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Comparing microbiological profiles, bioactivities, and physicochemical and sensory properties of donkey milk kefir and cow milk kefir

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Abstract: Consumption of fermented milk products especially kefir is accelerated in the population due to their high nutritional value and other health benefits. These health benefits are rooted in the composition of source material and flora of kefir grains. Therefore, the utilization of different kinds of milk as source material in kefir production directly affects the properties of kefir. In this study, changes in the physicochemical properties, microbiological profiles, antibacterial effects, antioxidant activities, sensory evaluation, and total phenolic and total flavonoid contents of kefirs obtained from donkey milk (DM) and cow milk (CM) were compared. It was found that donkey milk kefir (DMK) and cow milk kefir (CMK) have different physicochemical properties and microbiological profile. DMK showed antibacterial activity against seven bacterial strain used in this study. The antioxidant activity was increased with fermentation in both kefir samples. The total phenolic content of DMK was higher than that of CMK whereas the total flavonoid content of CMK was higher than that of DMK. Sensory analysis showed that participants prefer CMK to DMK. It can be concluded that with its higher flavonoid content and antibacterial activity, DMK might be an alternative nutrient for consumers.

Key words: Kefir, donkey milk kefir, antibacterial activity, antioxidant capacity, total phenolic content, total flavonoid content

1. Introduction

Milk is an important element in human diet since its unique components which promote nutritional, immunological, and developmental requirements of young mammals [1]. As a result of the intolerance and allergic reactions due to consumption of cow's milk (CM) by people, there has been an emerging need for alternative milk sources like horse and donkey milk (DM) in recent years [2] and DM is defined as "pharmafood" which is highly preferred by the consumers [3]. Nowadays, the economic value of DM has been noticed not only for its nutritional value but also for its therapeutic and functional properties due to likeliness of its chemical composition to human milk, especially for infants who have cow's milk protein allergy [4]. In addition, DM has a specific protein composition as well as high polyunsaturated fatty acid, essential amino acid, and lactose content [5]. In addition to its unique composition, DM has antiageing, antioxidant, antimicrobial, antiinflammatory, and antiaggregant properties [6].

Although raw milk has been mostly preferred for consumption, fermented milk products have also had an upward trend due to their therapeutic effects and positive influence on health [7]. Kefir is a fermented milk product originating from the Caucasus Mountains of Russia, Tibet, or Mongolia and is composed of a unique blend of useful microorganisms [8]. It is shown that fermented milk products mostly contain lactic acid bacteria (LAB) such as *Acetobacter*, *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Lactococcus* spp., and yeasts such as *Saccharomyces*, *Torula*, *Kluyveromyces*, and *Candida* spp. [9]. All these microorganisms coexist in a water-soluble branched glucogalactan polysaccharide matrix called kefiran, which may strengthen consumers' immune system and increase the resistance against specific diseases such as neoplasia and infections [10]. In order to produce kefir, small clusters of microorganism mixtures placed in a specific polysaccharide matrix called kefir grains can be inoculated into milk and fermentation may occur in approximately 24 h [11]. Fermentation of milk is not only a traditional preservation method but also a practice used to improve the quality or the taste of dairy products [12]. The textural characteristics of fermentation process influence the consumers' acceptance of the product via sensory attributions and physicochemical properties [13]. For kefir fermentation, the composition of source material is the main determinant. Therefore, properties of kefir such as probiotic, prebiotic, antimicrobial, anticarcinogenic, antidiabetic, antiallergic, antitumor, and antioxidant

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activities are directly related to milk used for fermentation [14].

In this study, CM and DM were used as source material for fermentation of kefirs in order to analyse the physicochemical and microbial profile of both milk and kefir samples. Additionally, antibacterial activities of these fermented milk products were investigated on both well-defined bacterial strains and two clinical isolates. Antioxidant activities, total phenolic and total flavonoid contents of kefirs were also detected. This study compares physicochemical and sensory properties, microbial profiles, and bioactivities of donkey milk kefir with cow milk kefir for the first time.

2. Materials and methods

2.1. Content of milk and kefir grains

CM used in kefir production was obtained from Cattle Farm affiliated to Akdeniz University Faculty of Agriculture. DM was obtained from local Donkey Farm, Antalya, Turkey. Milk samples were collected between June 2019 and December 2019. Kefir grains were obtained from Akdeniz University Faculty of Engineering, Department of Food Engineering.

