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Antimutagenic and Antioxidant Properties of Milk−Kefir and Soymilk−Kefir

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This study was aimed at evaluating the antimutagenic and antioxidant properties of milk−kefir and soymilk−kefir. Such antimutagenic activity was determined by means of the *Salmonella* mutagenicity assay, whereas the antioxidant properties of kefir were evaluated by assessing the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, lipid peroxidation inhibition activity, ferrous ion chelating ability, reducing power, and antioxidative enzyme activity. Both milk−kefir and soymilk−kefir demonstrated significantly greater antimutagenic activity than milk and soymilk. Milk−kefir and soymilk−kefir also displayed significantly greater scavenging activity upon DPPH radicals, an inhibition effect upon linoleic acid peroxidation, and more substantial reducing power but displayed a reduced glutathione peroxidase activity than was the case for milk and soymilk. Milk and soymilk fermented by kefir grains did not alter the ferrous ion chelating ability and superoxide dismutase activity of the original materials. These findings have demonstrated that milk−kefir and soymilk−kefir possess significant antimutagenic and antioxidant activity and suggest that milk−kefir and soymilk−kefir may be considered among the more promising food components in terms of preventing mutagenic and oxidative damage.

KEYWORDS: Antimutagenicity; antioxidant; kefir; soymilk; reducing power; scavenging effect; chelating effect; antioxidant enzyme

INTRODUCTION

Several reactive oxygen species, including the superoxide radical, hydroxyl radical, hydrogen peroxide, and the peroxide radical, are known to cause oxidative damage not only to food systems but also to living systems. Accumulating evidence suggests that reactive oxygen species and their subsequent modification of cellular macromolecules play a significant pathological role in human diseases such as cancer, atherosclerosis, hypertension, and arthritis (*1*, *2*). Although the human body has an inherently antioxidative system (i.e., superoxide dismutase, glutathione peroxidase, and uric acid) to protect itself from damage caused by peroxidants, these systems are not sufficiently effective to totally prevent such damage (*3*). Hence, there is an increasing interest in finding natural antioxidants from food, because it is believed that they can protect the human body from the attack of free radicals and retard the progress of many chronic diseases, as well as retarding the lipid oxidative rancidity in foods (*4*). Antioxidants from natural sources are likely to be more desirable than those chemically produced, because some synthetic antioxidants have been reported to be carcinogenic (*5*).

Mutagens and carcinogens in food are of great concern to human health. There is often a high degree of correlation between *in vitro* mutagenicity and *in vivo* carcinogenicity (*6*). Many of these mutagens act on the cell via its active metabolites or by generating free radicals; therefore, the use of certain antimutagens dietarily may contribute to the body's capability of preventing or inhibiting the development of human cancer and genetic diseases (*7*).

Soybeans are an excellent source of low-cost protein and have been an important nutritional component in the typical diets of many countries for many generations. Many nutritional and medical investigations have revealed the great potential of soy foods for lowering blood cholesterol levels and the incidence of heart disease and cancer (*8*, *9*). Soybeans contain many kinds of polyphenols, the main polyphenols including isoflavone analogues, such as daidzin, genistin, daidzein, and genistein. Genistein, which has been found to be a potent inhibitor of DNA topoisomerases and also protein tyrosine kinases and other critical enzymes involved in signal transduction is believed to demonstrate anticarcinogenic activity (*10*, *11*). Further, genistein also exhibits antioxidant properties by preventing the hemolysis of red blood cells by dialuric acid or hydrogen peroxide and by protecting microsomal lipid peroxidation induced by a ferrous ion−ADP complex (*12*, *13*). Furthermore, dietary genistein enhances the activities of antioxidant enzymes in various organs of mice (*14*). It is interesting to note that fermented foods from

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soybeans (such as tempeh, miso, and natto) do not lose their antioxidative properties as a consequence of the fermentation process but, in fact, exhibit increased antioxidative activity (*15*).

Fermented milk has been studied for possible beneficial physiological effects, such as antimutagenicity, immune-potentiating activity, antitumor activity, and the prevention of pathogens (*16*). Kefir is an acidic and mildly alcoholic fermented milk product that is believed to contain many functional substances, and it has been postulated that the longevity of Bulgarian peasants is partially due to their frequent consumption of this fermented milk (*16*). Kefir differs from other milk products in that it is not the result of the metabolic activity of a single species of microflora, but it is the product of fermentation with a mixed group of microflora confined to a matrix of discrete "kefir grains", which are recovered after fermentation (*17*). In the kefir grains, lactic acid bacteria and yeasts are embedded in a slimy polysaccharide matrix named kefiran, thought to be produced by the lactobacilli in the grain (*18*). Various lactic acid bacteria and yeasts have been identified as being present in kefir grains, including *Lactobacillus breris*, *L. helveticus*, *L. kefir*, *Leuconostoc mesenteroides*, *Kluyveromyces lactis*, *K. marxianus*, and *Pichia fermentans* (*19*, *20*). The microorganisms contained within the kefir grains produce lactic acid, antibiotics, and several kinds of bactericides, such products inhibiting the proliferation of both degrading and pathogenic microorganisms in kefir milk (*19*). Furthermore, kefiran is reported to possess some antitumor activity (*21*).

