Protective Effects of the Fermented Milk Kefir on X-Ray Irradiation-Induced Intestinal Damage in B6C3F1 Mice

Kiichiro Teruya,*[,a] Yuki Myojin-Maekawa,[b,#] Fumio Shimamoto,[b] Hiromitsu Watanabe,[c] Noboru Nakamichi,[a] Koichiro Tokumaru,[d] Sennosuke Tokumaru,[d] and Sanetaka Shirahata[a]

[a] *Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University; 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan:* [b] *Department of Health Science, Faculty of Human Culture and Science, Prefectural University of Hiroshima; 1–1–71 Ujina-higashi, Minami-ku, Hiroshima 734–8558, Japan:* [c] *Department of Experimental Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8558, Japan: and* [d] *Nihon Kefir Co., Ltd.; 13–16 Asahi-machi, Fujisawa, Kanagawa 251–0054, Japan.*

Received August 13, 2012; accepted December 3, 2012; advance publication released online December 8, 2012

Gastrointestinal damage associated with radiation therapy is currently an inevitable outcome. The protective effect of Kefir was assessed for its usefulness against radiation-induced gastrointestinal damage. A Kefir supernatant was diluted by 2- or 10-fold and administered for 1 week prior to 8 Gray (Gy) X-ray irradiation at a dose rate of 2 Gy/min, with an additional 15 d of administration post-irradiation. The survival rate of control mice with normal drinking water dropped to 70% on days 4 through 9 post-irradiation. On the other hand, 100% of mice in the 10- and 2-fold-diluted Kefir groups survived up to day 9 post-irradiation ($p<0.05$ and $p<0.01$, respectively). Examinations for crypt regeneration against 8, 10 and 12 Gy irradiation at a dose rate of 4 Gy/min revealed that the crypt number was significantly increased in the mice administered both diluted Kefir solutions ($p<0.01$ for each). Histological and immunohistochemical examinations revealed that the diluted Kefir solutions protected the crypts from radiation, and promoted crypt regeneration. In addition, lyophilized Kefir powder was found to significantly recover the testis weights ($p<0.05$), but had no effects on the body and spleen weights, after 8 Gy irradiation. These findings suggest that Kefir could be a promising candidate as a radiation-protective agent.

Key words Kefir; crypt survival; X-ray; probiotic; reactive oxygen species; mouse

Gastrointestinal complications (GICs) frequently occur following radiation therapy for malignancies in the thoracic, abdominal and pelvic areas.[1,2] Such complications are experienced by up to approximately 80% of radiation-treated patients.[3–5] GICs are caused by damage to ionizing radiation (X-rays, γ-rays)-sensitive tissues that contain rapidly regenerating cells, as exemplified by the gastrointestinal, reproductive and hematopoietic systems.[4–6] Above all, the small intestine is considered to be the most radiosensitive organ.[7] The absorptive epithelial mucous lining of the small intestine bears finger-like structures termed villus projections on its lumen. The base of each villus intrudes inward toward the intestinal base to form a unique structure called the crypt, and the crypt-villus unit represents the functional unit of the small intestine.[8,9] Stem cells are situated in the crypt base and give rise to transit amplifying cells, which continuously divide, move upward along the villous lining and then die out.[8,9] The rapidity of the division rate makes transit cells susceptible to ionizing radiation.[5] Furthermore, radiation-induced degeneration of the functional units in the small intestinal mucosa leads to reductions in the villous height and number, thereby causing GICs such as diarrhea as well as other malfunctions.[7,10] Therefore, the development of drugs and/or foodstuffs for reducing or preventing the side effects inherent to radiation therapy is highly anticipated. To this end, a variety of agents against GICs have been developed from different sources, and tested in experimental animals with varying levels of efficacy.[7,11–19] In addition to these prospective agents, accumulating data suggest that probiotics are promising candidates for the prevention and control of GICs.[20] Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" according to the Food and Agriculture Organization (FAO)/World Health Organization (WHO) guideline.[21] Representative live microorganisms used as probiotics are bacteria, such as *Bacilli* and *Cocci*, yeasts and molds.[20] For therapeutic purposes against radiation-induced GICs, probiotics can be administered singly or as combinations of several strains selected for their anti-diarrheal and anti-inflammatory effects.[3–5,20,22] However, the effectiveness of probiotics against GICs remains inconclusive to date.[4]

A probiotic known as Kefir has become increasingly popular in recent years for its beneficial effects on human health.[23] Kefir was described to originate in the Caucasus mountain areas of Georgia.[23] It is a fermented milk drink produced from Kefir grains, which commonly contain *lactobacilli*, *lactococci*, *streptococci*, acetic acid bacteria and yeasts at various proportions depending on the origin of the grains.[23,24] Owing to this complexity of microorganisms in Kefir grains, more distinctive properties are expected compared with yogurt. The accumulated data on Kefir present a variety of functional properties. For example, Kefir has been evaluated for its anti-cancer effects on breast and colorectal cancers.[25,26] Kefir and other probiotics exhibit antiapoptotic effects through their antioxidative properties.[5,27] Moreover, an attractive property of Kefir is its modulatory effect on the gastrointestinal system.[23,24,28] Protective effects of Kefir on radiation-induced colonic crypt cell apoptosis[27] and freeze-dried Kefir powder on small intestinal crypt survival[29] have been reported.

In the present study, we aimed to extend the previous

[#] Equal contribution with first author.

* To whom correspondence should be addressed. e-mail: kteruya@grt.kyushu-u.ac.jp

studies and to strengthen the foundation of the efficacies inherent to Kefir drink and dried Kefir powder on mouse small intestinal crypt survival and organ weights.

MATERIALS AND METHODS

Reagents Lyophilized Kefir powder and fermented milk product, Kefir, were supplied by Nihon Kefir Co., Ltd. (Kanagawa, Japan). A certified MF (standard) diet was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). A mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Dako-PCNA; PC 10; Code No. M879) was obtained from Dako Co. (Kyoto, Japan). Other materials (hematoxylin, eosin, ethanol, hydrogen peroxide, *etc.*) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals Six-week-old male Crj:B6C3F1 mice were purchased from Charles River Japan Inc. (Hino, Japan). For acclimation, the MF diet and tap water were administered *ad libitum*. Groups of mice were housed in polycarbonate cages and maintained under constant conditions of temperature (24±2°C) and humidity (50±10%) with a 12-h light/12-h dark cycle, following the Guidelines for the Care and Use of Laboratory Animals established by Hiroshima University.

