Comparison of antioxidant capacity of cow and ewe milk kefir

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ABSTRACT

This research aimed to evaluate the effects of using either grain or commercial starter culture on the antioxidative capacity of cow and ewe milk kefir. The antioxidant capacity of kefir samples during fermentation and 21 d of storage was assessed by using 3 assays: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation decolorization; 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) radical scavenging activity assay; and Fe⁺³-reducing power (ferric reducing antioxidant power assay, FRAP). Vitamin E and β -carotene contents were also quantified. All kefir samples exhibited varying values for DPPH, ABTS, and FRAP assays depending on the starter culture and milk type. Vitamin E and β -carotene contents were similar in all kefir samples during storage. The results of this study suggest that milk type (cow or ewe) and culture type (kefir grains or commercial starter) were the significant parameters for the antioxidative activity of kefir.

Key words: antioxidant capacity, cow, ewe, milk, kefir

INTRODUCTION

Some chronic diseases including cancer, cardiovascular disease, and degenerative disease of aging have been related to the oxidative stress caused by excessive production of reactive oxygen species and oxygen-free radicals (Pizzino et al., 2017). Oxidative stress can also increase oxidation of membrane phospholipids, proteins, and DNA, as well as modification of low-density lipoproteins (López-Pedrera et al., 2016). Aerobic organisms are protected from oxidative stress by various enzymatic and nonenzymatic antioxidant defense systems. When the endogenous defense system cannot prevent the damage of reactive radicals, artificial antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole, and *tert*-butylhydroquinone, and natural antioxidants are used to impede the detrimental

effects of oxidative stress. Because artificial antioxidants have been reported to be toxic, carcinogenic, and unsafe, most consumers prefer natural antioxidants as they believe these are not chemicals and are not subjected to any legislative restrictions of use (Boskou and Elmadfa, 2010; Bartosz, 2013; Landete, 2013).

Milk is a unique food that contains valuable macro- and micronutrients for the growth and development of breastfed infants; it is also a basic source of energy and nutritional components for mammals. It has naturally occurring nonenzymatic antioxidants such as vitamins A, C, and E, carotenoids, uric acid, CLA, and enzymatic antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase. Some proteins in milk (e.g., casein, lactoferrin, α -LA, β -LG) and milk amino acids such as tyrosine, cysteine, tryptophan, and lysine are known to have antioxidant potential. Therefore, the antioxidant defense system of different animal milk and milk products can be considered to have health-beneficial effects against free radical damage (Lindmark-Månsson and Akesson, 2000; Kullisaar et al., 2003; Kitts, 2005; Zulueta et al., 2009).

Kefir has been manufactured and consumed since ancient times for its nutritional, taste, and flavor properties, as well as its potential health benefits. Consumption of kefir is increasing among consumers because of its profound antimicrobial, antibacterial, antitumor, antihypertensive, antioxidative, anticytotoxic, and hypocholesterolemic effects (de Oliveira Leite et al., 2013; Arslan, 2015; Chen et al., 2015; Prado et al., 2015). Kefir is obtained by the fermentation of milk with mixed microflora, including various species of lactic acid bacteria, acetic acid bacteria, and yeasts. The acid-alcoholic fermentation of milk with these microorganisms results in a refreshing and slightly acidic taste. Kefir is produced by either traditional or industrial methods. Traditional kefir production involves addition of kefir grains, whereas industrial kefir has been produced by commercial starter cultures of pure or mixed-strain cultures in powder form. Different animal milks such as cow, ewe, and goat, and plant-based milks obtained from soy, coconut, and rice can be used for production. The type of milk, type and quantity of starter culture, time and temperature-related conditions of fermenta-

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tion, and storage conditions all affect the microbiological, chemical, and sensorial characteristics of kefir (Gao and Li, 2016; Barukčić et al., 2017; Rosa et al., 2017).

In previous studies, milk and milk products (i.e., yogurt, cheese, whey) were shown to have antioxidant properties depending on the presence and activity of natural bioactive components (Taylor and Richardson, 1980; Korpela et al., 1995; Chen et al., 2003; Pulido et al., 2003; Smet et al., 2008; Gupta et al., 2009; Kesenkas et al., 2011; Perna et al., 2013; Shori and Baba, 2013; Chen et al., 2015; Sabokbar et al., 2015; Sah et al., 2015; Yilmaz-Ersan et al., 2015). Although ewe and cow milks are widely used in the manufacture of different fermented milk products, little information is available on the antioxidative characteristics of kefir produced with these milk types. This study had 2 main aims. First, we determined the contents of vitamin E, β -carotene, and total phenolics, as well as total antioxidant capacity of kefir during different fermentation stages. Second, we investigated the varied roles of milk and starter culture type on kefir characteristics.