Because both kefir and soybeans are beneficial to health, we attempted to produce soymilk−kefir and subculture kefir grains in soymilk in previous studies (*22*, *23*). The microorganisms in kefir grains produce sufficient lactic acid and ethanol for this process; however, the quantity of polysaccharide produced is relatively small when grown in soymilk. The composition of the polysaccharide produced from soymilk−kefir grains is also different from that produced from the milk−kefir grains. Furthermore, we demonstrated that orally administered soymilk−kefir not only inhibited tumor growth and induced the apoptotic form of tumor-cell lysis but also increased the total IgA levels in tissue extracts from the wall of the small intestine of mice (*24*). To the best of our knowledge, however, the antimutagenicity and antioxidant properties of soymilk−kefir have not been reported on previously.

The aim of this study was to investigate the antimutagenic and antioxidant properties of soymilk−kefir. The *Salmonella* mutagenicity assay was used to determine the antimutagenic activity of soymilk−kefir, whereas reducing power, the scavenging of radicals, the chelating of ferrous ions, and antioxidative enzyme activities were used to evaluate the antioxidant properties of soymilk−kefir.

MATERIALS AND METHODS

Chemicals. A direct-acting mutagen, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and an indirect-acting mutagen, 4-nitroquinoline-*N*′-oxide (NQNO), were purchased from Sigma Chemical Co. (St. Louis, MO). A rat-liver S9 microsomal fraction induced by Aroclor 1254 was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). All other reagents used were of analytical grade.

Preparation of Soymilk. A total of 1 kg of dry, mature, whole soybeans was soaked in 3 L of distilled water at 25 °C for 24 h. The soak water was then decanted, and the beans were washed and then ground in 6 L of boiling distilled water in a blender (Waring Division, Dynamics Corporation, New Hartford, CT). The resulting suspension was filtered through three layers of cheesecloth, autoclaved for 15 min at 121 °C, and then stored at 4 °C until required.

Kefir Grains. Kefir grains were collected from households in northern Taiwan. In the laboratory, these were inoculated (5 wt %/volume) and propagated in sterilized (121 °C for 15 min) reconstituted milk (10 wt %/volume) or soymilk at 20 °C for 20 h with 2 or 3 times weekly transfers and kept at 4 or −80 °C for short- or long-term storage, respectively.

Milk−Kefir and Soymilk−Kefir Manufacture. Milk−kefir and soymilk−kefir were manufactured by sterilized (121 °C for 15 min) reconstituted milk (10 wt %/volume) and soymilk inoculated with 5% (w/v) kefir grains and incubated at 20 °C for 32 h, respectively. During the fermentation process, samples were taken every 8 h and filtered through three layers of cheesecloth to remove the kefir grains. Samples were then treated with 6% lactic acid to a pH level of 4.6 and centrifuged at 4 °C and 17000*g* for 45 min to remove precipitated proteins. The resulting supernatants were then freeze-dried and stored at −80 °C. Prior to the antimutagenicity and antioxidant properties assay, the 10 mg/mL solutions of milk, soymilk, milk−kefir, and soymilk−kefir were prepared by dissolving the freeze-dried powder into sterilized, distilled water.

Antimutagenicity Assay. The antimutagenicity of milk−kefir and soymilk−kefir against different mutagensis in the *Salmonella typhimurium* strains TA98 were assessed using the standard mutagenic protocol described by Maron and Ames (*25*). For the plate incorporation inhibition assay, 0.1 mL of a 10−11-h-old nutrient broth culture containing the tester strain, 0.1 mL of milk, soymilk, milk−kefir, or soymilk−kefir sample (10 mg/mL), 50 μg of MNNG or 10 μg of NQNO (both in 100 μL of DMSO), and S9 mix (500 μL) when NQNO was used as a mutagen were added to 2 mL of molten top agar containing 0.05 mM biotin-histidine and dispersed onto minimal glucose agar plates. The plates were incubated at 37 °C for 48 h, after which the number of developed revertants was scored. As a control experiment, 0.1 mL of distilled water was used instead of 0.1 mL of kefir and all other procedures were the same as those described above. Antimutagenicity was expressed as follows: inhibition (%) = [1 − (number of revertants in the presence of the sample − spontaneous revertants)/(number of revertants in the absence of the sample − spontaneous revertants] × 100.