Kefir Supernatant Preparation The maker provided Kefir milk is referred to 1× starter solution in its original concentration. The starter Kefir solution was centrifuged at 12000×***g*** for 30 min. The supernatant was carefully recovered avoiding sediment contamination and referred to as the Kefir stock solution. The Kefir stock solution was diluted 10- and 2-fold with distilled water (D.W.). At 1 week prior to X-irradiation, one group was administered D.W. only as a control, a second group was administered 10-fold-diluted Kefir solution and a third group was administered 2-fold-diluted Kefir solution. Three groups were administered D.W. or Kefir solutions *ad libitum*.

Mixed MF diets were also prepared by adding 0.25%, 0.5%, 1.0% and 2.0% (w/w) lyophilized Kefir powder to the basic MF diet.

Irradiation Radiation effects were assessed by previously reported protocol.[11,12] Briefly, a group of five mice without anesthesia were whole-body-irradiated between 09:00 and 12:00 using an X-ray irradiator (Shinai-go; 200 kV; 25 mA; no filter; Shimadzu, Kyoto, Japan). The irradiation doses were monitored during each exposure using a Radocon 555 dosimeter (Victreen Inc., Cleveland, OH, U.S.A.). The exposure factors were 200 kVp and a half-value layer of 1.18 mm Cu. The X-ray air dose (in R) was converted to the absorbed dose (in cGy) using a factor of 0.95 cGy/R.

For animal survival experiments with diluted Kefir solutions, a group of 10 mice were exposed to 8 Gy of whole-body X-ray irradiation at a dose rate of 2 Gy/min. The mice were observed every day at 08:00, 12:00 and 18:00, and dead mice were recorded. After the X-ray irradiation, the animals were administered the same solutions (D.W. and diluted Kefir solutions) for 15 d. To examine the effects of lyophilized Kefir powder on the body, testis and spleen weights, the irradiated mice (10 mice/group) were observed three times a day for 15 d until all mice died out. Whenever a dead mouse was found, the body weight was recorded and an autopsy was performed to weigh the testis and spleen. One week prior to 8 Gy X-irradiation, the animals were administered MF alone or mixed MF diet containing 0.25, 0.5, 1.0 and 2.0% lyophilized Kefir powder and feeding was continued with the same diets for 15 d post-irradiation.

For the crypt survival (regenerated crypt) experiment, a group of five mice were exposed once to whole-body X-ray irradiation with 8, 10 or 12 Gy at a dose rate of 4 Gy/min. The animals were kept for 3.5 d after irradiation and euthanized for crypt examinations.

Assessment of Crypt Survival and Cellular Proliferation For histological and immunohistochemical evaluations, a segment of the jejunum from the ileocecal junction (30–40 cm) was removed immediately after euthanasia and fixed for 1 h in Carnoy's solution which was prepared right before use by mixing methanol and acetic acid with 3 to 1 ratio. The samples were cut into pieces of approximately 8 mm in length, and formed into a bundle of 10–15 pieces parallel to one another so that the faces of the cross-sections could be seen. The bundled specimens were embedded in paraffin, cross-sectioned at a thickness of 3 μm and subjected to H&E staining. To quantify regenerating crypts, the numbers of crypts per circumference in 10 cross-sections were counted for each mouse.[30]

To examine the cellular proliferation levels in the crypts, the anti-PCNA antibody was used in combination with the avidin-biotin complex method. Briefly, tissue sections were deparaffinized with xylene, hydrated through a graded series of ethanol solutions and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The section were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining, followed by incubation with the mouse monoclonal anti-PCNA antibody (diluted 1 : 200) for 1 h at room temperature. The PCNA-positive cells were observed microscopically and photographed.

Statistical Analysis Statistical significance was determined with Dunnett's method and the Cox proportional hazard model for multiple comparisons using logarithmic transformation and Student's *t*-test. For mice surviving 10 d or longer, survival was compared between any two groups with the two-sided log-rank test. p values of less than 0.05 were regarded as significant.

RESULTS

Effects of Kefir on the Survival of 8 Gy X-Ray-Irradiated Cri B6C3F1 Mice First, we investigated the effects of Kefir on the survival rate of mice monitored for 15 d after 8 Gy irradiation. In this experiment, the MF diet was fed as a basal food in all groups, and the mice administered 10- or 2-fold-diluted Kefir were compared with those given D.W. Mice in the D.W. group started to die as early as day 4 post-irradiation, while the other mice survived up to day 9. The survival rates in the Kefir-administered groups started to decline rather dramatically after day 10 and all the mice had died by day 14 (Fig. 1). All mice given the two types of diluted Kefir survived up to day 9. Mice given 10-fold-diluted Kefir started to die after day 11 and had all died by day 13 (Fig. 1). The survival rate was significantly higher in the 10-fold-diluted Kefir group than in the D.W. group ($p<0.05$). In the 2-fold-diluted Kefir group, the mice started to die after day 10, 10% of mice survived days 13 through 14, and all mice had died by day 15 (Fig. 1). The survival rate was significantly higher in the

2-fold-diluted Kefir group than in the D.W. group ($p<0.01$). The survival rates between the 10- and 2-fold-diluted Kefir groups did not differ significantly ($p=0.0546$). These findings demonstrate that the diluted Kefir solutions contributed to extension of the survival of the X-ray-irradiated mice.

