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Antioxidant Properties of Kefir Produced from Different Cow and Soy Milk Mixtures

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ABSTRACT

The aim of this study is to determine the antioxidant properties of kefir samples produced from different cow/soy milk mixtures. Antioxidative activities such as the inhibition of ascorbate autoxidation, reducing activity, the scavenging effect of superoxide anion radicals and hydrogen peroxide of kefir samples were determined. Kefirs produced from whole soymilk had the highest inhibition rate of ascorbate autoxidation. Reducing activities of kefir samples, expressed as equivalent amounts of cysteine, were found statistically different and elevated by increased soymilk ratio. Results of the inhibition of superoxide radical generation of cow, cow/soy and soymilk kefir samples were found statistically different. However, the effect of fermentation on this activity neither with kefir grain nor culture was significant. Results indicated that none of kefir samples exhibit a hydrogen peroxide scavenging activity.

Keywords: Antioxidant; Kefir; Soymilk; Reducing activity; Scavenging activity

Farklı İnek ve Soya Sütü Karışımlarından Üretilen Kefirlerin Antioksidan Özellikleri

ESER BİLGİSİ

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ÖZET

Bu çalışmanın amacı inek, soya sütü ve bunların farklı karışımlarından üretilen kefir örneklerinin antioksidan kapasitelerini belirlemektir. Bu amaçla, üretilen kefir örneklerinde askorbat otooksidasyonu, süperoksit anyon radikali ve hidrojen peroksit tutuklayıcı etkileri ile indirgen aktivite gibi antioksidatif özellikler incelenmiştir. Sadece soya sütü ile üretilen kefirler en yüksek askorbat otooksidasyon oranına sahip olmuştur. Kefir örneklerinin sistein eşdeğer miktarı olarak ifade edilen indirgen aktiviteleri istatistiksel olarak farklı bulunmuş ve soya sütü oranının artmasıyla yükselmiştir. İnek, inek/soya ve soya sütlerinden üretilen kefirlerle ait süperoksit radikal oluşu-

munu durdurma sonuçları istatistiksel olarak farklı bulunmuştur. Ancak, gerek kefir tanesi ile gerekse kefir kültürü ile gerçekleştirilen fermentasyonun söz konusu aktivite üzerine etkisi önemsizdir. Elde edilen sonuçlara göre kefir örneklerinin hiçbirisi hidrojen peroksit tutuklayıcı etki göstermemiştir.

Anahtar sözcükler: Antioksidan; Kefir; Soya sütü; İndirgeme aktivitesi; Tutuklayıcı aktivite

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1. Introduction

The oxidative damage caused by free radicals and other reactive oxygen species plays a significant pathological role in human diseases (Wang et al 2006). Among these diseases cancer has an important place. According to observational epidemiological studies, diets containing mainly fruits and vegetables are related to a lower cancer incidence, especially cancers from the gastrointestinal tract. This is due in part to the dietary antioxidant content of fruit and vegetables (Serrano et al 2007). So it is clear that natural antioxidants from foods may reduce the oxidative damage on the human body (Lin & Yen 1999).

Soybeans are the most important food source of isoflavones that exhibit antioxidant activity. Isoflavones have been associated with beneficial health effects in humans, including prevention of cancer, cardiovascular diseases, osteoporosis and relief of menopausal symptoms (Callou et al 2010).

Soymilk is obtained by aqueous extraction from whole soybeans; it contains no cholesterol or lactose and only small quantities of saturated fatty acids. Chemical compounds in soymilk, however, often impart a distinctive flavor with beany and grass-like characteristics which is considered displeasing by some consumers. These defects may be improved by lactic acid fermentation (Liu et al 2002) or mixing with cow milk.

Kefir is a soured, frothy and mildly alcoholic dairy drink produced by the result of acid and alcohol fermentation. Kefir preparation involves natural fermentation of cow's milk with kefir grains (Chandan 2006). It was produced and used in Middle Asian countries, Russia and Caucasia for many years. In these countries, kefir has been widely considered as a beverage and a medication for treatment of various illnesses (Kılıç et al 1999). Its microbiological and chemical

composition provides a complicated probiotic effect due to the inherent lactic acid bacteria and yeast (Güzel-Seydim et al 2011).