Scavenging Effect of Kefirs upon 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radicals. The effect of kefirs upon DPPH radicals was estimated according to the method of Shimada et al. (*26*). The milk, soymilk, milk−kefir, or soymilk−kefir sample (0.8 mL, 10 mg/mL) was mixed with 0.2 mL of methanolic solution containing DPPH radicals, resulting in a final concentration of the DPPH of 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was than measured at 517 nm. The capability of the test material to scavenge DPPH radicals was calculated as (%) = [1 − (absorbance of the sample at 517 nm)/(absorbance of the control at 517 nm] × 100.

Inhibition Effect of Kefirs upon Lipid Peroxidation. The antioxidant activity of kefirs was determined by application of the linoleic acid system (*27*). The linoleic acid emulsion was prepared by mixing equal volumes of linoleic acid, Tween 20, and phosphate buffer (0.02 M at pH 7.0). After this, the milk, soymilk, milk−kefir, or soymilk−kefir sample (0.5 mL, 10 mg/mL) was mixed with 2.5 mL of linoleic acid emulsion (0.002 M) and 2 mL of phosphate buffer (0.2 M at pH 7.0). The reaction mixture was incubated at 50 °C in the dark, and the degree of oxidation was measured according to the thiocyanate method (Yen et al.), which involved the sequential addition of 4.7 mL of ethanol (75%), 0.1 mL of ammonium thiocyanate (30%), 0.1 mL of sample solution, and 0.1 mL of ferrous chloride (20 mM) in HCl (3.5%). After the mixture had been stirred for a period of 3 min, the peroxide value was determined by reading the absorbance at 500 nm. The relative inhibition of linoleic acid peroxidation was calculated as (%) = [1 − (absorbance of the sample at 500 nm)/(absorbance of the control at 500 nm] × 100. A greater percentage figure indicates a greater level of antioxidant activity.

Chelating Effects of Kefirs upon Ferrous Ions. The ferrous ion chelating ability of kefirs was determined according to the method of Decker and Welch (*28*). The milk, soymilk, milk−kefir, or soymilk−kefir sample (5 mL, 10 mg/mL) was mixed with 0.1 mL of ferrous chloride (2 mM) and 0.2 mL of ferrozine (5 mM). The mixture was shaken and left to stand for 10 min at room temperature. The absorbance of the resulting solution was measured at 562 nm. The relative capability

of the kefir sample to chelate the ferrous iron was calculated as (%) = [1 − (absorbance of the sample at 562 nm)/(absorbance of the control at 562 nm] × 100. A greater percentage figure indicates a greater chelating ability.

Reducing Power of Kefirs. The reducing power of kefirs was determined according to the method of Oyaizu (*29*). The milk, soymilk, milk−kefir, or soymilk−kefir sample (2.5 mL, 10 mg/mL) was mixed with an equal volume of sodium phosphate buffer (200 mM at pH 6.6) and potassium ferricyanide (1%). The mixture was incubated at 50 °C for a period of 20 min. After this, an equal volume of thrichloro acetic acid (1%) was added to the mixture, which was then centrifuged at 1400*g* for 10 min. The upper layer (5 mL) was mixed with 5 mL of distilled water and 1 mL of ferric chloride (0.1%), and the absorbance of the incident radiation by the solution was measured at a wavelength of 700 nm, with a greater value for the absorbance indicating a greater reducing power.

Superoxide Dismutase (SOD) Activity of Kefirs. The SOD activities of milk−kefir and soymilk−kefir were determined according to the method of Granelli et al. (*30*). The milk, soymilk, milk−kefir, or soymilk−kefir sample (0.1 mL, 10 mg/mL) was mixed with 3 mL of reaction solution containing Tris-HCl (50 mM at pH 8.5), cytochrome *c* (24 μM), xanthine (100 μM), diethylentriaminpentaacetic acid (DTPA, 100 μM) and xanthine oxidase (33 milliunits). After the mix was held for 20 min at room temperature, the absorbance of the mixture was determined at 550 nm. The SOD activities of milk−kefir and soymilk−kefir were calculated from the standard curve of purified bovine erythrocyte SOD. A total of 1 unit of SOD activity was defined as the quantity of SOD required to cause a 20% inhibition of the cytochrome *c* reduction activity of the tested sample using the described conditions.