MATERIALS AND METHODS

Kefir Grains and Inoculum Preparation

Two types of starter cultures were used for manufacturing: traditional kefir grains (obtained from Department of Food Engineering, University of Uludag, Bursa, Turkey) and freeze-dried, direct vat set (DVS) culture (obtained from Danisco, Poznan, Poland). The DVS culture contains *Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus diacetylactis*, *Candida kefir*, and *Saccharomyces unispora*. For reactivation, the kefir grains were transferred 3 times into pasteurized milk, incubated at 25°C for 18 h, separated from the milk, and washed with sterile distilled water. These active grains (5%, wt/vol) were used to inoculate the fermentation medium. Upon receipt, the DVS culture was added 2% (vol/vol) directly to the fermentation medium.

Kefir Production

Raw cow and ewe milks were obtained from the Uludag University Dairy Farm (Bursa, Turkey), heated to 90°C, and kept at that temperature for 10 min. Then, the milks were cooled to the inoculation temperature (25°C) and inoculated with active grains or DVS culture. After inoculation, milks were incubated at 25°C until the pH reached 4.60 to 4.70. From each kefir batch, samples were collected at 4-h intervals during fermentation. At the end of the fermentation, the kefir grains were separated by use of a sterilized plastic

sieve (2-mm pore size). After being transferred into 200-mL bottles, the kefir beverages were cooled to 4 to 6°C and stored at $4 \pm 1^\circ\text{C}$ until analysis. The samples were analyzed on d 1, 7, 14, and 21 of cold storage. Cow (C) and ewe (E) milk kefir made using kefir grains (GR) and starter culture (SC) are designated C-GR, C-SC, E-GR, and E-SC, respectively. Two replications of all batches and 3 replications of the analyses were performed. The pH was monitored using a digital pH meter (Analyzer model 315i/SET, WTW, Weilheim, Germany) throughout fermentation.

Chemicals

2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-2-triazine (TPTZ), Folin-Ciocalteu phenol reagent, potassium persulfate, gallic acid, tocopherol standards (α -tocopherol), HPLC-grade vitamin E and β -carotene were purchased from Sigma-Aldrich (St. Louis, MO); Trolox ([$\pm$]-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich Chemical Co. (Steinheim, Germany). All standard solutions were prepared in HPLC-grade methanol (Merck, Darmstadt, Germany).

Extraction of Kefir Samples

Kefir samples (2 g) were blended with 20 mL of aqueous methanolic solution (70:30%, vol/vol) at room temperature in the dark for 4 h with magnetic stirring. The extracts were centrifuged at $1,420 \times g$ for 10 min and filtered through qualitative filter paper (Whatman grade 2, 8- μm thickness); the supernatants were used for spectroscopic analysis.

Two grams of kefir was centrifuged and 5 mL of supernatant was saponified with 2 mL of an aqueous KOH (50%, vol/vol) at 65°C for 30 min in a water bath at 40 kHz. After cooling on ice for 15 min, 4 mL (2×2) of hexane and ethyl acetate solution (80:20%, vol/vol) was added to extract vitamin E. The organic fractions (3 mL) were evaporated to dryness. Finally, the residue was dissolved in 1 mL of hexane for HPLC-diode array detection (DAD) analysis of vitamin E. The kefir samples were extracted following the same method but without saponification for β -carotene analysis.

Determination of Antioxidant Capacity

ABTS Assay. Total antioxidant capacity of the samples was determined by using ABTS $^{\bullet+}$ radical cation decolorization assay (Sahin et al., 2012). The ABTS $^{\bullet+}$ radical stock solution was produced by re-

acting 20 mM ABTS^{•+} with 2.45 mM potassium persulfate solution (1:1, vol/vol) and storing in the dark at room temperature for 12 to 16 h before use. The ABTS^{•+} working solution was prepared by dilution of stock solution with ethanol at a ratio of 1:10 to obtain an absorbance of 0.7 ± 0.02 at 734 nm. The ABTS^{•+} decolorization assay procedure for kefir samples was as follows: 0.25 mL of kefir extract, 3.75 mL of ethanol, and 1 mL of the ABTS^{•+} radical cation solution were mixed; the mixture was allowed to stand for 3 min at room temperature; and the absorbance at 734 nm was recorded by using a spectrophotometer (Varian Cary UV-Vis Spectrophotometer; Varian Inc., Palo Alto, CA) against a reagent blank. A standard curve was prepared using different concentrations of Trolox, and the results were expressed as milligrams of Trolox equivalents (TE) per 100 mL of sample.

DPPH Assay. The DPPH decolorization assay was executed as described by Sahin et al. (2012). First, 0.25 mL of extract and 0.18 mL of DPPH (1×10^{-3} M stock solution) were mixed, and methanol was added to bring the final volume to 3 mL (6×10^{-5} M) in a test tube. The absorbance readings were taken using a spectrophotometer (Varian Cary UV-Vis Spectrophotometer, Varian Inc.) at 515 nm against a reagent blank after being kept in dark for 30 min. Different concentrations of Trolox were used as standards for calibration, and the results were expressed as milligrams of TE per 100 mL of sample.