Effects of Kefir on the Intestinal Crypt Numbers in 8 Gy X-Ray-Irradiated Mice The above-described positive effects of the Kefir solutions on the survival of mice after X-ray irradiation prompted us to investigate the protective effects of Kefir on intestinal damage. The number of surviving crypts in one circumference in the D.W. group with 0 Gy irradiation was 114.20±15.95, and referred to as the standard crypt number (Table 1, MF+D.W.). Compared with the standard, the numbers of surviving crypts in the D.W. groups with 8, 10 and 12 Gy irradiation were significantly decreased to 83.32±11.26, 58.04±9.05 and 33.42±5.80, respectively, in a radiation dose-dependent manner (Table 1, MF+D.W. column, $p<0.01$). These findings indicated that the irradiation conditions were appropriate, and we judged that any one of the X-ray doses could be used successfully in the subsequent experiments. When the diluted Kefir solutions were administered in place of D.W., the numbers of surviving crypts were increased significantly for both doses compared with the D.W. group (Table 1, $p<0.01$). These findings demonstrate that the diluted Kefir solutions have protective effects on the crypt damage caused by X-ray irradiation. Unexpectedly, significant differences for the crypt numbers were not observed between the 2- and 10-fold-diluted Kefir solutions.

Histological and Immunohistochemical Examinations of Surviving Crypts As described above, the diluted Kefir solutions were found to protect against the crypt degeneration induced by X-ray irradiation at doses as high as 12 Gy. To correlate the crypt regeneration with histological changes, we visualized the crypt areas using H&E and PCNA staining methods after 10 Gy irradiation (Fig. 2). The results of the histological and immunohistochemical examinations (Figs. 2A–C, 3A–C) were well correlated with the regenerated crypt numbers before and after irradiation (Table 1, 58.04±9.05 *vs.* 70.96±8.25, $p<0.01$). Furthermore, the anti-PCNA antibody detected proliferating cell nuclei (Figs. 3A–C), demonstrating that the Kefir solutions promoted regeneration of the damaged intestinal crypts by X-ray irradiation.

Effects of Lyophilized Kefir Powder on the Body, Testis and Spleen Weights We previously reported that MF diets containing 0.5–2.0% 6-d-old fresh lyophilized Kefir powder significantly improved the crypt survival rate.[29] In addition, animal survival experiments showed that the MF diet containing 2% Kefir powder significantly increased the survival rate compared with the MF diet alone.[29] To further establish the effects of Kefir *in vivo*, we measured three parameters after irradiation. During the 15-d observation period after irradiation, the body weights of dead mice and their isolated testis and spleen weights were measured (Table 2). The body, testis and spleen weights of irradiated mice were significantly decreased in a manner that was independent of the Kefir powder supplementation ($p<0.01$ or $p<0.05$). Addition of Kefir powder to the MF basal diet at various amounts (0.25–2.0%) did not recover the body and spleen weights from the decreased states. However, the testis weight of mice fed Kefir powder at 0.25–2.0% recovered significantly from the decreased state after irradiation ($p<0.01$). This finding was more evident when the ratios of the testis and body weights were examined (Table 2, $p<0.01$). Although a significant increase in the body weight of 0.25% Kefir-fed mice was noted sporadically ($p<0.01$), correlations between the body and spleen weights and the amount of Kefir powder were not evident. Comparisons of the body and spleen weights with or without Kefir supplementation did not show significant changes (Table 2, compare 0% *vs.* 2% Kefir without irradiation). Based on these findings, Kefir powder was demonstrated to increase the testis weight from the decreased state caused by irradiation.

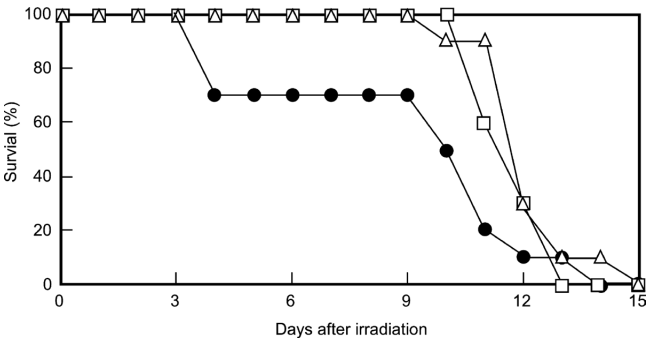

Fig. 1. Effects of Diluted Kefir Solutions on Mouse Survival after 8 Gy Irradiation

All groups of mice (10 mice/group) were administered the standard MF diet, and supplemented with distilled water (D.W.), and 10-fold- or 2-fold-diluted Kefir solutions for 7 d prior to 8 Gy whole-body X-irradiation. The surviving mice were counted every day for 15 d after the irradiation. ●: D.W. group. □: 10-fold-diluted Kefir group. △: 2-fold-diluted Kefir group. D.W. *vs.* 10-fold-diluted Kefir: $p<0.05$; D.W. *vs.* 2-fold-diluted Kefir: $p<0.01$; 10-fold-diluted Kefir *vs.* 2-fold-diluted Kefir: $p>0.05$.

Effects of Lyophilized Kefir Powder on the Food and Water Consumption by 8 Gy-Irradiated Mice As described above, lyophilized Kefir powder was shown to recover the testis weights, but not the body and spleen weights. To examine whether these findings were related to food and water consumption, we measured these parameters. As shown in Table 3, food consumption was nearly constant among the

Table 1. Effects of Diluted Kefir Solutions on the Regeneration of Small Intestinal Crypt Numbers

Diets	Radiation doses (Gy)			
	0	8	10	12
D.W.	114.20±15.95	83.32±11.26*	58.04±9.05*	33.42±5.80*
10-Fold-dil		97.14±11.02**	70.50±8.07**	53.24±7.01**
2-Fold-dil		101.90±12.17**	70.96±8.25**	50.92±5.15**

All groups of mice (5 mice/group) were administered the standard MF diet, and supplemented with distilled water (D.W.), and 10-fold- or 2-fold-diluted Kefir solutions. *$p<0.01$: crypt numbers in the 8, 10 and 12 Gy-exposed groups are significantly reduced compared with the 0 Gy-exposed D.W. group. **$p<0.01$: crypt numbers in the 10-fold- or 2-fold-diluted Kefir groups are significantly increased compared the D.W. group after 8, 10 and 12 Gy exposure at a dose rate of 4 Gy/min. Values are expressed as means±S.D.