Glutathione Peroxidase (GSHPx) Activity of Kefirs. The GSHPx activity of kefirs was measured using a coupled enzymatic assay as described by Lindmark-Mansson et al. (*31*). The milk, soymilk, milk−kefir, or soymilk−kefir sample (0.2 mL, 10 mg/mL) was mixed with 0.8 mL of reaction solution containing reduced glutathione (GSH, 0.63 mM), *tert*-butylhydroperoxide (BHPx, 0.1 mM), glutathione reductase (GR, 5 μg/mL), and NADPH (0.25 mM) in phosphate buffer (50 mM at pH 7.6). After incubation for a period of 5 min at 37 °C, the absorbance of the test solution was measured at 340 nm. A total of 1 unit of GSHPx activity was defined as 1 nmol of NADPH oxidized per minute.

Statistical Analysis. All results were analyzed using the general linear-model procedure available from the Statistical Analysis System software package version 8.1 (*32*). Duncan's multiple range test (*33*) was used to detect differences between treatment means. Each experiment was conducted in triplicate.

RESULTS AND DISCUSSION

Antimutagenicity Assay. The number of revertant colonies and the percentage of inhibition of milk−kefir and soymilk−kefir against the mutagenicity induced by ′MNNG or ′NQNO are shown in **Table 1**. Both milk−kefir and soymilk−kefir displayed characteristic antimutagenic effects upon the mutagenicity of MNNG or NQNO under the assay conditions of this test. For both cases of direct- or indirect-acting mutagens, the antimutagenic activities of milk−kefir and soymilk−kefir were significantly greater than those of unfermented milk and soymilk.

Fermented milk products prepared with lactic acid bacteria are known to exhibit antimutagenicity toward a large spectrum of mutagens, such as MNNG, NQNO, and 3,2′-dimethyl-4-aminobiphenyl (DMAB) (*34*, *35*). It has also been reported previously that milk proteins, such as casein, α-lactalbumin, and β-lactoglobulin, are able to bind mutagenic heterocyclic amines at high percentage (*36*, *37*). Further, van Boekel et al. (*38*) observed strong inhibitory activity by casein against NQNO and found that the anti-NQNO activity increased when casein was hydrolyzed by pepsin. Matar et al. (*39*) reported that antimutagenic compounds are produced in milk during fermentation by *L. helveticus*, and these authors further suggested that the release of peptides is one possible contributing mechanism. In a previous study, we found that *L. helveticus* isolated from kefir grains possessed highly proteolytic activities (*20*). Thus, we suggest that the antimutagenicity of milk−kefir maybe attributable, in part, to peptides released from milk proteins during fermentation.

Table 1. Effect of Milk, Soymilk, Milk–Kefir, and Soymilk–Kefir upon ′MNNG and ′ NQNO Mutagenesis in the Ames Assay

sample[a]	number of *his*$^+$ revertant colonies/plate	inhibition rate (%)
	N-Methyl-*N*′-nitro-*N*-nitrosoguanidine	
control	373.2 ± 4.8^b	
milk	329.0 ± 3.0^c	12.3 ± 0.8^c
soymilk	343.3 ± 7.7^d	8.3 ± 2.1^c
milk–kefir	149.7 ± 14.1^e	62.0 ± 3.9^b
soymilk–kefir	208.3 ± 8.3^f	45.7 ± 2.3^d
	4-Nitroquinoline-*N*′-oxide	
control	690.0 ± 11.4^b	
milk	605.3 ± 6.5^d	14.7 ± 1.1^f
soymilk	530.7 ± 12.5^c	27.7 ± 2.2^c
milk–kefir	176.3 ± 23.5^e	89.3 ± 4.1^b
soymilk–kefir	294.0 ± 13.1^f	68.8 ± 2.3^d

[a] The concentration was 10 mg/mL. [b−f] Means in a column with different superscripts are significantly different ($p < 0.05$).

Soybeans are a unique dietary source of isoflavone phytoestrogens, such as daidzein and genistein. Miyazawa et al. (*40*) reported that daidzein and genistein from soybean seeds were antimutagenic compounds with their activity directed against chemical mutagens. Park et al. (*41*) noted that the most effective antimutagenic compound in Doenjang (Korean fermented soypaste) was genistein, which was formed from genistin during the fermentation in the soybeans. In our study, the antimutagenic activity of soymilk−kefir proved to be significantly greater than that of soymilk; however, in a previous study, we found that the fermentation process did not affect the concentration of genistein in soymilk significantly (*24*). Therefore, we suggest that genistein was not the major antimutagenic compound in soymilk−kefir. As stated above, milk-protein-derived peptides have been reported to demonstrate some antimutagenic activity (*38*, *39*). To the best of our knowledge, from a thorough search of the literature, there would not appear to be available any published studies pertaining to research conducted in the area of the antimutagenic activity of peptides derived from soybean proteins. Clearly thus, it would appear appropriate if future studies in this realm could focus upon the antimutagenicity or other bioactivities of peptides derived from soybean proteins.