Ferric Reducing Antioxidant Power Assay. The ferric reducing antioxidant power (FRAP) assay was performed using the method of Benzie and Strain (1996) using a UV-Vis spectrophotometer (Varian Cary 50) with slight modifications. The method, using iron as a redox sensor, is based on the reduction of the Fe³⁺-TPTZ (ferri-tripyridyl-triazine) complex to the Fe²⁺-TPTZ at low pH in the presence of antioxidants. This reduction results in a blue color, which can be monitored by measuring the absorption change at 593 nm. The working FRAP reagent was prepared by mixing 10 volumes of 1.0 M acetate buffer, pH 3.6, with 1 volume of 10 mM TPTZ in 40 mM HCl and with 1 volume of 20 mM FeCl₃. In a test tube, 0.25 mL of sample was mixed with 2.75 mL of the FRAP reagent and incubated at 37°C for 30 min. The solution was then centrifuged at $1,050 \times g$ for 10 min. The supernatant (0.5 mL) was mixed with 0.5 mL of distilled water and 0.1 mL of 0.1% (wt/vol) FeCl₃. Absorbance was measured after 8 min. A standard curve was prepared using different concentrations of FeSO₄·7H₂O (100–1,000 μM). The antioxidant efficiency of the sample solution was calculated with reference to the standard curve given by a Fe²⁺ solution of known concentration. The results were expressed as milligrams of TE per 100 mL of sample.

Determination of Total Phenolic Content

The total phenolic content (TPC) was determined by Folin–Ciocalteu method as described by Sahin et al. (2013). The absorbance was measured using a spectrophotometer (Varian Cary UV-Vis spectrophotometer) at 750 nm against a reagent blank after 30 min at room temperature. A standard calibration curve was plotted using different concentrations of gallic acid and TPC was expressed in milligrams of gallic acid equivalents (GAE) per 100 mL of sample.

Determination of Vitamin E and β-Carotene Contents

Vitamin E and β-carotene contents in kefir samples were determined by HPLC-diode array detection (Agilent 1200 HPLC System, Agilent Technologies, Waldbronn, Germany) analysis. The HPLC system consisted of a vacuum degasser, binary pump, autosampler, and YMC Carotenoid C30 column (4.6×250 mm internal diameter, 5-μm film thickness) column. The mobile phase was 95% methanol and 0.05% triethylamine in water (solvent A) and methyl *tert*-butyl ether (solvent B). The gradient profile of the mobile phase was as follows: 0–5 min 5% B, 15–20 min 20% B, 20–30 min 30% B, 30–40 min 40% B, 40–75 min 75% B, 40–45 min 5% B; the total run time was 45 min. The column was equilibrated for 10 min before each analysis at 25°C. The flow rate was 1.0 mL/min and injection volume was 20 μL. The monitoring wavelength was 225 nm for vitamin E and 450 nm for β-carotene. The peaks were identified and quantified based on comparison of retention times of standard solutions.

Statistical Analysis

Statistical analyses was performed with Minitab 13.0 for Windows (Minitab Inc., State College, PA), and the data were compared using ANOVA at 1 and 5% significance levels. The results are presented as means $\pm$ standard errors. Differences between means of the kefir samples were controlled using the least significant difference multiple range test, as the level of significant difference was used to determine the effect of fermentation and storage time on the antioxidant properties. Hierarchical cluster analysis, which gives intuitive similarity relationships between one sample and the entire data set, was used to discriminate kefir samples with different culture and milk types and the antioxidant properties based on their similarities with respect to predetermined selection criterion. Two-way clustering and Ward's linkage method were performed for clustering (JMP Software Version 12, SAS Institute Inc., Cary, NC).

Table 1. Total antioxidant capacity (TAC) and total phenolic content (TPC) of raw and heat-treated cow and ewe milks

Item	Ewe milk		Cow milk	
	Raw	Heat-treated	Raw	Heat-treated
TAC method ¹				
DPPH	8.70	5.03	3.14	2.66
ABTS	33.18	24.13	21.48	48.60
FRAP	5.82	6.43	1.41	6.15
TPC ²	121.63	115.82	68.91	152.14

¹DPPH = 2,2-diphenyl-1-picrylhydrazyl assay; ABTS = 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay; FRAP = ferric reducing antioxidant power assay; results were expressed as milligrams of Trolox equivalents (TE) per 100 mL of sample.

²Expressed in milligrams of gallic acid equivalents (GAE) per 100 mL.