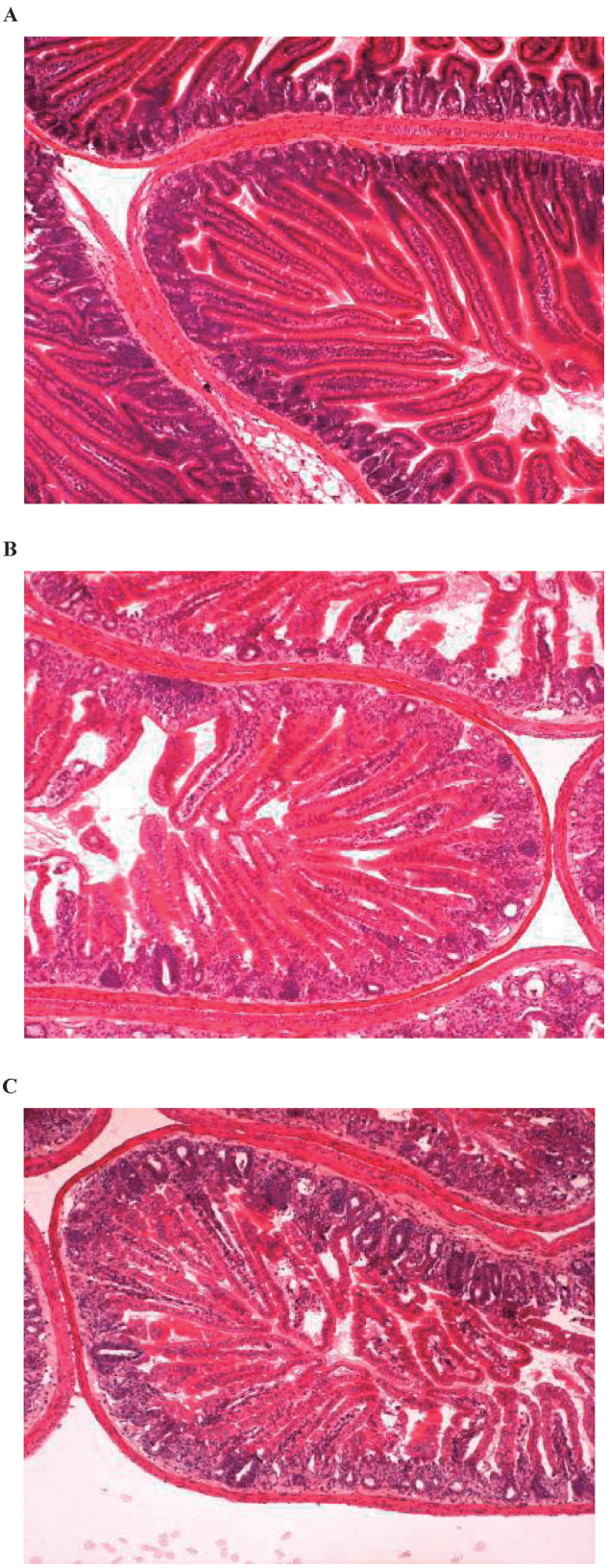

Fig. 2. Histological Examination of Surviving Crypts in 10 Gy-Irradiated Mice

The mice were kept for 3.5 d after 10 Gy irradiation and euthanized for crypt examination. Cross-sections were subjected to H&E staining for microscopic observation. (A) Normal intestine; (B) 10 Gy-irradiated intestine; (C) 2-fold-diluted Kefir intestine exposed to 10 Gy irradiation.

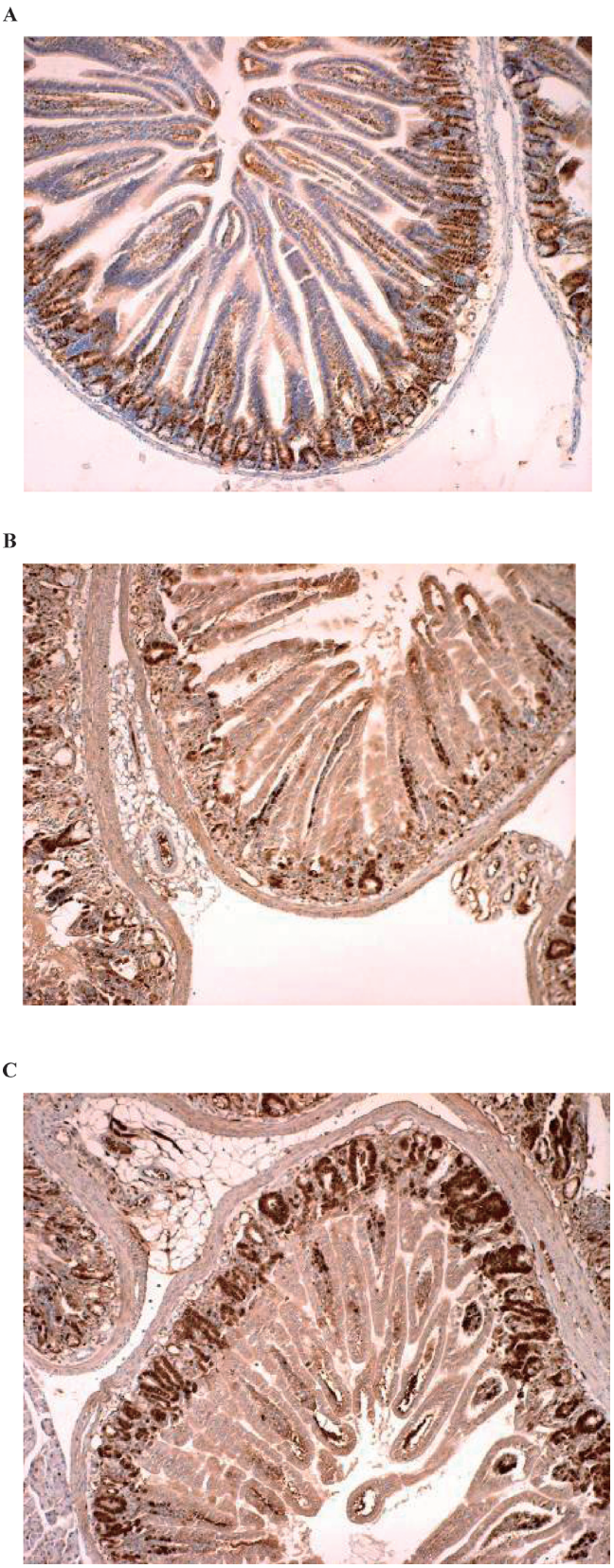

Fig. 3. Immunohistochemical Examination of Surviving Crypts in 10 Gy-Irradiated Mice

The mice were kept for 3.5 d after 10 Gy irradiation and euthanized for crypt examination. The cellular proliferation levels in the crypts were examined by immunohistochemical staining with an anti-PCNA antibody. (A) Normal intestine; (B) 10 Gy-irradiated intestine; (C) 2-fold-diluted Kefir intestine exposed to 10 Gy irradiation.