2.2. Kefir preparation

For kefir production, milk samples were pasteurized at 65 °C for 30 min and then cooled down to 25 °C. One-gram kefir grains were inoculated into 100 mL of cooled milk samples and stored at room temperature (RT) until pH of the product reached 4.6. The produced kefirs were then filtered via plastic sieve and the grains were collected.

2.3. Physicochemical analyses

Total dry matter (%) of milk samples used in kefir production and kefirs was measured with the gravimetric method described in TS 1018 Raw Milk Standard [15]. For investigation of fat content, Gerber method [16] and ash content gravimetric method were used [15]. The protein content of the samples was detected using the Kjeldahl method [17]. For titration acidity measurements, Soxhlet-Henkel method described in TS1018 Raw Milk Standard was used and the results were calculated in % lactic acid equivalent [15].

2.4. Microbiological profiles of the kefir samples

For microbiological analysis of the produced kefir samples, decimal serial dilutions were prepared with 1/4 strength Ringer solution in aseptic conditions. Yeast Extract Glucose Chloramphenicol Agar (YGC) (Merck, Germany) for yeast count, Plate Count Agar (PCA) (Merck, Germany) for total mesophilic aerobic bacteria count, De Man Rogosa Sharp Agar (MRS) (Merck, Germany) for *Lactobacillus* count, M17 Agar (Merck, Germany) for *Lactococcus* count were used. For *Leuconostoc* count, Mayeux, Sandline and Elliker (MSE) Agar (Biolife, Italia) were prepared. For coliform count, Violet Red Blue (VRB) Agar (Merck, Germany) was used. Colonies were counted after 4-day incubation and colony forming unit per millilitre (cfu/mL) was calculated according to Equation 1 and converted to log cfu/mL:

$$N = \frac{C}{[V(n_1+(0.1\times n_2))]} \times d \quad (1)$$

where N means total microorganism amount in 1 g or 1 mL of food sample, C indicates total colony amount from all counted petri dishes, V refers to volume transferred to counted petri dishes in mL, n_1 states the number of counted petri dishes from first dilution, n_2 denotes the number of counted petri dishes from second dilution, and d represents the most concentrated dilution ratio of two consecutive dilutions in which the count is made [18].

2.5. Antibacterial activity test and bacterial strains

Kefir samples were prepared for antibacterial activity test according to the previous study [19]. Filtered kefir samples were centrifuged at 3200 × *g* for 10 min. Supernatants were collected and sterilized using a 0.45-µm pore-size syringe filter (Sartorius Stedim, Germany).

Antibacterial activity of milk and kefir samples was determined with the disc diffusion method defined by National Committee for Clinical Laboratory Standards [20]. As controls, discs incubated with Ampicillin (Sigma Aldrich, USA) (25 µg/mL) and Kanamycin (Cayman Chemical Co., ABD) (50 µg/mL) were used.

Klebsiella pneumoniae ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* DSM 22648, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 35218, and clinical isolates *Proteus mirabilis* and *Listeria monocytogenes* were inoculated into nutrient agar (Merck KGaA, Germany) and incubated overnight at 37 °C. Next day, the cells were inoculated into sterile 0.85% NaCl (Sigma Aldrich, USA) and turbidity was adjusted to 0.5 McFarland. Two hundred microlitres of bacterial solution was inoculated into new agar plate. Discs were placed on top of inoculated agar plates and incubated at 37 °C for 24 h. After incubation, the zone diameters were measured. All antibacterial tests were performed in triplicate.

2.6. Antioxidant activity assay

Protocol described by von Gadow et al. (1997) was performed for the antioxidant activity assay based on the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH) [21]. Briefly, 100 µL of 1/100 diluted filter-sterilized kefir samples was mixed with 4 mL 6 × 10^{-5} M methanolic DPPH (Sigma Aldrich, USA) solution. After 30 min of incubation, the absorbance of mixtures was measured at 516 nm (SOIF, China) and expressed as ascorbic acid equivalents (AAE) in µM/mL.

2.7. Total phenolic content assay

Total phenolic content of the samples was determined using the Skerget et al. (2005) protocol [22]. FC reagent

was prepared according to Singleton and Rossi (1965) [23]. Five hundred microliters of filter-sterilized samples was mixed with 2.5 mL of FC reagent which was 10 times diluted with ddH_2O. After 2 min of incubation, 2 mL of 7.5% Na_2CO_3 (Merck, Germany) solution was added to the mixture and vortexed for 30 s and incubated at 50 °C in water bath for 5 min. The samples were then cooled down to room temperature (RT) and absorbance was measured at 760 nm. Total phenolic content of samples was calculated as gallic acid equivalent (GAE) in µL/mL.