Previous studies have shown that fermented soy products performed better antioxidative activity (Wang et al 2006). Furthermore, soymilk bioprocessing by active kefir cultures has been shown to cause phenolic antioxidant mobilization (McCue & Shetty 2005).

Therefore the aim of this study was to investigate the antioxidant properties of kefir samples produced from different cow/soy milk mixtures by inoculation kefir culture or kefir grain. For this purpose, antioxidative activities including the inhibition of ascorbate autoxidation, reducing activity, the scavenging effect of superoxide anion radicals and hydrogen peroxide were evaluated.

2. Materials and Methods

2.1. Preparation of soymilk

Whole soybeans were first washed and soaked overnight in distilled water containing sodium bicarbonate (0.8%). After decanting the water, the soaked soybeans were mixed with 10 times their weight of distilled water for 3 min and comminuted to get soymilk in a lab type soymilk machine (Soypower, MJ820, NY, USA) (Kamaly 1997). The total solids, fat and protein content of this unstandardised soymilk was 7.75, 2.35 and 3.75% respectively.

2.2. Production of kefir samples

Both cow milk and soymilk were standardized to 3 fat-8.5% non-fat solids for full fat kefir production and to 1.5 fat-8.5% non-fat solids for half fat kefir production. Soymilk powder and vegetable oil were used during standardization process of soymilk. The cow milk and soymilk were pasteurized for 10 min at 90°C. Then they were divided into 5 different portions (100% cow

milk, 75% cow milk–25% soymilk, 50% cow milk–50% soymilk, 25% cow milk–75% soymilk and 100% soymilk) and cooled to 25°C. Afterward, ten different milk portions were obtained by inoculation of 3% kefir grains (Ege University, Turkey) or 3% kefir culture (Danisco-Biolacta, Poland) respectively. All inoculated samples were incubated at 25°C until pH reached 4.7. After separating the grains, stirring and glass bottling, all kefir samples were stored at 4±1°C until being used. The same production steps were applied for both full and half fat kefir. The microbial population of both kefir grains and culture is given in Table 1.

Table 1-Microbial population in kefir grains and kefir culture (n=3)

Çizelge 1-Kefir taneleri ve kültürlerinde mikrobiyal populasyon

	<i>Kefir grain</i>	<i>Kefir culture</i>
	log cfu ml ⁻¹	
<i>Lactobacillus</i> spp.	10.30±0.01	9.66±0.62
<i>Lactococcus</i> spp.	8.29±0.01	8.29±0.01
<i>Acetobacter</i> spp.	6.12±0.03	5.21±0.02
Yeasts	5.18±0.04	4.17±0.04

2.3. Measurement of inhibition of ascorbate autoxidation

The method described by Rekha & Vijayalakshmi (2008) was used to determine the inhibition of ascorbate autoxidation. A 0.1 ml of ascorbate solution (5.0 mM, Sigma, USA) and 9.8 ml phosphate buffer (0.2 M, pH 7.0) were mixed with 0.1 ml of kefir sample. The mixture was incubated at 37°C for 10 min and then the absorbance was measured at 265 nm. The same steps were carried out for 0.1 ml of distilled water as control. The ascorbate autoxidation inhibition rate of kefir samples were measured by the help of the formula defined in Equation 1:

$$\text{Inhibition effect (\%)} = \left[\frac{A_{\text{sample}}}{A_{\text{control}}} - 1 \right] \times 100 \quad (1)$$

2.4. Measurement of reducing activity

A 0.5 ml of sample or distilled water was mixed with 0.5 ml of potassium ferricyanide (1.0%) and

0.5 ml of sodium phosphate buffer (0.02 M, pH 7.0). 10% trichloroacetic acid was added to this mixture following the incubation at 50°C for 20 min. The mixture was centrifuged at 780 g for 5 min. Later 1.5 ml was taken from the upper layer and mixed with ferrichloride (0.1%) and then the absorbance was measured at 700 nm. The reducing activity of cysteine was used as a standard. The reducing activity is directly proportional with the absorbance obtained. In other words higher absorbance values mean higher reducing activity (Lin & Yen 1999; Wang et al 2006).