RESULTS AND DISCUSSION

Acidification Kinetics

The manner of pH decline is an important parameter to evaluate the fermentation capacity of starter microorganisms used in dairy products. The activity and growth rate of starter cultures are known to vary with lactose and protein contents of milk, fermentation conditions, and the type of milk (Rogelj and Perko, 1998). Figure 1 presents the variation in pH values of kefir samples during fermentation as well as the average acidification time needed to reach a pH of 4.60 to 4.70. We detected significant differences in pH values among the samples depending on the culture and milk type used ($P < 0.01$). At the beginning of the acidification process, the pH values of kefirs were similar, ranging from 6.46 to 6.20, and then decreased gradually throughout the acidification period to reach the target pH. The final pH was higher in kefir made using kefir grains, independent of milk type, indicating that the commercial starter culture was more effective in decreasing the pH to the target. The final pH of all kefirs averaged 4.6, illustrating the effective process control obtained during acidification. Time to reach the desired pH was 4 h for C-SC, whereas it was almost 24 h for E-SC. In the samples inoculated with grains, pH decreased to 4.74 after 12 h of the fermentation for E-GR and to 4.57 during 24 h of fermentation for C-GR. The acidification time observed in the present study was similar to that reported by Wszolek et al. (2001), who investigated the properties of kefir made from cow, goat, and ewe milks with different starter cultures. In the present study, variations in pH of the kefir samples were due to the different buffering capacities of the milks and differential microbial populations during fermentation. The largest decrease in pH was observed for kefir samples made with grains because they have higher metabolic activity than commercial starter culture.

Antioxidant Capacity of Milk Used for Kefir Manufacture

The efficiency of antioxidants in biological systems may differ, thus, various methods based on different principles should be used to evaluate TAC. There is increasing interest in measuring total antioxidant activity rather than simply the contents of different antioxidants. The total antioxidant capacity (TAC) of raw cow and ewe milks used for kefir manufacture was evaluated by 3 methods in the current study: DPPH radical scavenging, ABTS radical scavenging, and ferric reducing antioxidant potential. The antioxidant capacity of raw and heated milks showed significant differences in relation to the method of estimation, as shown in Table 1.

Phenolic compounds are present both in soluble form and combined with proteins in milk. The temperature and duration of heat treatment, pH, protein type and concentration, and the type and structure of phenolic compounds may cause reversible or permanent breakdown of the protein-polyphenolic bonds (O'Connell and Fox, 2001; Rashidinejad et al., 2015). In the current study, milks were heated to 90°C for pasteurization before their antioxidant capacity was tested. The heat-

Figure 1. Changes of pH of kefir samples during fermentation. C-GR = cow milk kefir made using kefir grains; C-SC = cow milk kefir made using starter culture; E-GR = ewe milk kefir made using kefir grains; E-SC = ewe milk kefir made using starter culture.

ing process was effective on TAC and TPC in both cow and ewe milk (Table 1).

The DPPH, ABTS, and TPC values were lower for heat-treated ewe milk than for raw milk, whereas the FRAP value was higher. The TPC values of ewe milk decreased during heat treatment but increased for cow milk, indicating the instability of phenolics to heat, depending on the differences of biologically active components present in both milk species. Although heat treatment decreased the DPPH value in cow milk, it resulted in higher ABTS, FRAP, and TPC values. Calligaris et al. (2004) stated that the reducing properties of milk were strongly affected by heating; heat treatment could increase its pro-oxidant activity, probably as a consequence of both the loss of natural antioxidants and the formation of novel oxidative molecules in the early stages of the Maillard reaction. However, only the application of severe heat treatments, associated with the formation of brown melanoidins, allowed a recovery, and even a possible increase in antioxidant properties. In addition to their nutritive ingredients, dairy products such as milk, yogurt, cheese, and kefir contain antioxidative factors (Songisepp et al., 2004; Liu et al., 2005; Aloglu and Oner, 2011). Milk proteins, such as caseins, β -LG, α -LA, immunoglobulins, lactoferrin, and serum albumin, can exert their antioxidant activity directly or after hydrolysis to different peptides by proteolytic enzymes (Lindmark-Månsson and Akesson, 2000; Virtanen et al., 2007).

The ABTS assay gave higher values than the DPPH and FRAP methods for ewe milk, because some milk components such as cysteine, glutathione, and protein sulfhydryls, depending on the AA sequence, can scavenge ABTS^{•+} radicals (Cao and Prior, 1998; Suetsuna et al., 2000; Rival et al., 2001), in agreement with Chen et al. (2003).

Milk antioxidant components should be considered as lipophilic and hydrophilic. Vitamin E and carotenoids contribute to antioxidant activity in the lipophilic phase, whereas ascorbic acid contributes in the hydrophilic phase (Pulido et al., 2003). Therefore, differences in antioxidative capacity between cow and ewe milks could be explained by their relative contents of fat, protein, vitamin, minerals, phenolic compounds, antioxidative enzymes, and fatty acid and AA compositions. The TPC values of ewe milk decreased during heat treatment but those of cow milk increased (Table 1). In many studies, the effects of heat treatment on TPC in foods are contradictory. Some researchers reported an increase in the phenolic content, whereas others observed a decrease. Ertan et al. (2017) stated that heat treatment conditions and sample matrix could be responsible for these contradictory results.