2.8. Total flavonoid content assay

Aluminium chloride method described by Chang et al. (2006) was used for total flavonoid content measurement [24]. Five hundred microliters of filter-sterilized kefir sample, 2.5 mL of ddH_2O, and 150 µL 5% $NaNO_2$ (Merck, Germany) solution were mixed by vortex for 30 s and incubated at RT for 5 min. Then, 300 µL of 10% $AlCl_3$ (Merck, Germany) solution, 1 mL of 1 M NaOH (Sigma Aldrich, USA) solution, and 550 µL ddH_2O were added to the mixture and incubated for 5 min. Absorbance was measured at 510 nm. Total flavonoid content of the samples was expressed as quercetin equivalent (QE) in µL/mL.

2.9. Sensory analysis

In order to evaluate the sensory properties of fermented milk products, protocol previously described was used with slight modifications [8]. In this analysis, acidity, flavour, odour, viscosity, and overall assessment were evaluated by participants. Forty-five individuals between the ages of 18 and 45 were selected from the Akdeniz University campus for analysis. Analysis was designed as a blind test. Participants were not informed about the contents of the drinks and drinks were evaluated by them from 1 to 5 which are equal to very bad to very good and expressed degree of acceptability in 5-point hedonic scale.

2.10. Statistical analysis

All values were expressed as mean ± SD. All statistical analyses were evaluated with one-way ANOVA by using IBM SPSS 22 software [25], and Tukey's HSD and Tamhane's T2 tests were applied. Statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Physicochemical analyses

Total dry matter (%), total protein content, total fat content, titration acidity (% lactic acid equivalent), and total ash content measurements for both milk and kefir types were shown in Table 1.

It was found that while total fat content and titration acidity was increased via fermentation, total solid matter amounts decreased in all samples. Although the total protein amount of CMK was higher than that of CM, the total protein amount of DMK was lower than that of DM. It was also observed that ash content of CM was highest among all samples.

There are different studies showing that the physiochemical properties of CM and DM can be varied. In their study, El-Hatmi et al. (2015) reported that total dry matter, total fat, total protein, and ash amounts of CM were 8.87%, 2.15%, 2.59%, and 0.710%, respectively [26]. In another study, Guo et al. (2007) stated that total dry matter, total fat, total protein, and ash amounts of CM were 12.5–13.0%, 3.5–3.9%, 3.1–3.8%, and 0.7–0.8% for CM [5]. For CMK, the results of the aforementioned properties ranged as follows: 10.70–11.15%, 3.30–3.5%, 3.09–3.91%, 0.61–1.068%, and 0.64–0.81% [11,27,28]. Based on these, our CMK results of total dry matter, total protein content, and ash were consistent with the literature whereas total fat content and titratable acidity results were slightly higher. It was observed that the physicochemical property values of CMK were higher than those of CM except for lower ash content. On the other hand, there is no defined standard for physicochemical properties of DM. In their studies, Salimei et al. (2004) and Martini et al. (2014) determined the total dry matter content of DM

Table 1. Physicochemical analysis of CM, CMK, DM, and DMK.

	Cow		Donkey	
	Milk	Kefir	Milk	Kefir
Total dry matter (%)	11.45 ± 0.03^{a}	$10.92 \pm 0.05^{a,b}$	$8.93 \pm 0.01^{a,b,c}$	$8.32 \pm 0.004^{a,b,c}$
Total fat content (%)	3.66 ± 0.36^{d}	$4.25 \pm 0.35^{d,e}$	$0.45 \pm 0.07^{d,e}$	$0.55 \pm 0.07^{d,e}$
Total protein content (%)	3.66 ± 0.04^{f}	3.75 ± 0.03^{g}	$2.41 \pm 0.01^{f,g}$	$1.87 \pm 0.02^{f,g}$
Ash (%)	0.77 ± 0.04^{h}	0.64 ± 0.04^{i}	$0.36 \pm 0.05^{h,i}$	$0.37 \pm 0.02^{h,i}$
Titration acidity (% lactic acid)	0.3 ± 0.05^{k}	$0.9 \pm 0^{k,l}$	$0.17 \pm 0^{l,m}$	$0.8 \pm 0.01^{k,m}$