2.5. Measurement of superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity was estimated according to method described by Wang et al (2006). All reagents were prepared in 100 mM phosphate buffer (pH 7.4). The kefir sample or distilled water (control) (50 µl) was mixed with 50 µl of nitrobluetetrazolium (300 µM), 50 µl of NADH (936 µM) and 50 µl of phenazine methosulfate (120 µM). The mixture was then left to stand for 5 min at room temperature. After this, the absorbance was measured at 560 nm. The superoxide anion radical scavenging activities of kefir samples were calculated as follows (Equation 2):

$$\text{Scavenging activity (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (2)$$

2.6. Measurement of hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity assay was performed by the method of Büyükbacı & El (2008). Briefly, 1 ml of sample was mixed with 2.4 ml of 0.1 M phosphate buffer (pH 7.4), and then 0.6 ml of 43 mM solution of H₂O₂ in the same buffer were added. The mixture was incubated at room temperature for 40 min and the absorbance values of the reaction mixtures at 230 nm were recorded against a blank solution containing phosphate buffer without H₂O₂ for each sample. The hydrogen peroxide scavenging activity was measured according to the following

Equation 3:

$$\text{H}_2\text{O}_2 \text{ scavenging activity (\%)} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (3)$$

2.7. Statistical analysis

In 2 (fat level; full or half) \times 2 (inoculation; grain or culture) \times 5 (milk source; cow:soy ratios; 100:0, 75:25, 50:50, 25:75 or 0:100) factorial arrangement, analysis of variance (ANOVA) was applied, and Duncan's Multiple Range Test was used in order to determine the differences by using SPSS[®] 15.0 for Windows. If *P* value is ≤ 0.05 , it was considered statistically significant. All experiments were conducted in triplicate.

3. Results and Discussion

Antioxidative activities including inhibition of ascorbate autoxidation, scavenging effect for superoxide anion radicals and hydrogen peroxide and, also the reducing activity exerted by kefir samples produced from full and half fat cow/soy milk mixtures are shown in Table 2.

3.1. Inhibition of ascorbate autoxidation

It was found that the inhibition rate of cow/soy milk kefir samples to inhibit ascorbate autoxidation ranged from 8.34–17.00% depending on soymilk ratio. As shown in Table 2, kefir samples produced from 100% soymilk (0:100) had the highest inhibition rates whereas the cow milk kefir samples (100:0) had the lowest values. According to statistical analysis and Duncan test, a significant increase was determined in kefir samples reaching to 50% of soymilk ($P < 0.05$). It is clear that the significant increase in inhibition rates may be related to the action of isoflavones and tocopherols, the main phenols found in soy bean (Wang et al 2006). Similarly with milk source, inoculation type was also affected the inhibition rate of ascorbate autoxidation ($P < 0.05$) because culture inoculated kefir samples consistently had higher values than grain inoculated ones. This result can be explained by intensive effect of intracellular antioxidants of microorganisms in kefir culture rather than in kefir grain. However fat content has not affected the inhibition rate of ascorbate autoxidation.

3.2. Reducing activity of kefir

The determination of reducing ability of kefir samples produced from different cow/soy milk mixtures could explain the relationship between their antioxidative effect and their reducing activity. Because reducing capacity of a compound can be an indicator for its own potential antioxidant activity (Meir et al 1995; Liu et al 2002). As shown in Table 2, reducing activities of kefir samples expressed as an equivalent amount of cysteine (μM) were increased significantly with elevated soymilk ratio ($P < 0.05$). The differences were noted after the soymilk ratio is reached 50% in kefir samples. Contrarily both fat level and milk source have not affected the reducing activity of samples.

Although milk-derived proteins and peptides demonstrate some level of antioxidative activity which may contribute the reducing activity of cow milk kefir samples (100:0) and despite to intracellular antioxidants sourced from kefir microflora, soymilk kefir samples (0:100) had approximately 3 times higher reducing activity. This result can be due to the reductones generally formed during fermentation of soymilk which could react with free radicals to stabilize and terminate radical chain reactions (Yang et al 2000; Wang et al 2006).

