at 95% confidence ($P<0.05$). Statistical analyses were performed using Minitab version 17.3.1 (Minitab, Inc., State College PA, USA).

RESULTS AND DISCUSSION
Proximate composition and chemical properties

Proximate composition (moisture, protein, fat and ash), chemical properties (pH and total acidity) of hazelnut milk (HM), Ayran and fermented hazelnut milk (FHM) are presented in Table 1. HM had remarkable higher content of fat and slightly lower content of protein in comparison with those of a standardized cow milk (3.2 and 3.3 g/100 mL of fat and protein, respectively) (Wijesinha and Burlingame 2013). The findings of this study were comparable with those reported in the literature (Bernat et al. 2015; Ilyasoğlu et al. 2015). There were no huge differences between the properties of HM and FHM except for large significant differences in their pH and total acidity values ($P=0.000$). The reason to this change is attributed to decreasing the pH value and increasing titratable acidity during fermentation where lactic acid bacteria used glucose to form lactic acid and CO_2 gas. Small significant differences were also obtained in moisture and fat contents ($P=0.039$ and $P=0.012$, respectively). However significant differences ($P<0.05$) were obtained between the properties of Ayran and FHM. The results of this work indicated that SS of FHM was lower than that of Ayran (Table 1). This might be attributed to the differences in pH, titratable acidity and properties of protein matrix which are important factors affecting SS (Gursoy et al. 2016).

Table 1. Characteristics of HM and FHM (n =3), (Mean±sd)

Characteristics	HM	Ayran[d]	FHM	Level of significance (Ayran-FHM)	Difference (%) for Ayran[a]
Proximate composition					
Dry matter[b]	11.63±0.25	7.75±0.83	12.56±0.47	* (*P*=0.001)	5.21
Protein[b]	2.34±0.09	1.14±0.34	2.60±0.20	* (*P*=0.003)	-128.07
Fat[b]	7.48±0.10	2.09±0.34	7.03±0.15	* (*P*=0.000)	-236.52
Ash[b]	0.54±0.07	0.48±0.04	0.66±0.04	* (*P*=0.006)	-36.80
Chemical properties					
pH	6.93±0.03	4.48±0.02	4.95±0.02	* (*P*=0.000)	-10.41
Titratable acidity[c]	0.33±0.03	0.82±0.10	1.25±0.05	* (*P*=0.003)	-52.44
SS (mL/50 mL)	-	28.50±0.10	14.00±0.42	* (*P*=0.000)	50.87

[a] Difference (%) Ayran = [(Ayran value - FHM value)/Ayran value] x 100,
[b] g/100g product,
[c] g lactic acid/100 mL,
[d] (Özünlü and Koçak 2010; Gursoy et al. 2016)
* significantly different (*P*< 0.05)
HM: hazelnut milk, FHM: fermented hazelnut milk, SS: serum separation

The results of this study revealed that FHM contained monounsaturated and polyunsaturated fatty acids in its lipid fraction (Table 2). As it can be seen in Table 2, FHM contained around 89 g mono and poly-unsaturated fatty acids and 11 g saturated fatty acids per 100 g of lipid fraction. The oleic acid concentration (approximately 81%) in FHM was similar to the one reported previously (Ilyasoğlu et al. 2015). They found around 85% oleic acid in yogurt -like product from hazelnut slurry. They also reported the linoleic acid and palmitic acid concentrations as approximately 7% and 5%, which agrees with the data obtained in this study (around 7.1% and 6.8%).

Table 2. Fatty acid composition of FHM (%) (n=3) (Mean±sd)

Fatty Acid Composition	%
Lauric Acid (C12:0)	0.25±0.12
Miristic Acid (C14:0)	0.21±0.05
Miristoleic Acid (C14:1 Cis-9)	0.12±0.00
Pentadecenoic Acid (C15:1 Cis-10)	0.30±0.06
Palmitic Acid (C16:0)	6.76±0.56
Palmitoleic Acid (C16:1 Cis-9)	0.17±0.01
Margaric Acid (C17:0)	0.06±0.00
Heptadecenoic Acid (C17:1 Cis-10)	0.60±0.75
Stearic Acid (C18:0)	3.11±0.11
Oleic Acid (C18:1 Cis-9)	80.97±0.15
Linoleic Acid (C18:2 Cis-9.12)	7.07±0.52
Linolenic Acid (C18:3 Cis-6.9.12)	0.20±0.12
Eicosenoic Acid (C20:1 Cis-11)	0.39±0.54
ɣ- Linoleic Acid (C18:3 Cis-9.12.15)	0.12±0.01

FHM: fermented hazelnut milk

Colloidal stabilization of HM

The ultrasound homogenization had significant effect on the size distribution of plydispersed fat droplets as the size of bigger fat droplets was reduced almost 10 folds. These findings are in agreement with those reported previously (O'Sullivan et al. 2015).