Sreekumar and Hosono (*42*) reported that polysaccharides produced by *Bifidobacterium longum* revealed high levels of antimutagenicity. Kefir differs from other milk products, in that it is fermented with a mixed microflora confined to a matrix of discrete kefir grains. Within the kefir grains, lactic acid bacteria and yeasts are embedded in a slimy polysaccharide matrix, called kefiran, which is the result of the microbial metabolism of milk lactose (*17*, *18*). The composition of the polysaccharide deriving from soymilk−kefir grains is different from that of the polysaccharide deriving from milk−kefir grains (*23*). To the best of our knowledge, information specific to the antimutagenic properties of kefiran is lacking, however. Further investigation is required to verify the antimutagenic properties of kefiran and the effect of polysaccharide composition upon the antimutagenic properties of kefiran.

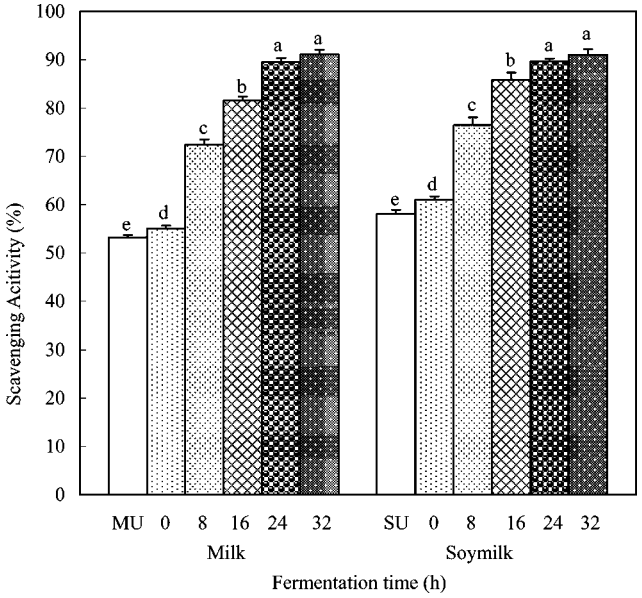

Figure 1. Effect of fermentation time upon the DPPH radical-scavenging activity of milk and soymilk fermented by kefir grains. MU and SU represent milk and soymilk uninoculated with kefir grains, respectively. Vertical bars represent the standard deviation for each data point. Locations marked by the same letter are not significantly different ($n = 3$, $p < 0.05$).

Scavenging Effect of Kefirs upon DPPH Radicals. Proton-radical scavenging has been reported to be an important mechanism for antioxidation. The assay for assessment of proton-radical-scavenging activity with DPPH is relatively simple and quite reproducible, with this compound having been reported to be stoichiometrically decolorized by antioxidants. The reduction in the concentration of the DPPH is allowed to monitor the decrease in its absorbance at a characteristic wavelength when it encountered proton-radical scavengers (*43*). **Figure 1** depicts the DPPH radical-scavenging activity of milk−kefir and soymilk−kefir. As revealed in **Figure 1**, unfermented soymilk demonstrated a greater DPPH radical-scavenging activity than unfermented milk. Immediately following the addition of kefir grains to the milk and soymilk, the DPPH radical-scavenging activity appeared to have increased, indicating that some components of the antioxidants contained in the kefir grains were transferred to milk and soymilk. After the incubation for 32 h, the DPPH radical-scavenging activities of milk−kefir and soymilk−kefir were significantly greater than those of milk and soymilk.

Soybeans are recognized as being a good source of several required food nutrients, including many kinds of polyphenols, with the main types being isoflavone analogues such as daidzin, daidzein, genistin, and genistein. The other polyphenols, such as α-, γ-, and δ-tocophenols, saponins, chlorogenic acid isomers, caffeic acid, and fenolic acid, are also contained. These polyphenols demonstrate the potential ability to scavenge free radicals related to oxygen (*15*). Several workers have found that fermented soybean foods produced by using microorganisms do not lose their antioxidative properties after fermentation has occurred but, in fact, reveal increased antioxidative activity (*15*). During the process of soybean fermentation, at least a partial cleavage or change in the contained soybean glucosides takes place. Further, the level of genistein in the fermented soybean products has been observed to be greater than the corresponding value for unfermented soybeans (*15*), although we observed in a previous study that the concentrations of genistein in soymilk and soymilk−kefir did not differ significantly between natural and fermented soybean products (*24*). The increase in the DPPH radical-scavenging activity of soymilk−kefir as compared to soymilk might be attributable to antioxidative compounds other than genistein.