3.3. Scavenging of superoxide anion radical

Superoxides are biologically quite toxic and are oxygen-centered radicals with selective reactivity. They might be produced by a number of enzyme systems in autoxidation reactions and by non-enzymatic electron transfers that univalent reduces molecular oxygen (Gulcin et al 2010). These radicals indirectly initiate lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radical which have potential to react with biological macromolecules and thereby inducing tissue damage (Wang et al 2006; Gulcin et al 2010).

Table 2 shows the percentage of superoxide anion radical scavenging activity of cow/soy and soymilk kefir samples. The effect of milk

Table 2-Antioxidant properties of kefir samples produced from different cow and soymilk mixtures
Çizelge 2-Farklı inek ve soya sütü karışımlarından üretilen kefir örneklerinin antioksidan özellikleri

Fat level, F	Inoculation, I	Milk source, M, cow:soymilk ratio [†]	Inhibition of ascorbate autoxidation, %	Reducing activity, equivalent cysteine, μ M	Superoxide anion radical scavenging activity, %	Hydrogen peroxide scavenging activity, %	
Full Fat	Grain Inoculated	100:0	8.50±0.14	3.65±0.08	27.42±0.65	-8.75±1.52	
		75:25	8.90±0.41	4.02±0.09	27.65±0.70	-9.07±1.08	
		50:50	10.71±0.33	5.84±0.10	39.21±0.80	-29.32±4.11	
		25:75	12.94±0.33	7.33±0.12	43.05±0.52	-49.61±1.32	
		0:100	16.34±0.32	10.58±0.07	56.04±0.50	-68.74±1.40	
	Culture Inoculated	100:0	8.62±0.17	3.50±0.07	27.55±0.09	-8.37±0.50	
		75:25	8.93±0.21	4.00±0.08	27.90±0.10	-9.00±0.92	
		50:50	10.94±0.32	5.65±0.07	41.22±0.07	-31.44±1.32	
		25:75	13.22±0.36	7.07±0.07	43.61±0.79	-50.23±1.62	
		0:100	17.00±0.09	11.02±0.06	55.27±0.65	-70.62±1.53	
	Half Fat	Grain Inoculated	100:0	8.34±0.20	3.72±0.07	28.41±1.09	-9.02±0.62
			75:25	8.55±0.22	4.11±0.07	29.07±1.27	-9.35±0.55
50:50			11.07±0.36	5.66±0.10	40.22±1.32	-32.11±1.80	
25:75			12.36±0.08	7.29±0.21	44.33±1.36	-50.52±1.32	
0:100			15.97±0.27	10.42±0.32	57.68±1.39	-70.63±1.90	
Culture Inoculated		100:0	8.46±0.32	3.66±0.08	29.41±1.32	-9.00±0.31	
		75:25	8.66±0.08	4.02±0.06	29.49±1.36	-9.23±0.60	
		50:50	11.26±0.09	5.52±0.32	41.07±1.37	-33.94±1.38	
		25:75	12.98±0.23	7.00±0.30	45.27±1.81	-51.58±1.60	
		0:100	16.67±0.36	10.86±0.30	58.37±1.90	-71.38±1.90	
Main effects (means)							
Full			11.61	6.26	38.56	-33.51	
Half			11.43	6.22	40.33	-34.67	
	Grain		11.36	6.26	39.30	-33.71	
	Culture		11.67	6.22	39.58	-34.48	
		100:0	8.48 ^a	3.63 ^a	28.19 ^a	-8.79 ^a	
		75:25	8.75 ^a	4.03 ^a	28.52 ^a	-9.16 ^a	
		50:50	10.99 ^b	5.66 ^b	40.43 ^b	-31.70 ^b	
		25:75	12.87 ^c	7.16 ^c	44.06 ^c	-50.48 ^c	
		0:100	16.49 ^d	10.72 ^d	56.00 ^d	-70.34 ^d	
<i>P</i> values							
F			0.211	0.827	<0.001	0.001	
I			0.035	0.833	0.355	0.025	
M			<0.001	<0.001	<0.001	<0.001	
F×I			0.768	0.965	0.093	0.846	
F×M			0.452	0.978	0.006	0.177	
I×M			0.623	0.688	0.022	0.189	
F×I×M			0.994	1.000	0.033	0.943	