Table 2. Effects of Lyophilized Kefir Powder on the Body, Testis and Spleen Weights Measured for 15 d after 8 Gy Irradiation

Kefir (%)	X-Ray (8 Gy)	B.W. (g)	Testis (g)	Testis/B.W. ×100 (%)	Spleen (g)
0.0	−	26.6±0.7	0.173±0.009	0.65±0.04	0.102±0.017
0.0	+	18.7±1.7*	0.070±0.016*	0.37±0.08*	0.033±0.011*
0.25	+	19.4±2.3*	0.100±0.012*,***	0.53±0.10**,***	0.027±0.006*
0.5	+	16.4±1.3*	0.098±0.026*,***	0.56±0.20***	0.033±0.011*
1.0	+	17.0±1.4*	0.101±0.014*,***	0.59±0.08***	0.029±0.013*
2.0	+	17.0±1.1*	0.095±0.012*,***	0.56±0.06***	0.025±0.012*
2.0	−	26.6±1.2	0.187±0.020	0.70±0.06	0.100±0.008

*$p<0.01$, **$p<0.05$: the body, testis and spleen weights in the MF alone group differ significantly with (+) and without (−) 8 Gy irradiation at a dose rate of 2 Gy/min. ***$p<0.01$: three parameters in the MF alone (0% Kefir)-fed group differ significantly from those in the 8 Gy-exposed Kefir-fed groups. Each group consisted of 10 mice. Values are expressed as means±S.D.

Table 3. Daily Consumption of Food and Water after 8 Gy Irradiation

Kefir (%)	Before irradiation		After 8 Gy irradiation	
	Food (g)	Water (mL)	Food (g)	Water (mL)
0.0	4.0±0.3	5.0±0.4	2.7±0.4*	3.5±1.0*
0.25	4.2±0.4	6.2±0.6	3.1±0.3*	4.7±0.9*
0.5	4.4±0.4	6.6±0.7	3.2±0.8*	3.9±1.2*
1.0	4.3±0.5	6.2±0.5	3.0±0.5*	4.1±0.9*
2.0	4.0±0.6	5.9±0.5	3.1±0.6**	3.5±0.9*

The MF basic diet was supplemented with 0.0–2.0% Kefir powder. *$p<0.01$, **$p<0.05$: significant reductions in food and water consumption after 8 Gy irradiation at a dose rate of 2 Gy/min. Each group consisted of 5 mice. Values are means±S.D.

mice fed the different amounts of Kefir before irradiation. After the mice were irradiated, averaged food consumption measured for 3 d was significantly reduced in all the Kefir-supplemented groups ($p<0.01$ or $p<0.05$). The food consumption in the MF diet (0% Kefir)-fed group was most affected ($p<0.01$), suggesting a protective effect of Kefir. However, notable differences in the food consumption were not observed among the mice fed the various amounts of Kefir after 8 Gy irradiation. Furthermore, the trends for averaged water consumption measured for 3 d were similar to those for food consumption. Therefore, reduced daily food and water consumption after 8 Gy irradiation can be interpreted as a major cause of the reduced body and spleen weights.

DISCUSSION

In this study, we have demonstrated a significant increase in the regeneration of small intestinal crypts and an improved average lifespan of mice receiving diluted Kefir solutions. The present results further strengthen previously reported data.[27,29)]

Radiation therapies in combination with surgical and/or chemotherapy are commonly used to accomplish maximum efficacy against malignancies. However, such efforts concomitantly bring about damage to radiosensitive normal tissues. Upon radiation, rapidly proliferating normal tissues, such as the intestinal mucosa and reproductive organs, are affected acutely and chronically.[6,31,32)] The major function of the small intestine is the absorption of nutrients from ingested foods through the crypt-villus units, which are critically important for the maintenance of health.[8,9)] However, functional crypt-villus units are susceptible to radiation because they contain rapidly dividing transit cells.[5)] The degeneration of such cells leads to subsequent reductions in the villous height and number.[7,10)] In addition, the degeneration of small intestinal mucosa cells is linked to massive amounts of free radicals generated by the interactions of radiation energy with intracellular water molecules.[5,33,34)] Free radicals attack a variety of intracellular biomolecules that eventually kill the cells by apoptosis.[4,5)] Kefir is known to exhibit beneficial effects on the gastrointestinal tract when consumed as a drink or dried powder.[3,4,20,28,35)] Kefir drinks as a whole would contain live bacteria, yeasts, milk components and fermentation metabolites. The milk components include proteins, carbohydrates and vitamins among others. In addition, the fermentation metabolites comprise amino acids, bacteriocins, polysaccharides and others.[24)] In this study, we centrifuged Kefir milk to prepare a Kefir supernatant, which was expected to contain two major components, *i.e.*, milk constituents and fermentation metabolites. Thus, the usage of a Kefir supernatant allows us to limit the prospective functional ingredients to be within the two major components. We demonstrated that diluted Kefir solutions regained the reduced crypt numbers caused by X-ray irradiation. The present findings suggest possible mechanisms to explain the observed effects. One possibility is that the Kefir components contain antioxidants that directly scavenge radiation-induced reactive oxygen species.[5,36–39)] Several antioxidative enzymes have been described to exist in milk.[39)] However, further study demonstrated that fermentation of Kefir for 32 h did not increase the superoxide dismutase levels and rather decreased the glutathione peroxidase levels.[39)] In addition, catalase activity was not detected in either milk or Kefir.[39)] Therefore, it seems that such antioxidants in milk are less likely to be involved in the antioxidative activity observed in the present study. On the other hand, Liu *et al.*[39)] reported that the reducing power of Kefir was significantly increased during 32 h of fermentation. They suggested that certain metabolites are involved in the reducing power. Amino acids, one of the Kefir metabolites, were reported to exhibit repairing effects on the irradiated colonic wall of rats.[14,18)] Another study demonstrated that amino acids exert antiapoptotic

effects on radiation-induced intestinal damage.[7)]