Antioxidant Capacity of Kefir During Fermentation

The TPC and antioxidative capacity values of kefir samples showed significant variations during fermentation, probably due to inhibition of enzymes of the microorganisms present in kefir grains and commercial starter, which were activated at the initial stages of fermentation (Figures 2 and 3). The TPC data were comparable to previous findings that reported values between 74.67 and 148.84 mg of GAE/100 mL for ewe kefir and between 66.70 and 176.87 mg of GAE/100 mL for cow kefir. The vitamin E and β -carotene contents of the kefir samples during fermentation showed no detectable significant differences, and therefore, vitamin E and B-carotene values were not considered for TAC.

We detected significant differences by kefir type and fermentation time for TAC values ($P < 0.01$). The values for DPPH throughout fermentation in E-GR and E-CS were 1.68 and 9.05 mg of TE/100 mL, respectively, whereas DPPH values in C-GR and C-CS were 3.99 and 9.57 mg of TE/100 mL. The average ABTS scavenging capacities in E-GR and E-CS were 11.34 and 32.93 mg of TE/100 mL and in C-GR and C-CS were 10.25 and 22.44 mg of TE/100 mL, respectively. The average FRAP values of ewe milk kefir by grains and commercial starter culture were 5.09 and 15.27 mg of TE/100 mL, whereas for cow milk kefir they were 145 and 12.34 mg of TE/100 mL, respectively (Figure 3a-d).

After the addition of kefir grains to the milks at 0 h of fermentation, ABTS values were highest of all TAC assays. At the end of fermentation, DPPH and ABTS values of E-GR kefir samples had decreased significantly ($P < 0.01$), whereas FRAP values had increased ($P < 0.01$). As shown in Figure 3a, the ABTS assay for E-GR sample had greater values than other TAC methods. The DPPH assay is a reproducible and simple method in which the reduction in the concentration of this stable radical is measured by decolorization with an antioxidant component present in milk such as AA including cysteine, tocopherol, and glutathione peroxidase. This decolorization is stoichiometric with respect to the number of electrons captured (Nishino et al., 2000). This assay was used by Sabokbar et al. (2015), who stated that fermentation with kefir grains had a positive influence on scavenging activity of DPPH radical.

The ABTS and FRAP values of E-SC kefir were highest after 8 h of fermentation (Figure 3b). The variations in antioxidative capacity of kefir samples during fermentation might be the result of differences of microorganisms and their activity in grains and commercial starter culture. Because kefir has high protein content,

Figure 2. Changes of total phenolic content [TPC; in mg of gallic acid equivalents (GAE) per 100 mL] of kefir samples during fermentation. C-GR = cow milk kefir made using kefir grains; C-SC = cow milk kefir made using starter culture; E-GR = ewe milk kefir made using kefir grains; E-SC = ewe milk kefir made using starter culture.

the free AA and oligopeptides formed by microbial proteases during fermentation could also account for antioxidant activity. The synergistic effect of phenolics with other components present could contribute to the overall observed antioxidant capacity. Therefore, the fermentation period is critical to ensure optimal breaking down of compound linkages but not permit free compounds to be used as substrates for microbial growth (Ehsan et al., 2010).

The antioxidative capacity values of C-GR and C-SC samples are shown in Figure 3c and 3d. We detected significant differences ($P < 0.01$) in DPPH, ABTS, and FRAP values throughout fermentation. The ABTS and FRAP values in C-GR were highest at 12 h of the fermentation. This might be attributed to the population and metabolites formed by the activity of complex microorganisms present in the grains and bacterial culture. The FRAP values increased progressively during the first 8 h of fermentation for E-GR, E-SC, and C-GR samples in contrast to the decrease in pH, which allowed us to assess the color change by reduction of ferric ion to ferrous at low pH. The FRAP values of E-GR were highest at 24 h of fermentation. Bioactive peptides in milk, the lactic acid bacteria present, and their Fe^{2+} chelating ability might contribute to the high reducing activity of the E-GR sample. Lin and Yen (1999) demonstrated that *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium longum* had chelating ability due to intracellular chelators such as penicillamine. These chelators could capture metal ions that catalyze oxidation reactions.

Kefir grains and commercial culture consist of different species of lactic acid bacteria, acetic acid bacteria, yeasts, and filamentous molds in varying numbers. Because the phenomenon of associations of microbial populations in the grains has not been completely elucidated, commercial cultures containing various lactic acid bacteria or yeast species isolated from kefir grains are used to standardize kefir production. However, kefir manufactured with a pure culture is differentiated from that manufactured with kefir grains by sensorial and chemical attributes, depending on the activity of microorganisms present, as some species are absent in commercial cultures (Wszolek et al., 2001; de Oliveira Leite et al., 2013; Arslan, 2015; Prado et al., 2015).

The instability of antioxidant activity observed in kefir samples might be related to various factors such as the type of milk, chemical composition, processing conditions, starter culture type/amount, microbial population of the grains, types of enzymes in starter culture, hydrolysis of proteins, polarity, and ability to donate atoms and electrons of antioxidant biofactors (Sun et al., 2009; Perna et al., 2013). Some natural bioactive components in kefir exhibit a relatively slow scavenging potential, because large peptides and proteins hydrolyze slowly, and thus display a lower antioxidant activity. Furthermore, low-molecular-weight casein hydrolysates could have strong radical scavenging activities due to differences in characteristics of the starter culture used (Kim et al., 2007; Sah et al., 2015). Virtanen et al. (2007) noted that antioxidant activity in fermented products could be attributed to the development of proteolysis.