Nishino et al. (*44*) reported that heat treatment enhanced the DPPH radical-scavenging activity of skim milk, with the activity being further increased by fermentation with *Lactobacillus casei* strain Shirota. These authors also suggested that casein hydrolysate present in the fermented milk might be one of the factors enhancing radical-scavenging activity. Suetsuna et al. (*45*) also demonstrated that some peptides deriving from the pepsinic hydrolysate of casein demonstrated DPPH radical-scavenging activities. In addition to milk protein-derived peptides, soybean protein-derived peptides have also been reported to exhibit radical-scavenging activity. Chen et al. (*46*) reported that a total of 22 peptides that derived from proteolytic digests of a soybean protein, β-conglycinin, exhibited some DPPH radical-scavenging activity. In this study, we demonstrated that the DPPH radical-scavenging activity of milk−kefir and soymilk−kefir was significantly greater than that of milk and soymilk, suggesting that this activity may be attributable, in part, to the peptides deriving from milk proteins and soybean proteins.

Inhibition Effect of Kefirs upon Lipid Peroxidation. Lipid peroxidation of mono- and polyunsaturated fatty acids during food processing and storage is a major concern to the food industry. In the human body, lipid peroxides are toxic and capable of damaging most body cells. The process of lipid peroxidation is initiated by an attack upon a fatty acid or fatty acyl side chain by any chemical species that features sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. The resulting lipid radicals then undergo molecular rearrangement, followed by reacting with oxygen to produce peroxyl radicals, which are capable of abstracting hydrogen from adjacent fatty acid side chains and thus propagating a chain reaction of lipid peroxidation (*1*). Therefore, the inhibition of lipid peroxidation is of great importance to the prevention of the deterioration of food quality to an inferior status and the certain human disease processes involving free radicals. As shown in **Figure 2**, soymilk demonstrates a more substantial inhibitory effect upon linoleic acid peroxidation than does milk. After incubation for a period of 32 h, the inhibitory effects of milk−kefir and soymilk−kefir upon linoleic acid peroxidation were significantly greater than was the case for milk and soymilk.

It has been previously reported that proteins deriving from dairy products reveal some antioxidant potential (*47*). Pena-Ramos and Xiong (*50*) found that peptides deriving from milk protein hydrolysates inhibited lipid oxidation, suggesting that the specific amino acid residue side-chain groups or the specific peptide structure of the antioxidative peptides may be attributable to chelation of prooxidative metal ions and termination of the radical chain reactions. Not only milk protein-derived peptides but also soybean protein-derived peptides were reported to contain antioxidant activity against peroxidation of lipids or fatty acids. Chen et al. (*46*) reported that protease hydrolyses of a soybean protein, β-conglycinin, yielded antioxidative activity directed against the peroxidation of linoleic acid. Further, more detailed subsequent studies have pointed to His and Pro in the peptide sequence playing important roles in the antioxidative activity as demonstrated by β-conglycinin-derived peptides (*49*). Thus, we suggest that the peptides deriving from milk and soymilk proteins might contribute to the inhibitory effects of milk−kefir and soymilk−kefir upon lipid peroxidation. It is interesting to note that fermented foods from soybean

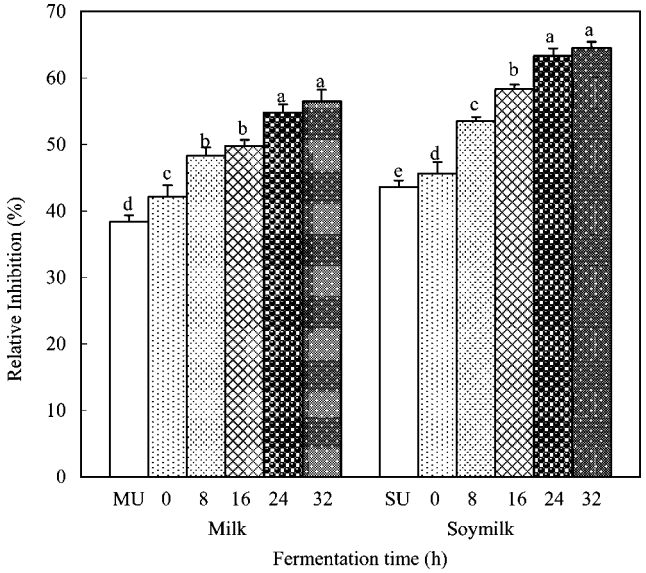

Figure 2. Effect of fermentation time upon the inhibition of linoleic acid peroxidation activity of milk and soymilk fermented by kefir grains. MU and SU represent milk and soymilk uninoculated with kefir grains, respectively. Vertical bars represent the standard deviation for each data point. Locations marked by the same letter are not significantly different ($n = 3$, $p < 0.05$).