An alternative mechanism is that Kefir components enhance reactive oxygen species-scavenging enzyme levels in the actively dividing cells. In this regard, a previous study showed that Kefir activates antioxidative enzyme systems, such as reduced glutathione, glutathione peroxidase, glutathione *S*-transferase, lipid peroxidation and catalase, in the liver and kidney.[40)] There is also a report that the antioxidative activity of Kefir toward neural stem cells in culture is mediated by a microbial fermentation product with a low molecular weight (MW) of <3,500 that is heat- and acid/base-stable. This molecule is suggested to be distributed throughout the body after absorption *via* the intestine.[37)] Therefore, such a molecule could act as an enhancer for the production of antioxidative enzymes.

Whole-body irradiation gave us the opportunity to investigate the protective effects of Kefir on radiation-sensitive male reproductive organs. Our study demonstrated that the testis weight was significantly reduced after 8 Gy whole-body irradiation. In agreement with this finding, decreased testicular weights were reported after exposure of rat testes to X-ray irradiation.[6)] Similarly, significant testicular weight losses were observed in mice after total-body irradiation[41)] and focal pelvic gamma irradiation.[42)] These findings are interpreted to indicate that irradiation of the testes induces germ cell apoptosis, particularly in actively dividing spermatogonia and preleptotene spermatocytes, thereby causing infertility in the irradiated testes.[42,43)] As a consequence, dead cells are removed from the testicular tubules, resulting in reduced testis weights.[34)] It is of importance to find ways to protect against radiation-induced germ cell loss. Otala *et al.*[41)] reported that sphingosine-1-phosphate (S1P) could protect against germ cell loss caused by radiation. Among other functions, S1P inhibits ceramide-mediated apoptosis. S1P is formed by phosphorylation of sphingosine by sphingosine kinase. Sphingosine is an acylation product of ceramide, which is formed from more complex sphingolipids, such as sphingomyelin (SPM). Regulation of apoptosis is exerted *via* conversion of SPM to ceramide mediated by sphingomyelinases, which are upregulated by ionizing radiation and oxidative stress.[41)] SPM is distributed in the plasma membrane and intracellular membrane structures in all eukaryotic cells.[44)] In addition, intracellular sphingosine in the colonic cells is thought to be derived from more complex sphingolipids like SPM or from dietary sources.[44)] Germ cell apoptosis started soon after irradiation, and protection by S1P was observed at 21 d after irradiation.[41)] Therefore, sphingolipids and their metabolites appear to play roles in germ cell biogenesis. Indeed, a Kefir powder extract was reported to contain SPM, comprising a mixture of four SPM species, as the active substance responsible for enhancing interferon-β production.[45)] The origin of the SPM in Kefir is milk, because bovine milk contains SPM and SPM is not synthesized by the microorganisms contained in Kefir grains.[44,45)] Combining these data together, the significant recovery of the testis weights could be partly attributable to the SPM contained in Kefir.

It is well recognized that overloading of an energy-rich diet is likely to bring about obesity and is causally linked to metabolic syndrome.[46)] From this point of view, we considered whether Kefir consumption affected the body weight. However, comparisons of the body and spleen weights with or without Kefir supplementation did not show any significant differences. In addition, the daily consumption of food and water before 8 Gy irradiation was similar among the Kefir-supplemented groups. Another study also showed that Kefir supplementation did not affect the body weight, body growth index or weights of the liver, intestine, stomach and pancreas.[28)] Thus, daily and prolonged consumption of Kefir does not appear to induce adverse effects like obesity or its related complications. In this study, we considered the effects of Kefir on the intestinal mucosa and testis in relation to its antioxidative properties. In addition, there are reports on the anti-inflammatory[47,48)] and mucosal immunomodulatory[24,29)] effects of Kefir, suggesting that these efficacies may have cooperatively contributed to the present results.

We previously suggested that the factor responsible for the antioxidative activity has a MW of less than 3500,[37)] while that for the thymine dimer repair enzyme activity has a MW of less than 5000[36)] and that for the glucose uptake enhancing activity has a MW of less than 1000.[38)] Based on these data, one of our ongoing tasks is to identify the substances responsible for the anti-GIC activity, and to relate such substances with those described above.

In summary, we have demonstrated experimentally that a Kefir supernatant and dried Kefir powder both exert radiation-protective effects on the small intestinal mucosa and male reproductive organs without body weight gain. Therefore, daily intake of the fermented milk Kefir is strongly anticipated to exhibit protective effects against the damage associated with therapeutic radiation. In addition, such intake habits could be beneficial in reducing the damage caused by irradiation from unexpected nuclear accidents like the Fukushima Daiichi nuclear plant disaster, which took place in Japan on 11 March, 2011.

Conflict of Interest This study was supported by Nihon Kefir Co., Ltd. K. Tokumaru and S. Tokumaru are Employees of Nihon Kefir Co., Ltd.