Antioxidant Capacity of Kefir During Storage

It is known that kefir has antioxidant components such as proteins, phenolics, vitamins; however, to our knowledge, there is no comparative study on antioxidant properties of kefir made from cow and ewe milk with kefir grains and commercial starter culture. The

TAC results for kefir samples as a function of storage time are presented in Table 2. The differences between the results of all TAC methods for all kefir samples were statistically significant ($P < 0.01$). The highest antioxidant capacity values were observed using the ABTS assay and lowest for the DPPH assay. In terms of antioxidative capacities, kefir made from ewe milk had

Figure 3. Changes of antioxidative capacity of ewe milk kefir made using kefir grains (E-GR; a), ewe milk kefir made using starter culture (E-SC; b), cow milk kefir made using kefir grains (C-GR; c), and cow milk kefir made using starter culture (C-SC; d) during fermentation. Antioxidant capacity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH, black bars), 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, gray bars), and ferric reducing antioxidant power (FRAP, white bars) assays. Different lowercase letters (a–c) indicate significant differences between assays ($P < 0.01$); different uppercase letters (A–E) indicate significant differences between means for fermentation time ($P < 0.01$).

higher levels than kefir made from cow milk, and kefir samples fermented by grain gave higher antioxidant capacity for DPPH, and the highest ABTS results were obtained in kefir samples fermented by starter culture at the end of storage. In general, differences in antioxidant capacity observed among kefir samples with grains and pure culture could be due to the specific AA sequence of the milk proteins or the lipid contents of milks, which affects the reactivity of lipid-soluble antioxidants and fat globule membrane proteins (Chen et al., 2003). More likely, the TAC values of kefir samples represent a complex mixture of numerous compounds with antioxidant activities functioning via different chemical reactions.

The DPPH values increased notably in ewe kefir samples during refrigerated storage ($P < 0.01$). This might be explained by proteolysis and increased organic acid content as a result of post-acidification during storage (Lourens-Hattingh and Viljoen, 2001; Correia et al.,

2004). Maximum levels of the DPPH values were observed on d 7 of storage for cow kefir samples. Extended storage to 21 d resulted in a decrease in DPPH value relative to that at d 7. Shori and Baba (2013) reported that DPPH scavenging activity was highest on d 7 and 14 for plain yogurt, after which values decreased until d 28, to levels below that on d 1 of storage. Similarly, Halah and Mehanna (2011) reported that DPPH values of milk fermented by *Lactobacillus rhamnosus* declined after 7 d of storage.

A significant increase in ABTS value was observed until d 14 of storage for E-GR, E-SC, and C-SC samples, which decreased thereafter until d 21. Among the samples, E-SC exhibited the highest ABTS⁺ inhibition rate (Table 2). Our results were in agreement with those reported by Perna et al. (2013), who observed a significant increase in ABTS until d 15 in yogurt made from cow milk. Gupta et al. (2009) reported that ABTS and DPPH values for Cheddar cheese manufactured

Table 2. Changes in antioxidant capacity, total phenolic content, and contents of vitamin E and β -carotene in kefir samples during storage (means $\pm$ SE)