Table 2. Ferrous Ions Chelating Ability of Milk and Soymilk Fermented by Kefir Grains[a]

	chelating effect (%)	
fermentation time (h)	milk	soymilk
uninoculated	25.0 ± 0.6	13.2 ± 0.8
0	24.9 ± 0.7	13.8 ± 0.4
8	25.9 ± 0.9	12.9 ± 0.7
16	25.5 ± 1.0	12.5 ± 0.7
24	25.8 ± 0.8	12.7 ± 0.9
32	25.6 ± 1.0	12.9 ± 0.7

[a] The concentration was 10 mg/mL.

(i.e., tempeh, miso, natto, shoyu) do not lose their antioxidative properties but, in fact, show increased antioxidative activity. Several antioxidative substances, including 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoic acid, 8-hydroxidaidzein, and 8-hydroxygenistein, have been demonstrated to have been produced in fermented soybean products (*15*). Whether soymilk−kefir contains other kinds of potent antioxidative isoflavones or not remains to be elucidated.

Chelating Effects of Kefirs upon Ferrous Ions. Iron, the most abundant transition-metal ion in our body, may work as a catalyst for the generation of reactive oxygen species in pathological conditions. The reduced form of iron potentiates oxygen toxicity by converting, via the Fenton reaction, the less reactive hydrogen peroxide to the more reactive oxygen species, the hydroxyl radical and the ferryl ion. Therefore, it is not surprising that iron overload has been shown to be associated with carcinogenesis and cardiovascular disease. It has been assumed that the strict regulation of iron assimilation prevents an excess of free intracellular iron, which leads to oxidative stress (*48*). The ferrous ion chelating activities of milk−kefir and soymilk−kefir are depicted in **Table 2**. It can be seen that milk and soymilk fermented by kefir grains did not reveal a significant increase in their ferrous ion chelating activity as compared to their unfermented forms. Several researchers have investigated the ability of milk proteins to bind ferric or ferrous ions, e.g., lactoferrin, serum albumin, casein, and a high molecular-weight fraction of whey, all of which have been reported to demonstrate some iron-chelating activity (*51*, *52*) In general, milk fractions containing a greater number of phosphoseryl serine groups reveal a greater affinity for iron, although the carboxyl group of the amino acids asparagine and glutamine can bind iron as well (*47*). To the best of our knowledge, only a few studies have previously focused on the iron-chelating activity of soybeans. For example, Moran et al. (*51*) reported that phenolic compounds deriving from soybeans were able to chelate ferrous ion into a safe, catalytically inactive form. Chen et al. (*49*) reported that His-containing peptides deriving from proteolytic digests of a soybean protein could act as a metal ion chelator. Yang et al. (*54*) also noted that fermented soybean broth proved to be a good source of chelators for ferrous ions. Contrasting such results, however, we have found herein that milk and soymilk fermented by kefir grains did not demonstrate any apparent increased level of ferrous ion chelating ability as compared to natural milk and soymilk.

Table 3. Reducing Power of Milk and Soymilk Fermented by Kefir Grains[a]

	equivalent cysteine (μM)	
fermentation time (h)	milk	soymilk
uninoculated	508.9 ± 7.2^b	604.1 ± 7.9^c
0	560.3 ± 6.8^c	638.9 ± 7.5^d
8	597.0 ± 4.3^d	698.6 ± 2.6^e
16	611.3 ± 9.4^e	709.1 ± 5.3^e
24	688.2 ± 7.7^f	749.8 ± 3.2^f
32	753.4 ± 9.7^g	790.3 ± 9.3^g

[a] The concentration was 10 mg/mL. $^{b-g}$Means in a column with different superscripts are significantly different ($p < 0.05$).

Reducing Power of Kefirs. From our review of the literature, some studies have reported that the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*55*). Thus, it appears necessary to determine the reducing power of kefirs to elucidate the relationship between their antioxidant effect and their reducing power. **Table 3** illustrates the reducing power of milk−kefir and soymilk−kefir. The reducing power of both milk and soymilk was increased significantly by kefir fermentation. It has been reported previously that some milk-derived proteins and peptides demonstrate some level of antioxidative activity (*56*). Wong and Kitts (*47*) found that the reducing activity of certain buttermilk solids was mainly attributed to the sulfhydryl content of the group and that free hydroxyl groups could have also contributed, in part, to the observed reducing activity. Further, it has also been previously reported that some lactic acid bacteria may exhibit excellent reducing power (*57*). Yang et al. (*54*) observed that fermented soybean broth exhibited rather high reducing activity, with these authors suggesting that the reducing activity of fermented soybean broth might be due to its hydrogen-donating ability. In the present study, we found that the reducing power of milk−kefir and soymilk−kefir was significantly greater than that of either milk or soymilk and suggest that certain metabolites that demonstrate superior reducing power might be produced during kefir fermentation and that they could possibly react with free radicals to stabilize and terminate radical chain reactions.