Rheological properties of FHM
Steady-state shear properties

In this study, rheological properties of FHM (Fig. 1) was determined after fermentation of HM, therefore, FHM was characterized in terms of steady and dynamic shear properties. The change of shear stress versus the shear rate shows that the FHM was a non-Newtonian pseudoplastic flow behavior with a beginning resistance against the flow, as revealed by the calculated yield stress. This behavior of FHM is in agreement with the findings of previous studies (Koksoy and Kilic 2003; Janhøj et al. 2008; Kök 2010). Herschel-Bulkley model parameters of FHM, R^2, the yield stress, consistency coefficient and flow behavior index values were found to be 0.99, 0.21, 0.06 and 0.57 respectively, the R^2 value was very close to 1, which means that the Herschel-Bulkley function well fitted to the flow behavior of FHM.
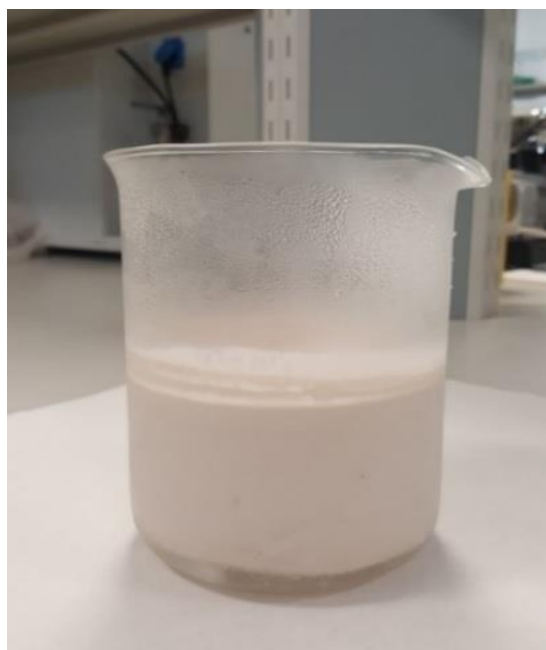

Figure 1. A view of fermented hazelnut milk

Viscoelastic properties

The viscoelastic properties of the FHM were measured under 0.5% strain using the frequency screening test. The magnitude of the energy stored in the material or recoverable per cycle of deformation is called as storage (or elastic) modulus G', while the energy that is lost at viscous dissipation per cycle of deformation is mentioned as the loss (or viscous) modulus G''. Therefore, all the energy is stored in a perfectly elastic solid, which results in zero loss modulus and the stress and strain will be in phase whereas all the energy is dissipated as heat in a liquid with no elastic properties; G' is zero and the stress and strain will be out of phase by $-90°$ (Sharoba et al. 2005).

The changes in storage modulus (G'), loss modulus (G'') and and complex viscosity (η^*) as a function of frequency for FHM is presented in Table 3. The magnitudes of G' and G'' increased with increase in frequency, revealing viscoelastic nature of FHM. Plots of frequency versus G' and G'' dynamic rheological data were subjected to non-linear regression and the magnitudes of intercepts, slopes and R^2 values were studied (Table 3). The storage and loss moduli and the complex viscosity plotted versus frequency indicated that the viscoelastic behavior of the FHM could be well described by Eqs. (2) and (3) with high determination coefficients (0.995–0.988).

Table 3. Values for the power law model for storage (G') and loss (G'') modules of fermented hazelnut milk (n=3) (mean± sd)

$G' = K'(\omega)^{n'}$		$G'' = K''(\omega)^{n''}$	
K'	2.099±0.110	K''	0.390±0.025
n'	0.245±0.014	n''	0.463±0.013
R^2	0.995±0.006	R^2	0.988±0.001

As far as the structure point of view of FHM was concerned, for true gels (G', G'') versus ω plots have zero slope; however, positive slopes show that the storage (elastic) modulus (G') is greater than the loss (viscous) modulus (G'') over a whole frequency ranges of ω studied for weak gels and highly concentrated dispersions (Ross-Murphy 1984). The dynamic oscillatory test data in this

study revealed that the slopes were positive ($n' = 0.245$; $n'' = 0.463$) and almost parallel to each other and G' ($K' = 2.099$) was higher than that of G'' ($K'' = 0.390$) ($G'>G''$) without exhibiting any cross point of G' and G'' along the whole frequency range studied (Fig. 2). These results suggest that the FHM was more predominantly elastic than viscous. Similar behavior has been reported for some food samples such as pulps, purees, juices and concentrates (Augusto et al. 2012). Ramchandran and Shah (2009) investigated textural and rheological properties of low-fat yogurt and they report that G'' was lower than G' for the samples indicating that they exhibited characteristics typical of a weak