Item ¹	Storage time (d)	Kefir sample ²			
		E-GR	C-GR	E-SC	C-SC
DPPH (mg of TE/100 mL)	1	3.03 $\pm$ 1.055 ^{c,C}	6.25 $\pm$ 0.010 ^{a,B}	2.10 $\pm$ 0.004 ^{d,C}	5.97 $\pm$ 0.016 ^{b,B}
	7	2.15 $\pm$ 0.018 ^{c,D}	6.76 $\pm$ 0.071 ^{a,A}	1.86 $\pm$ 0.006 ^{d,D}	6.55 $\pm$ 0.019 ^{b,A}
	14	3.76 $\pm$ 0.034 ^{b,B}	5.59 $\pm$ 0.003 ^{a,C}	2.34 $\pm$ 0.051 ^{d,B}	2.79 $\pm$ 0.006 ^{c,D}
	21	4.84 $\pm$ 0.000 ^{b,A}	5.41 $\pm$ 0.141 ^{a,C}	4.19 $\pm$ 0.008 ^{c,A}	3.40 $\pm$ 0.141 ^{d,C}
ABTS (mg of TE/100 mL)	1	12.79 $\pm$ 0.008 ^{b,C}	11.05 $\pm$ 0.066 ^{d,C}	13.62 $\pm$ 0.004 ^{a,C}	12.38 $\pm$ 0.006 ^{c,D}
	7	12.05 $\pm$ 0.069 ^{c,D}	10.19 $\pm$ 0.000 ^{d,D}	12.66 $\pm$ 0.007 ^{b,D}	13.85 $\pm$ 0.063 ^{a,C}
	14	13.98 $\pm$ 0.006 ^{c,A}	13.40 $\pm$ 0.001 ^{d,B}	16.42 $\pm$ 0.034 ^{a,A}	16.03 $\pm$ 0.037 ^{b,A}
	21	13.68 $\pm$ 0.020 ^{c,B}	14.08 $\pm$ 0.022 ^{b,A}	15.13 $\pm$ 0.048 ^{a,B}	15.05 $\pm$ 0.028 ^{a,B}
FRAP (mg of TE/100 mL)	1	11.68 $\pm$ 0.007 ^{a,A}	7.19 $\pm$ 0.141 ^{c,A}	5.05 $\pm$ 0.014 ^{d,B}	8.47 $\pm$ 0.240 ^{b,A}
	7	7.10 $\pm$ 0.141 ^{a,D}	7.16 $\pm$ 0.156 ^{a,A}	5.14 $\pm$ 0.006 ^{b,B}	6.81 $\pm$ 0.141 ^{a,B}
	14	8.41 $\pm$ 0.007 ^{a,C}	6.68 $\pm$ 0.007 ^{c,B}	7.53 $\pm$ 0.240 ^{b,A}	6.86 $\pm$ 0.141 ^{c,B}
	21	9.97 $\pm$ 0.000 ^{a,B}	6.40 $\pm$ 0.014 ^{c,B}	8.10 $\pm$ 0.141 ^{b,A}	6.04 $\pm$ 0.062 ^{c,C}
TPC (mg of GAE/100 mL)	1	77.74 $\pm$ 0.141 ^{b,C}	67.41 $\pm$ 0.141 ^{c,C}	80.89 $\pm$ 0.014 ^{a,B}	64.18 $\pm$ 0.255 ^{d,C}
	7	80.88 $\pm$ 0.010 ^{a,A}	66.81 $\pm$ 0.007 ^{b,D}	81.37 $\pm$ 0.522 ^{a,B}	59.09 $\pm$ 0.123 ^{c,D}
	14	79.84 $\pm$ 0.058 ^{b,B}	71.84 $\pm$ 0.054 ^{d,B}	84.79 $\pm$ 0.011 ^{a,A}	78.50 $\pm$ 0.004 ^{c,B}
	21	81.18 $\pm$ 0.013 ^{c,A}	73.65 $\pm$ 0.072 ^{d,A}	83.63 $\pm$ 0.044 ^{b,A}	85.69 $\pm$ 0.141 ^{a,A}
Vitamin E (mg/100 g)	1	0.037 $\pm$ 0.0001 ^{b,D}	0.025 $\pm$ 0.0002 ^{c,A}	0.045 $\pm$ 0.0000 ^{a,C}	0.018 $\pm$ 0.0001 ^{d,C}
	7	0.051 $\pm$ 0.0009 ^{b,B}	0.014 $\pm$ 0.0000 ^{c,C}	0.094 $\pm$ 0.0004 ^{a,B}	0.015 $\pm$ 0.0003 ^{c,D}
	14	0.084 $\pm$ 0.0002 ^{b,A}	0.008 $\pm$ 0.0001 ^{c,D}	0.121 $\pm$ 0.0001 ^{a,A}	0.084 $\pm$ 0.0000 ^{b,A}
	21	0.040 $\pm$ 0.0001 ^{a,C}	0.017 $\pm$ 0.0002 ^{d,B}	0.032 $\pm$ 0.0000 ^{b,D}	0.022 $\pm$ 0.0002 ^{c,B}
β -Carotene (mg/100 g)	1	0.004 $\pm$ 0.0000 ^{b,A}	0.005 $\pm$ 0.0005 ^{a,A}	0.003 $\pm$ 0.0004 ^{c,B}	0.002 $\pm$ 0.0000 ^{d,A}
	7	0.002 $\pm$ 0.0001 ^{b,C}	0.004 $\pm$ 0.0002 ^{a,A}	0.004 $\pm$ 0.0000 ^{b,A}	0.002 $\pm$ 0.0004 ^{b,A}
	14	0.003 $\pm$ 0.0001 ^{c,B}	0.003 $\pm$ 0.0000 ^{b,B}	0.004 $\pm$ 0.0001 ^{a,A}	0.002 $\pm$ 0.0001 ^{d,A}
	21	0.003 $\pm$ 0.0000 ^{b,B}	0.003 $\pm$ 0.0001 ^{b,B}	0.004 $\pm$ 0.0002 ^{a,A}	0.002 $\pm$ 0.0002 ^{c,A}

^{a-d}Different lowercase superscripts in the same row indicate significant differences between means for kefir samples ($P < 0.01$).

^{A-D}Different uppercase superscripts in the same column indicate significant differences between means for storage time ($P < 0.01$).

¹DPPH = 2,2-diphenyl-1-picrylhydrazyl [in mg of Trolox equivalents (TE) per 100 mL]; ABTS = 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP = ferric reducing antioxidant power assay; TPC = total phenolic content [in mg of gallic acid equivalents (GAE) per 100 mL].

