Arch Pharm Res Vol 29, No 9, 735-740, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Effect of Ginseng Polysaccharide on the Stability of Lactic Acid Bacteria during Freeze-drying Process and Storage

Seung-Hyun Yang, Sung-Hoon Seo, Sang-Wook Kim[1], Seung-Ki Choi[2], and Dong-Hyun Kim

College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea, [1]Kuan Industry Co., Ltd, Seoul, Korea, and [2]School of Medicine, Pochon Cha University, Kyungki-Do 463-712, Korea

(Received January 27, 2006)

Lactic acid bacteria (LAB) quickly attenuate or are killed during the freeze-drying process and storage. The effect of some natural polysaccharides, which are known as potent antitumor and immunomodulating substances, on the viability of the LAB, *Lactobacillus acidophilus* and *Bifidobacterium breve*, on freeze-drying and storage were investigated. Among the polysaccharides tested, red ginseng polysaccharide (RGP) and chitosan significantly inhibited the cell death of the LAB during freeze-drying, and fucoidan and RGP most potently protected the cell death of the LAB during storage. The stabilities of the LAB on the addition of RGP and fucoidan were comparable to that of skimmed milk. However, white ginseng polysaccharide (WGP) did not promote storage stability. When 5% skimmed milk/5% RGP treated LAB were freeze-dried and stored, their viabilities were found to be significantly higher those treated with 5% or 10% RGP. The stabilizing effect of 5% RGP/5% skimmed milk during LAB freeze-drying and storage stability was comparable to that of treatment with 10% skimmed milk. Based on these findings, we believe that RGP beneficially improves the stability of LAB during the freeze-dry process and storage.

Key words: Lactic acid bacterial, Ginseng, Polysaccharide, Stability

INTRODUCTION

Lactic acid bacteria (LAB) are regarded as safe microorganisms, with some having been claimed to enhance health when introduced into the diet (Collins *et al.*, 1999; Salminen *et al.*, 1974). The main reported beneficial effects of probiotics are related to alterations in the expressions of indigenous microflora (Campieri and Gionchetti, 1999; Perdigon *et al.,* 1991), the amelioration of their growth (Collins *et al.*, 1999; Salminen *et al.*, 1974), their antidiabetic and antihyperlipidemic effects (Tabuchi *et al.*, 2003; Taranto *et al.*, 1998), health enhancement through the inhibition of carcinogenesis (Adachi, 1992; Perdigon *et al.*, 1991) and via the non-specific activation of the host immune system (Adachi, 1992; Salminen *et al.*, 1974). Moreover, a favorable image and the elucidated biological effects of LAB have led to the probiotic concept.

Many compounds have been examined with a view toward improving the survival of LAB during freeze-drying and storage, including polyols, polysaccharides, disaccharides, amino acids, proteins, vitamins and salts (Champagne *et al.*, 1991 and 1996). Some macromolecules, such as polyethylene glycol (PEG), dextran and bovine albumin, have been found to increase the survival of LAB during freeze-drying (Font de Valdez, 1983). Particularly, some natural polysaccharides are the best known and most potent herbal medicine-derived substances, with both antitumor and immunomodulating properties (Kim *et al.*, 1990; Kim *et al.*, 1996; Mizuno *et al.*, 1995). Nevertheless, milk-based formulae have been commercially used to improve the stability of LAB but these agents have not found application as they confer no suitable biological cost effective benefits.

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), is frequently used in Asian countries as a traditional medicine. Its major active components are polysaccharides and ginsenosides (Kim *et al.*, 1990 and 2000; Lee *et al.*, 2001). The ginseng polysaccharide (GP) has been reported to exhibit various biological activities, including anti-tumor effects and anti-hemagglutination induced by *Helicobacter pylori* (Belogortseva *et al.*, 2002;

Correspondence to: Dong-Hyun Kim, College of Pharmacy, Kyung Hee University, 1, Hoegi, Dongdaemun-ku, Seoul 130-701, Korea
Tel. 82-2-961-0374, Fax 82-2-966-3885
E-mail: dhkim@khu.ac.kr

Kim *et al.*, 1990). Recently, methods for the isolation of this polysaccharide have been developed (Ahn *et al.*, 2006; Lim *et al.*, 2002). However, the stabilizing effects of GP on the freeze-drying and storage on LAB remain to be studied.

During the screening program of natural products to discover a stabilizer for the storage of LAB red ginseng polysaccharide (RGP) was found to increase the stability of LAB survival during storage. Therefore, in this study, the inhibitory effects of RGP on the cell attenuation and cell death of LAB during freeze-drying and storage were examined.

MATERIALS AND METHODS

Materials and bacterial cells

General anaerobic medium (GAM) was purchased from Nissui Pharm. Co., Ltd (Japan). MRS broth and skimmed milk were purchased from Difco Co. (U.S.A.); fucoidan, apple pectin, chitosan and inulin from Sigma Co. (U.S.A.).

Lactobacillus acidophilus KCTC 3168 was purchased from the Korea Collection for Type Cultures (Korea), and *Bifidobacterium breve* K-110 KCCM 10097 from the Korea Culture Center of Microorganisms (Korea).