Antioxidative Enzyme Activity of Kefirs. Highly reactive free radicals formed by exogenous chemicals or endogenous metabolic processes in the human body or in food systems are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Almost all organisms are well-protected against

Table 4. Specific Activities of SOD and GSHPx from Milk and Soymilk Fermented by Kefir Grains[a]

	specific activities (units/mL)			
	milk		soymilk	
fermentation time (h)	SOD	GSHPx	SOD	GSHPx
uninoculated	5.19 ± 0.11	10.68 ± 0.48[b]	0.25 ± 0.06	3.14 ± 0.08[b]
0	5.22 ± 0.06	10.56 ± 0.41[b]	0.24 ± 0.08	3.16 ± 0.11[b]
8	5.26 ± 0.11	9.43 ± 0.20[c]	0.22 ± 0.06	2.86 ± 0.09[c]
16	5.24 ± 0.11	7.88 ± 0.29[d]	0.29 ± 0.02	2.57 ± 0.23[d]
24	5.22 ± 0.12	6.49 ± 0.40[e]	0.27 ± 0.08	2.19 ± 0.19[e]
32	5.23 ± 0.08	6.15 ± 0.12[e]	0.20 ± 0.08	1.91 ± 0.13[f]

[a] The concentration was 10 mg/mL. [b−f] Means in a column with different superscripts are significantly different ($p < 0.05$).

free-radical damage by antioxidative enzymes such as SOD, catalase, peroxidase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, GSHPx, and GR. Among a number of different antioxidative enzymes, catalase, GSHPx, and SOD have been demonstrated to be present in milk (*31*), although, to the best of our knowledge, no study has yet been published focusing on the area of antioxidative enzyme activity in soymilk. Catalase is one of the most heat-labile enzymes known, with most of the activity of the enzyme being destroyed by even modest heat treatment. In this study, the catalase activities of milk, soymilk, and kefirs were all virtually undetectable (results not shown). The SOD and GSHPx activities of milk−kefir and soymilk−kefir are indicated in **Table 4**, from which it can be seen that the SOD activity in milk and soymilk did not appear to change during kefir fermentation, while the GSHPx activity decreased significantly.

SOD catalyzes the conversion of singly electron-reduced species of molecular oxygen to hydrogen peroxide and oxygen. The decomposition of excess superoxides by SOD is an important physiological antioxidant defense mechanism for aerobic organisms. It has been reported previously that some lactic acid bacteria do expressed SOD activity (*58*), although from the results of this study, milk and soymilk fermented by kefir grains did not appear to demonstrate altered SOD activity as compared to their unfermented analogues.

GSHPx, a selenium-containing enzyme, catalyses the reduction of different peroxides aided by glutathione or other substrates, thus providing protection against oxidative damage (*59*). The antioxidative enzymes present in uncooked food are mostly inactivated during food processing (*60*). Hojo (*61*) reported that heating milk at 80 °C for 10 min inactivated all GSHPx activity. In this study, we found that milk and soymilk fermented by kefir grains demonstrated significantly decreased GSHPx activity as compared to their unfermented analogues.

There are many examples of biologically active food proteins, featuring physiological significance beyond their purely nutritional requirements that relate to the provision of available nitrogen for normal growth and maintenance. Moreover, in the dietary realm, there exist many physiologically active peptides that may be derived as a consequence of protease activity upon various food protein sources. These peptides can exert a wide range of effects, such as antimicrobial, antihypertensive, antithrombotic, and immunomodulatory (*62*). Therefore, exploiting new products from food-derived bioactive peptides not only increases the utilization of food proteins but also promotes the development of livestock, food, and health-care industries. In a previous study, we found that *L. helveticus* isolated from kefir grains exhibited highly proteolytic activity (*20*). For this reason, future research will be directed to the investigation of bioactive peptides derived from milk and soybean proteins digested by proteolytic enzymes deriving from kefir grains.

ABBREVIATIONS

DPPH, 1,1-diphenyl-2-picrylhydrazyl; GR, glutathione reducatase; GSHPx, glutathione peroxidase; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; NQNO, 4-nitroquinoline-*N*′-oxide; SOD, superoxide dismutase.

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