²E-GR = ewe milk kefir made using kefir grains; C-GR = cow milk kefir made using kefir grains; E-SC = ewe milk kefir made using starter culture; C-SC = cow milk kefir made using starter culture.

with *Lactobacillus casei* ssp. *casei* 300 and *Lactobacillus paracasei* ssp. *paracasei* 22 increased during the first 4 mo of ripening and decreased significantly thereafter.

We detected significant changes in antioxidant activity evaluated by FRAP assay during storage. The highest FRAP values were determined for E-GR, C-GR, and C-SC at the beginning of storage, whereas an increase in FRAP during storage was recorded in the E-SC sample. The results were in agreement with the study of Najgebauer-Lejko et al. (2014).

After 21 d of storage, the highest antioxidative capacity was recorded in C-GR samples for DPPH, in E-SC samples for ABTS, and in E-GR samples for FRAP (Table 2). The assays used in the present study are relatively simple, rapid, widely used, and applicable to both hydrophilic and lipophilic antioxidant systems. However, they are based on different compounds having differing antioxidant activities. In addition, differences in antioxidative capacity of kefir samples might be related partly to their proteolysis type, the resultant AA composition, and their vitamin and fat contents, which influence the solubilization and concentration of carotenoids.

Mean TPC increased significantly ($P < 0.01$, Table 2) throughout storage. The TPC in cow and ewe kefir samples ranged between 59.09 and 85.69 mg of GAE/100 mL and 77.74 and 84.79 mg of GAE/100 mL, respectively (Table 2). The addition of commercial starter culture to kefir milk resulted in slightly higher TPC values than when using kefir grains. On d 7, TPC values for both C-SC and C-GR declined but then increased thereafter until d 21. These variations in TPC for C-SC samples might be explained by further breakdown of phenolic compounds by enzymatic activity of starter culture during storage (Dalling, 1986). In the present study, ewe kefir samples had higher TPC than

cow samples. Similar to cow kefir samples, TPC values of E-SC were higher than those of E-GR during storage. A possible explanation for higher TPC is related to metabolic activities of commercial starter culture, which can hydrolyze complex phenolic compounds into free phenolics (Sabokbar et al., 2015). Further studies are required to characterize the polyphenolic content in ewe milk, which may be responsible for the elevated TPC.

Table 2 shows the contents of vitamin E and β -carotene, which, as fat-soluble antioxidants, may contribute to dietary antioxidant intake from milk. The vitamin E contents of kefir samples ranged from 0.008 to 0.121 mg/100 g, whereas β -carotene contents ranged from 0.002 to 0.005 mg/100 g. In general, a significantly higher content of vitamin E was found in ewe milk kefirs than in cow milk kefirs ($P < 0.01$). No significant variations were found for β -carotene in all kefir samples throughout storage.

Cluster Analysis

Cluster analysis was performed using the hierarchical clustering method with Ward's linkage and 2-way clustering based on similar antioxidant capacities (evaluated by measuring scavenging capacity for DPPH, ABTS, and FRAP), TPC, and vitamin E and β -carotene contents on the first day of storage at 4°C (Figure 4). Four kefir samples were grouped into 3 clusters based on the mean values generated from the unweighted pair group mean average method of analysis, and all analysis parameters were grouped into 2 clusters. The results showed that C-GR samples were very similar to both E-GR and E-SC on the measured variables compared with the C-SC kefir. However, 2 clusters were observed in the case of antioxidant properties. The DPPH and

Figure 4. Cluster analysis of kefir samples. C-GR = cow milk kefir made using kefir grains; C-SC = cow milk kefir made using starter culture; E-GR = ewe milk kefir made using kefir grains; E-SC = ewe milk kefir made using starter culture. The box indicates the distance of DPPH, ABTS, and FRAP from each other. DPPH = 2,2-diphenyl-1-picrylhydrazyl assay; FRAP = ferric reducing antioxidant power assay; ABTS = 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay; VitE = vitamin E; TPC = total phenolic content.

β -carotene clearly formed a distinct group and the others (FRAP, ABTS, vitamin E, and TPC) more closely connected to each other.

CONCLUSIONS

The current work was conducted to evaluate the effect of using grains versus commercial culture on the antioxidant capacity of kefir from cow and ewe milks. Culture type (grains or commercial starter) had a direct influence on the antioxidant capacity of kefir samples during fermentation and storage. Kefir made from ewe milk had higher antioxidative levels than that made from cow milk, and kefir samples fermented by grains gave higher antioxidant capacity for DPPH and the highest ABTS results were obtained in kefir samples fermented by starter culture at the end of storage. These findings require further investigations to purify and identify each potential antioxidant component in kefir (i.e., peptides, lipids, phenolic compounds, and enzymes). In addition, in vivo studies are necessary to evaluate possible beneficial health effects of kefir, such as protection from free radical damage.

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