REFERENCES

1) Rodríguez ML, Martín MM, Padellano LC, Palomo AM, Puebla YI. Gastrointestinal toxicity associated to radiation therapy. *Clin. Transl. Oncol.*, **12**, 554–561 (2010).
2) Naymagon S, Warner RRP, Patel K, Harpaz N, Machac J, Weintraub JL, Kim MK. Gastroduodenal ulceration associated with radioembolization for the treatment of hepatic tumors: an institutional experience and review of the literature. *Dig. Dis. Sci.*, **55**, 2450–2458 (2010).
3) Ciorba MA, Stenson WF. Probiotic therapy in radiation-induced intestinal injury and repair. *Ann. N. Y. Acad. Sci.*, **1165**, 190–194 (2009).
4) Maria-Aggeliki KS, Nikolaos KL, Kyrias GM, Vassilis KE. The potential clinical impact of probiotic treatment for the prevention and/or anti-inflammatory therapeutic effect against radiation induced intestinal mucositis. A review. *Recent Pat. Inflamm. Allergy Drug Discov.*, **3**, 195–200 (2009).
5) Spyropoulos BG, Misiakos EP, Fotiadis C, Stoidis CN. Antioxidant properties of probiotics and their protective effects in the pathogenesis of radiation-induced enteritis and colitis. *Dig. Dis. Sci.*, **56**, 285–294 (2011).
6) Schally AV, Paz-Bouza JI, Schlosser JV, Karashima T, Debeljuk L, Gandle B, Sampson M. Protective effects of analogs of luteinizing hormone-releasing hormone against X-radiation-induced testicular

damage in rats. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 851–855 (1987).
7) Erbil Y, Öztezcan S, Giriş M, Barbaros U, Olgaç V, Bilge H, Küçücük H, Toker G. The effect of glutamine on radiation-induced organ damage. *Life Sci.*, **78**, 376–382 (2005).
8) Shaker A, Rubin DC. Intestinal stem cells and epithelial-mesenchymal interactions in the crypt and stem cell niche. *Transl. Res.*, **156**, 180–187 (2010).
9) Simons BD, Clevers H. Stem cell self-renewal in intestinal crypt. *Exp. Cell Res.*, **317**, 2719–2724 (2011).
10) Andreyev J. Gastrointestinal symptoms after pelvic radiotherapy: a new understanding to improve management of symptomatic patients. *Lancet Oncol.*, **8**, 1007–1017 (2007).
11) Ohara M, Lu H, Shiraki K, Ishimura Y, Uesaka T, Katoh O, Watanabe H. Radioprotective effects of miso (fermented soy bean paste) against radiation in B6C3F1 mice: increased small intestinal crypt survival, crypt lengths and prolongation of average time to death. *Hiroshima J. Med. Sci.*, **50**, 83–86 (2001).
12) Kubo N, Myojin Y, Shimamoto F, Kashimoto N, Kyo E, Kamiya K, Watanabe H. Protective effects of a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia and *Agaricus blazei* murill against X-irradiation in B6C3F1 mice: Increased small intestinal crypt survival and prolongation of average time to animal death. *Int. J. Mol. Med.*, **15**, 401–406 (2005).
13) de Moraes Ramos FM, Schönlau F, Novaes PD, Manzi FR, Bóscolo FN, de Almeida SM. Pycnogenol protects against Ionizing radiation as shown in the intestinal mucosa of rats exposed to X-rays. *Phytother. Res.*, **20**, 676–679 (2006).
14) Diestel CF, Marques RG, Lopes-Paulo F, Paiva D, Horst NL, Caetano CER, Portela MC. Role of L-glutamine and glycine supplementation on irradiated colonic wall. *Int. J. Colorectal Dis.*, **22**, 1523–1529 (2007).
15) Matsuu-Matsuyama M, Shichijo K, Okaichi K, Nakayama T, Nakashima M, Uemura T, Niino D, Sekine I. Protection by polaprezinc against radiation-induced apoptosis in rat jejunal crypt cells. *J. Radiat. Res.* (Tokyo), **49**, 341–347 (2008).
16) Bhanja P, Saha S, Kabarriti R, Liu L, Roy-Chowdhury N, Roy-Chowdhury J, Sellers RS, Alfieri AA, Guha C. Protective role of R-spondin 1, an intestinal stem cell growth factor, against radiation-induced gastrointestinal syndrome in mice. *PLoS ONE*, **4** (e8014), 1–10 (2009).
17) Qiu W, Leibowitz B, Zhang L, Yu J. Growth factors protect intestinal stem cells from radiation-induced apoptosis by suppressing PUMA through the PI3K/AKT/p53 axis. *Oncogene*, **29**, 1622–1632 (2010).
18) de Aguiar Picanço E, Lopes-Paulo F, Marques RG, Diestel CF, Caetano CER, de Souza MVM, Moscoso GM, Pazos HMF. L-Arginine and glycine supplementation in the repair of the irradiated colonic wall of rats. *Int. J. Colorectal Dis.*, **26**, 561–568 (2011).
19) Leibowitz BJ, Qiu W, Liu H, Cheng T, Zhang L, Yu J. Uncoupling p53 functions in radiation-induced intestinal damage *via* PUMA and p21. *Mol. Cancer Res.*, **9**, 616–625 (2011).
20) Narayan SS, Jalgaonkar S, Shahani S, Kulkarni VN. Probiotics: current trends in the treatment of diarrhoea. *Hong Kong Med. J.*, **16**, 213–218 (2010).
21) FAO. "FAO/WHO Guidelines for the evaluation of probiotics in food.": <ftp://ftp.org/es/esn/food/wgreport2.pdf.>, cited 2002.
22) Henriksson R, Franzén L, Sandström K, Nordin A, Arevärn M, Grahn E. Effects of active addition of bacterial cultures in fermented milk to patients with chronic bowel discomfort following irradiation. *Support. Care Cancer*, **3**, 81–83 (1995).
23) Farnworth ER. Kefir—a complex probiotic. *Food Sci. Technol. Bull.: Functional Foods*, **2**, 1–17 (2005).
24) Guzel-Seydim ZB, Kok-Tas T, Greene AK, Seydim AC. Review: functional properties of Kefir. *Crit. Rev. Food Sci. Nutr.*, **51**, 261–268 (2011).
25) de Moreno de LeBlanc A, Matar C, Farnworth E, Perdigon G. Study of cytokines involved in the prevention of a murine experimental breast cancer by Kefir. *Cytokine*, **34**, 1–8 (2006).
26) Topuz E, Derin D, Can G, Kürklü E, Çınar S, Aykan F, Cevikbaş A, Dişçi R, Durna Z, Şakar B, Saglam S, Tanyeri H, Deniz G, Gürer Ü, Taş F, Guney N, Aydıner A. Effect of oral administration of Kefir on serum proinflammatory cytokines on 5-FU induced oral mucositis in patients with colorectal cancer. *Invest. New Drugs*, **26**, 567–572 (2008).
27) Matsuu M, Shichijo K, Okaichi K, Wen CY, Fukuda E, Nakashima M, Nakayama T, Shirahata S, Tokumaru S, Sekine I. The protective effect of fermented milk Kefir on radiation-induced apoptosis in colonic crypt cells of rats. *J. Radiat. Res.* (Tokyo), **44**, 111–115 (2003).
28) Urdaneta E, Barrenetxe J, Aranguren P, Irigoyen A, Marzo F, Ibáñez FC. Intestinal beneficial effects of Kefir-supplemented diet in rats. *Nutr. Res.*, **27**, 653–658 (2007).
29) Myojin Y, Kashimoto N, Kashiwabara S, Kamiya K, Watanabe H, Teruya K, Shirahata S. The protective effects of fermented milk in small intestinal crypt survival and probability of survival of mice. *Hiroshima Igaku*, **59**, 383–385 (2006), in Japanese.
30) Potten CS, Rezvani M, Hendry JH, Moore JV, Major D. The correction of intestinal microcolony counts for variation in size. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, **40**, 321–326 (1981).
31) Mason KA, Milas L, Peters LJ. Effect of paclitaxel (taxol) alone and in combination with radiation on the gastrointestinal mucosa. *Int. J. Radiat. Oncol. Biol. Phys.*, **32**, 1381–1389 (1995).
32) Baliga MS, Rao S. Radioprotective potential of mint: a brief review. *J. Cancer Res. Ther.*, **6**, 255–262 (2010).
33) Agrawal A, Choudhary D, Upreti M, Rath PC, Kale RK. Radiation induced oxidative stress: I. Studies in Ehrlich solid tumor in mice. *Mol. Cell. Biochem.*, **223**, 71–80 (2001).
34) Liu G, Gong P, Bernstein LR, Bi Y, Gong S, Cai L. Apoptotic cell death induced by low-dose radiation in male germ cells: hormesis and adaptation. *Crit. Rev. Toxicol.*, **37**, 587–605 (2007).
35) Umeda C, Sonoyama K, Yamaguchi N, Saito R, Akashi K, Motoshima H, Kawabata J. Oral administration of freeze-dried kefir reduces intestinal permeation of and oral sensitization to ovalbumin in mice. *Biosci. Biotechnol. Biochem.*, **69**, 249–251 (2005).
36) Nagira T, Narisawa J, Teruya K, Katakura Y, Shim S-Y, Kusumoto K, Tokumaru S, Tokumaru K, Barnes DW, Shirahata S. Suppression of UVC-induced cell damage and enhancement of DNA repair by the fermented milk, Kefir. *Cytotechnology*, **40**, 125–137 (2002).
37) Kusumoto K, Helmrich A, Mericko P, Chen L, Sato JD, Shirahata S, Tokumaru S, Barns D. The protective anti-oxidant effects of Kefir on SFME neural stem cells. (eds.) S. Shirahata *et al. Animal cell Technology: Basic & Applied Aspects*, **12**, 353–357 (2002).
38) Teruya K, Yamashita M, Tominaga R, Nagira T, Shim S-Y, Katakura Y, Tokumaru S, Tokumaru K, Barnes DW, Shirahata S. Fermented milk, Kefram-Kefir enhances glucose uptake into insulin-responsive muscle cells. *Cytotechnology*, **40**, 107–116 (2002).
39) Liu J-R, Chen M-J, Lin C-W. Antimutagenic and antioxidant properties of milk-Kefir and soymilk-Kefir. *J. Agric. Food Chem.*, **53**, 2467–2474 (2005).
40) Güven A, Güven A, Gülmez M. The effect of kefir on the activities of GSH-Px, GST, CAT, GSH and LPO levels in carbon tetrachloride-induced mice tissues. *J. Vet. Med. B Infect. Dis. Vet. Public Health*, **50**, 412–416 (2003).
41) Otala M, Suomalainen L, Pentikäinen MO, Kovanen P, Tenhunen M, Erkkilä K, Toppari J, Dunkel L. Protection from radiation-induced male germ cell loss by sphingosine-1-phosphate. *Biol. Reprod.*, **70**, 759–767 (2004).
42) Kim J, Lee S, Jeon B, Jang W, Moon C, Kim S. Protection of spermatogenesis against gamma ray-induced damage by granulocyte colony-stimulating factor in mice. *Andrologia*, **43**, 87–93 (2011).
43) Kanter M, Topcu-Tarladacalisir Y, Parlar S. Antiapoptotic effect of L-carnitine on testicular irradiation in rats. *J. Mol. Histol.*, **41**, 121–128 (2010).