Data with the same lowercase letters in each row have significant difference ($P < 0.05$).

as 8.84 g/100 mL and 9.47 g/100 mL, respectively, which were slightly higher than the values in our study [29,30]. Guo et al. (2007) showed that the total fat content of DM varied from 0.3% to 1.8% and ash content from 0.3% to 0.5%, which are consistent with our results [5]. The level of protein in DM ranges from 1.5% to 2.0% [5]. With 2.41%, the protein content was higher in our study. This increase in the protein amount of the milk may be depending on the stage of the donkeys' lactation period and the effects of the farming system on the produced milk. It is known that in the early stages of lactation, protein level and fat level can be higher [31].

3.2. Microbiological profile of kefir

Total mesophilic aerobic bacteria, yeast, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Coliform* counts of kefirs made from CM and DM were shown in Table 2. According to our results, CMK has a higher number of microorganisms than DMK. No coliforms were detected in the kefir samples. In their study, Perna et al. (2019) states 10.39 ± 0.41, 9.72 ± 0.68, and 7.28 ± 0.35 log cfu/mL for *Lactobacilli*, *Lactococci* and yeast, respectively [32]. These are slightly lower cell counts than in our DMK samples.

3.3. Antibacterial activity

It was observed that DMK showed antibacterial activity against all bacterial samples except *P. aeruginosa* (Table 3). According to our results, *S. epidermidis*, S. aureus, *K. pneumonia*, *B. cereus*, and clinical isolate *L. monocytogenes* can be classified as susceptible to both kefirs. *P. aeruginosa* was found to be resistant to all kefirs in this study. Although *E. coli* and clinical isolate *P. mirabilis* were susceptible to DMK, they showed resistance to CMK. This could be an evidence for the importance of source material in fermentation. For both milks, the largest inhibition zones were observed in *S. epidermidis* plates. Interestingly, *S. aureus* was resistant to CM but it was susceptible to DM. The β-lactamase producer strain *E. coli* was susceptible to DMK whereas it was resistant to CM, CMK, and DM. Clinical isolate *P. mirabilis* was resistant to all samples except DMK and ampicillin. Clinical isolate *L. monocytogenes* showed resistance to all milk samples. It was susceptible to kefirs and antibiotics in this experiment.

Among other features, this can be related to the presence of lysozyme in DM [33]. Our results are consistent with the literature for *S. epidermidis*, S. aureus, *K. pneumonia*, and *B. cereus* [34,35]. Although Rodrigues et al. (2005) showed the susceptibility of *P. aeruginosa* to kefir, there was no zone formation in our study [9]. As the study of Aspri et al. (2018) demonstrated that fermented DM with different strains of *Enterococcus faecium*, *Lactobacillus casei* DM214, and *Leuconoctoc mesenteroides* DM236, separately, showed antibacterial activity only on *L. monocytogenes* 33413 and 1078 [4].

Table 2. Microbiological characteristics of kefir made from cow and donkey milk (log cfu/mL).

	Kefir	
	Cow	Donkey
Total mesophilic aerobic bacteria	8.12 ± 0.07[a]	7.87 ± 0.02[a]
Yeast	7.07 ± 0.01	6.99 ± 0.03
Lactobacillus	10.09 ± 0.08[b]	8.38 ± 0.08[b]
Lactoccoccus	10.03 ± 0.05[c]	8.13 ± 0.01[c]
Leukonostoc	nd*	7.28 ± 0.05
Coliform	0	0

*nd: Not determined.
Data with the same lowercase letters in each row have significant difference ($P < 0.05$).

3.4. Antioxidant activity

The free radical scavenging activity of CMK was found to be the highest (6818.75 µM/mL AAE) and DM (1318 µM/mL AAE) to be the lowest among all samples (Table 4). The antioxidant activity in DMK sample (5318.75 µM/mL AAE) was found to be lower than that of the CMK samples (6818.75 µM/mL AAE). Both kefir samples had high antioxidant activities and there was statistically significant difference between CMK and DMK samples ($P < 0.05$).

The results of antioxidant capacity test show that fermentation causes increase in the antioxidant capacity of milks. Interestingly, in some studies, the antioxidant capacity of DM was found higher than that of CM [36]. However, in their study, Simos et al. (2011) showed that antioxidant activity of CM is higher than DM, and Oner et al. (2011) found a similar antioxidant capacity between CM and DM [37,38]. In this study, it was observed that fermentation causes significant increase in the antioxidant activity of DM (1318 to 5318.75 µM/mL AAE).