[†] 100:0; Kefir made from 100 % cow milk, 75:25; Kefir made from 75% cow - 25% soymilk, 50:50; Kefir made from 50% cow - 50% soymilk, 25:75; Kefir made from 25% cow - 75% soymilk, 0:100; Kefir made from 100% soymilk

^{a-d}: Means in the same column with different superscripts significantly differ ($P<0.05$).

source and fat level on this activity was statistically significant ($P < 0.05$). However, the effect of fermentation neither with kefir grain nor culture was significant ($P > 0.05$). Although contrary statistical results were found in other assays, the interaction effects of factors on superoxide anion radical scavenging activity were significant except the interaction between fat level and inoculation type (F×I). The nature of the interactions were that increased soymilk ratio had a stronger effect at lower fat content and/or inoculation with culture.

Similarly with the ascorbate autoxidation ability and reducing activity of kefir samples, the superoxide anion scavenging effect of samples increased as the soymilk ratio was raised (Table 2). According to Duncan grouping, this activity sharply increased in kefirs containing at least again 50% soymilk. It has been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical (Yen & Duh 1994). Thus, the higher superoxide anion scavenging effect values in those kefir samples can be attributed to soymilk originated isoflavones. Moreover, soybeans contain superoxide dismutase which possesses the superoxide anion scavenging effect by catalyzing the conversion of superoxides to hydrogen peroxide and oxygen (Liu et al 2002). Decomposition of excess superoxides by superoxide dismutase is an important physiological antioxidant defense mechanism in aerobic organisms. It has been indicated that some *Lactococcus* spp. (Sanders et al 1995; Lin & Yen 1999) and *S. thermophilus* (Archibald & Fridovich 1981) are able to express antioxidative enzyme superoxide dismutase activity. So the bacterial flora mainly the homofermentative lactic acid bacteria in kefir samples could positively affect the superoxide anion scavenging effect.

3.4. Scavenging of hydrogen peroxide

Hydrogen peroxide has strong oxidizing properties. It can be generated in biological and food systems by many oxidizing enzymes. It can cross membranes and may slowly oxidize a

number of compounds. For instance it can form a hydroxyl radical (the most highly reactive oxygen radical) in the presence of transition metal ions and participate in free-radical reaction (Wang et al 2006; Gulcin et al 2010). As seen in Table 2 kefir samples did not exhibit a hydrogen peroxide scavenging activity. Contrarily, accumulation of hydrogen peroxide was found in all kefir samples which can be understood with the results below zero. This may be attributed to the formation of hydrogen peroxide by the organisms mainly lactobacilli and leuconostocs in kefir microflora (Kesenkaş et al 2006). However the kefir samples produced from 100% soymilk (0:100) accumulated the highest amounts of hydrogen peroxide whereas the kefirs containing cow milk resulted in a reduced level of hydrogen peroxide as the cow milk ratio is increased ($P < 0.05$). Moreover fat content and inoculation type were also effect the hydrogen peroxide scavenging activity ($P < 0.05$). According to these results, it can be said that NADH peroxidase activity of bacteria mainly in kefir grains is getting higher in the presence of full fat cow milk. Because, this enzyme can degrade hydrogen peroxide and lead to lower accumulation of hydrogen peroxide (Wang et al 2006).

4. Conclusion

In conclusion, it can be said that there are significant variations among antioxidant properties of kefir samples produced from different cow/soy milk mixtures relation to soymilk ratio in kefir milk. The threshold soymilk level for significant antioxidative activities was 50%. Moreover only the reducing activity did not affect by fat content or inoculation type. These two factors were effective mainly on hydrogen peroxide scavenging activity. Soymilk products are recognized as beneficial to health however; improvement of their undesirable sensorial properties by using cow milk can make them good functional food candidates. In addition, fermentation of these milk mixtures with kefir grains or culture can also bring in the probiotic status.

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