viscoelastic gel structure. Shirkhani et al. (2015) studied the rheological behavior of fermented milk drink and they reported that G' values were greater than G'' ($G'>G''$), indicating the presence of gel-like structure. Similarly, Zhou (2018) found that G' values were greater than G'' ($G'>G''$) for drinking yogurt. However, according to Kök (2010)'s findings, yogurt drink samples showed non-Newtonian behaviour and viscous component predominated over elastic modulus ($G'' > G'$). The reason might be attributed to the variation in composition and structure of the samples studied as well as degree of homogenization.
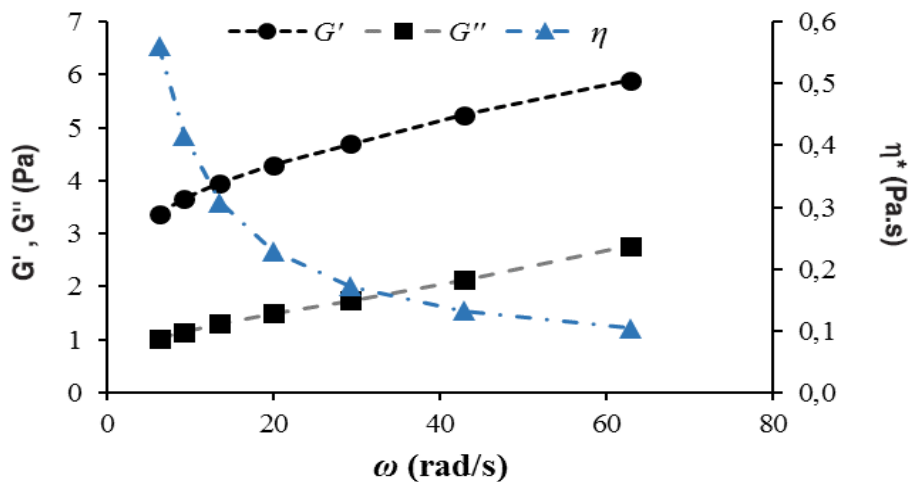

Figure 2. Mechanical spectra of fermented hazelnut milk

In the description of viscoelastic behavior, loss tangent (G''/G') is also used to indicate if the material of concern would have elastic or viscous properties. In the case of FHM, the larger the tan δ value, the more easily the FHM flows. The loss tangent values showed a predominantly elastic behavior (tan δ < 1 or $G' >G''$) for FHM over the increased frequency, which can be attributed to the existence of a phase lag between the input sinusoidal signal and the response one (Muñoz et al. 2007).

Sensory properties
The sensory attributes of FHM were compared with those of commercial full fat Ayran based on

the hedonic scale. According to results given in Table 4, the taste attribute of FHM was scored very close to that of Ayran (P=0.906) whereas the consistency of FHM was evaluated as less consistent (P=0.027). Acidity and mouthfeel attributes got similar scores with slight differences from the panelists (P=0.960 and P=0.313, respectively). According to panelists' evaluation, the score of overall acceptability attribute of FHM showed significant difference compared to that of Ayran (P=0.002) even though the gap between the scores are not relatively large (Table 4).

Table 4. Comparison of sensory attributes of FHM with those of commercial yoghurt drink. (20 panelists) (mean± sd)

Attributes	Ayran	FHM	Level of significance
Color	4.50±0.41	3.56±0.40	* (P=0.047)
Taste	4.10±0.95	4.02±0.65	(P=0.906)
Aroma	0.62±0.21	4.41±0.79	* (P=0.001)
Consistency	3.70±0.44	2.53±0.40	* (P=0.027)
Typical yoghurt odor	4.40±0.52	3.27±0.32	* (P=0.033)
Acidity	3.65±0.41	3.63±0.13	(P=0.960)
Mouthfeel	3.95±0.05	3.73±0.32	(P=0.313)
Overall acceptability	4.20±0.10	3.60±1.10	* (P=0.002)

FHM: fermented hazelnut milk, * significantly different (P< 0.05)

CONCLUSION

Fermented hazelnut milk by using yogurt starter culture including *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* strains has been successfully produced and its chemical, physico-chemical, rheological and sensorial attributes were analysed. The results of this study showed that FHM might be considered a novel fermented product with potential health-promoting properties, suitable for a wide range of consumer groups, such as vegetarians and the ones prone to lactose containing products or people allergic to animal proteins. In addition, the fatty acid distribution of the beverage implies better health-promoting nutritional properties than the animal based milk produtcs. The overall appearance of the fermented hazelnut milk was good and palatable. Although the sensory characteristic might require further research, this work underlines the technical feasibility and makes one step further to produce an alternative fermented drink, rich of nutritive components.

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