3.5. Total phenolic content

It is known that phenolic compounds are preferred by consumers in regular diet due to their antioxidant capacities [39]. Total phenolic content of all samples are shown in µL/mL gallic acid equivalent (GAE) (Table 4). We found that total phenolic content of milk samples was higher than that of kefirs. Data indicates that CM has higher total phenolic content (3068.61 µL/mL GAE) and with fermentation, this content decreased to 785.54 µL/mL GAE. The total phenolic compound in DM was 1873.54 µL/mL GAE and in DMK it was measured as 1132.77 µL/mL GAE. There was statistically significant difference between CM and CMK; DM and DMK ($P < 0.05$). The decrease of phenolic content in kefir samples in our study is probably because of the metabolic activities of microorganisms in kefir grain

Table 3. Average zone diameter (mm) of test organisms for antibacterial effect of kefir made of cow and donkey milk.

Zone diameter (mm)*						
	CM	CMK	DM	DMK	Amp	Kan
SA	6 ± 0	8 ± 1.4	11.5 ± 2	12 ± 1	23.4 ± 2.1	22.9 ± 2.8
PA	6 ± 0	6 ± 0	6 ± 0	6 ± 0	17.6 ± 2	13.8 ± 1.6
SE	11.6 ± 1.9	15.3 ± 1.5	14.8 ± 2.8	17.3 ± 1.5	29 ± 2.8	22.8 ± 2.4
KP	6.8 ± 0.7	11.7 ± 1.1	11.5 ± 1.9	13.5 ± 0.7	22.3 ± 1	12.5 ± 1.6
BC	6 ± 0	14 ± 1.4	6 ± 0	17 ± 1.4	17.9 ± 1.2	12.4 ± 1.5
EC	6 ± 0	6 ± 0	6 ± 0	8 ± 1	8.7 ± 0.5	24.5 ± 2.2
LM	6 ± 0	7 ± 0	6 ± 0	9.5 ± 0.7	21.1 ± 1.2	22.4 ± 2.1
PM	6 ± 0	6 ± 0	6 ± 0	9.5 ± 0.7	20.3 ± 0.8	6 ± 0

* SA: *S. aureus*, PA: *P. aeruginosa*, SE: *S. epidermidis*, KP: *K. pneumonia*, BC: *B. cereus*, EC: *E. coli*, LM: *L. monocytogenes,* PM: *P. mirabilis*. CM: Cow milk; CMK: Cow milk kefir; DM: Donkey milk; DMK: Donkey milk kefir. Amp: Ampicillin, Kan: Kanamycin

Table 4. Antioxidant activity of kefirs (µM/ mL AEE), Total phenolic compounds (µg/mL GAE) and Total flavonoid content (µg/mL QE).

	Antioxidant Activity (µM/ mL AAE)	Total phenolic compounds (µg/mL GAE)	Total flavonoid content (µg/mL QE)
CM	2318.75 ± 187.5^{a}	$3068.61 \pm 25.38^{d,f}$	$6886.33 \pm 12.60^{h,k}$
CMK	$6818.75 \pm 277.17^{a,c}$	$785.54 \pm 3.93^{d,g}$	$808.33 \pm 39.18^{h,l}$
DM	1318 ± 344.22^{b}	$1873.54 \pm 21.60^{e,f}$	$1560.33 \pm 16.22^{j,k}$
DMK	$5318.75 \pm 580.72^{b,c}$	$1132.77 \pm 8.99^{e,g}$	$629.66 \pm 16.01^{j,l}$

Data with the same lowercase letters in each column have significant difference ($P < 0.05$).

[32,40]. It is known that kefir is not the only product in which amount of phenolic compound decreases with fermentation. Du and Myracle (2018) also demonstrated that aronia kefir has less phenolic compound and high antioxidant activity than its nonfermented control [41]. Another study reported that acids and microbial enzymes produced during fermentation cause decomposition of phenolic compounds in kombucha [42]. Although DM had lower total phenolic content than CM, DMK had higher total phenolic content than CMK. This could be evidence for the composition of milk used in fermentation as one of the determinants of kefir properties. Furthermore, our DMK results were consistent with the literature [32,43].