44) Vesper H, Schmelz E-M, Nikolova-Karakashian MN, Dillehay DL, Lynch DV, Merrill AH Jr. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.*, **129**, 1239–1250 (1999).
45) Osada K, Nagira K, Teruya K, Tachibana H, Shirahata S, Murakami H. Enhancement of interferon-β production with sphingomyelin from fermented milk. *Biotherapy*, **7**, 115–123 (1994).
46) Farooqui AA, Farooqui T, Panza F, Frisardi V. Metabolic syndrome as a risk factor for neurological disorders. *Cell. Mol. Life Sci.*, **69**, 741–762 (2012).
47) Kwon O-K, Ahn K-S, Lee M-Y, Kim S-Y, Park B-Y, Kim M-K, Lee I-Y, Oh S-R, Lee H-K. Inhibitory effect of kefiran on ovalbumin-induced lung inflammation in a murine model of asthma. *Arch. Pharm. Res.*, **31**, 1590–1596 (2008).
48) Uchida M, Ishii I, Inoue C, Akisato Y, Watanabe K, Hosoyama S, Toida T, Ariyoshi N, Kitada M. Kefiran reduces atherosclerosis in rabbits fed a high cholesterol diet. *J. Atheroscler. Thromb.*, **17**, 980–988 (2010).
49) Vinderola G, Perdigón G, Duarte J, Farnworth E, Matar C. Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. *Cytokine*, **36**, 254–260 (2006).