3.6. Total flavonoid content

Total flavonoid content of milks and kefirs were determined via aluminium chloride assay and expressed in µL/mL QE (Table 4). Similar to total phenolic content results, milk samples had higher total flavonoid content than kefirs. According to the results obtained, CM had the highest flavonoid content (6886.33 µL/mL QE) and DMK had the lowest (629.66 µL/mL QE). There was a decrease in total flavonoid content when milks were fermented with kefir grain. Especially for CM samples, fermentation caused a dramatic decrease in total flavonoid content. There was statistically significant difference between CM and CMK; DM and DMK; CMK and DMK samples ($P < 0.05$). It has been shown that flavonoids accumulate in the lipid and the water-soluble parts of the milk of grazing animals thus affecting the properties of the milk [44]. Moreover, Irkin et al. (2015) states that phenolic compounds including flavonoids, consumed by lactic acid bacteria which could explain the dramatic decrease of total flavonoid content in kefir samples with bacterial growth [39].

3.7. Sensory analysis

Results of the sensory analysis were shown in Figure. CMK (2,2) was found to be more acidic than DMK (2,3) by participants but there was no significant difference ($P > 0.05$). Participants preferred milks to kefir samples in terms

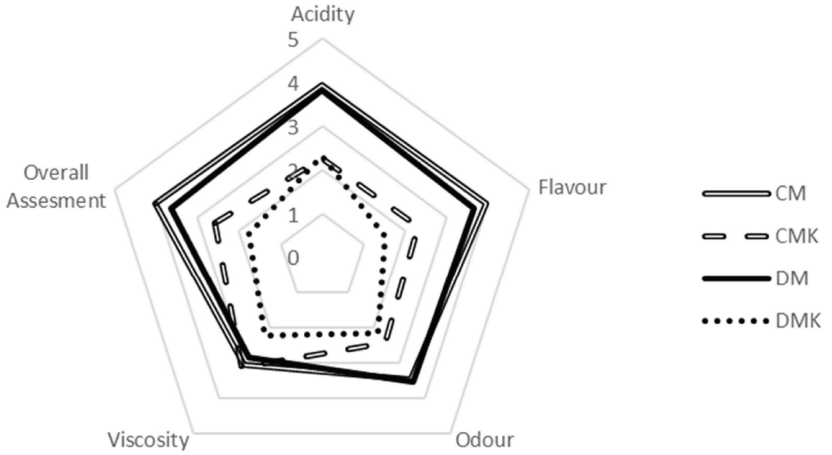

Figure. Five-point hedonic scale diagram demonstrating sensory evaluation of CM, CMK, DM, and DMK. Scores ranged from 1 to 5 with 1 indicating extreme dislike and 5 indicating extreme liking.

of flavour. CM (4,0) was found to be the most delicious beverage which is followed by DM (3,7), CMK (2,3), and DMK (1,5). It was found that DM had the best odour (3,6) among all samples and with fermentation the odour of the kefir samples was described as more obnoxious. As overall assessment participants found milks more delicious than kefirs. CM (4,0) was preferred more than DM (3,6) and CMK (2,6) was more preferred than DMK (1,8) among kefir samples. DMK was less preferred compared to CMK, but it might still be a good alternative to add to consumers' diet. According to Wszolek et al. (2001), kefir made from bovine milk was more preferred than ovine or caprine milk-based kefirs [45]. As Cais-Sokolińska et al. (2008) stated sensory characteristics of kefirs mainly based on milk and starter culture composition [46].

4. Conclusion

The aim of this study was to compare the physicochemical properties, microbiological profiles, and bioactivities of kefirs made from cow and donkey milk. Although the bioactivity of DMK fermented with single bacterium was examined before [4], in this study, the physicochemical activity, microbiological profile and bioactivity of DM fermented with a kefir grain were shown and was compared with CMK for the first time. To investigate the antibacterial activity of DMK, eight bacterial strains were used and only *P. aeruginosa* was resistant to DMK which was also resistant to CMK. Although total phenolic content and total flavonoid content decrease with the fermentation of milks via kefir grains according to our results, fermented milk products have high antioxidant activity and become a good alternative to enrich the diets of consumers. The antioxidant properties of the fermented product vary particularly depending on the content of the milk and the starting culture used. In conclusion, it can be said that DMK could be a good alternative fermented product to consume because of its high antibacterial and antioxidant activity.

Conflict of interest

The Authors declare that there is no conflict of interest.

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