Polysaccharide extraction

Dried white ginseng (WG) and red ginseng (RG) (1 kg) extracts were prepared from the roots of *Panax ginseng* C.A. Meyer (Araliaceae), by initially defatting three times with aqueous 70% ethanol for 2 h at 80°C. The remaining residues were extracted twice with 5L of water for 2 h at 60°C. Extracts were centrifuged to remove insoluble materials, dialyzed for 5 days at 4°C and then precipitated by the addition of the same volume of cold ethanol. The resulting precipitates were freeze-dried, and referred to as WG polysaccharide (WGP) and RGP, respectively. Their average molecular weights were determined to be >100-70 and 90-40 kDaltons, respectively, using Sephadex G-75 column chromatography.

LAB Culture and dehydration

The previously cultured LAB (10 mL) were inoculated into 300 mL of MRS broth and cultured at 37°C for 20 h. The LAB (2 g as a wet weight) were then collected by centrifugation at 4°C and 5,000×g for 30 min, washed with saline, resuspended in 100 mL of 2, 5 or 10% skimmed milk containing 5 or 10% WGP or RGP, and then frozen at -4 and -40°C or in liquid nitrogen. Frozen samples were lyophilized using a freeze-drier (Eyela, Tokyo) at -20°C and 1.5 mmHg for 20 h.

Storage of dried LAB

Each of the freeze-dried LAB was placed in both 15 mL closed and open polyester bottle, and stored at both 4 and 25°C. The viabilities of the test organisms and their moisture contents were measured at predetermined time intervals.

Enumeration of test organisms

To count the number of bacteria in the freeze-dried samples, 0.1 g of dried LAB was mixed with 0.9 mL of peptone water, vortexed for 15 s and then serially diluted with peptone water. Serially diluted samples (0.2 mL) were plated on GAM agar plates, and then anaerobically cultured for 72 h at 37°C in an anaerobic BBL GasPak™ (Becton, Dickinson and Company, MD, U.S.A.). The olonies appearing on the plates were counted, and colony-forming units (CFUs) per mg calculated.

Statistical analysis

All data were obtained in triplicate, and are expressed as the means ± standard deviation. Statistical significance was analyzed by a one way ANOVA followed by the Student-Newman-Keuls test. P values of <0.05 were considered statistically significant.

RESULTS

Effect of some natural polysaccharides on stability of LAB during freeze-drying process

Freeze-drying can either attenuate or kill LAB cells. Therefore, some polysaccharides were added as stabilizers of *L. acidophilus* and *B. breve* during the freeze-drying process, and their protective effect against attenuation or killing evaluated (Fig. 1). The percentage of LAB surviving without the addition of polysaccharides was dramatically reduced by the freeze-drying process. However, the addition of skimmed milk increased the survival of *L. acidophilus* the most, followed by the additions of chitosan and RGP. The addition of these polysaccharides to *B. breve* prior to freeze-drying also potently increased the stability, but WGP exhibited no survival effect.

Effect of some natural polysaccharides on LAB storage stability

The water content is an important parameter for the stability of dried LAB. To investigate the optimal conditions for storage, LAB were freeze-dried with skimmed milk and stored in both closed and open bottles, and the relationship between their viability and moisture content investigated (Table I). The viability of LAB stored in open bottles decreased more gradually with storage time than those stored in closed bottles, but the moisture content in the former increased more than that in the latter. The viability of freeze-dried LAB stored for 15 days in open bottles at 4°C was higher than for those stored at 25°C, but the

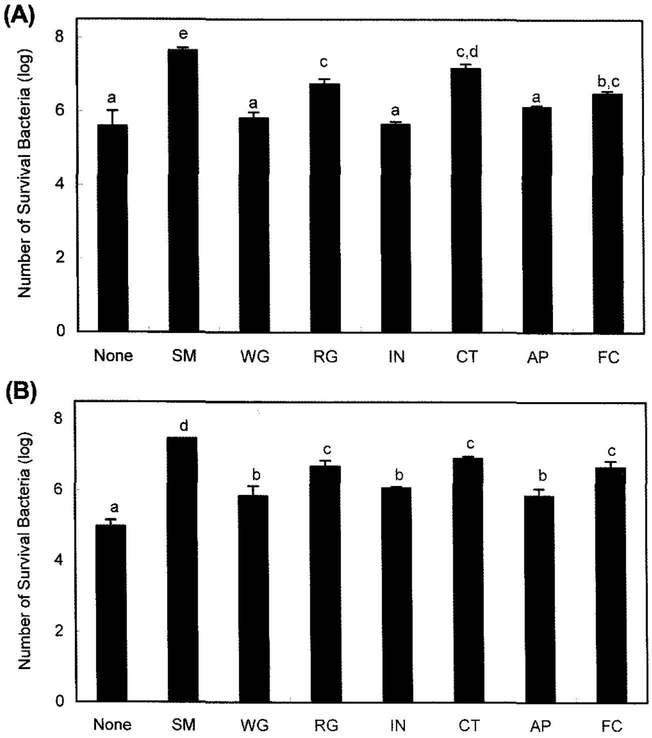

Fig. 1. The stabilizing effect of several polysaccharides on the viabilities of *L. acidophilus* (A) and *B. breve* (B) towards the freeze-drying process. Values represent the means ± S.D. of three experiments. *Significantly different compared to the non-treated control (*P<0.05). None, freeze-dried without additives; WG, freeze-drier with white ginseng polypolysaccharide RGP, freeze-dried with red ginseng polysaccharide; IN, freeze-dried with inulin; CT, freeze-dried with chitosan; SM, freeze-dried with skim milk; AP, freeze-dried with apple pectin; FC, freeze-dried with fucoidan.

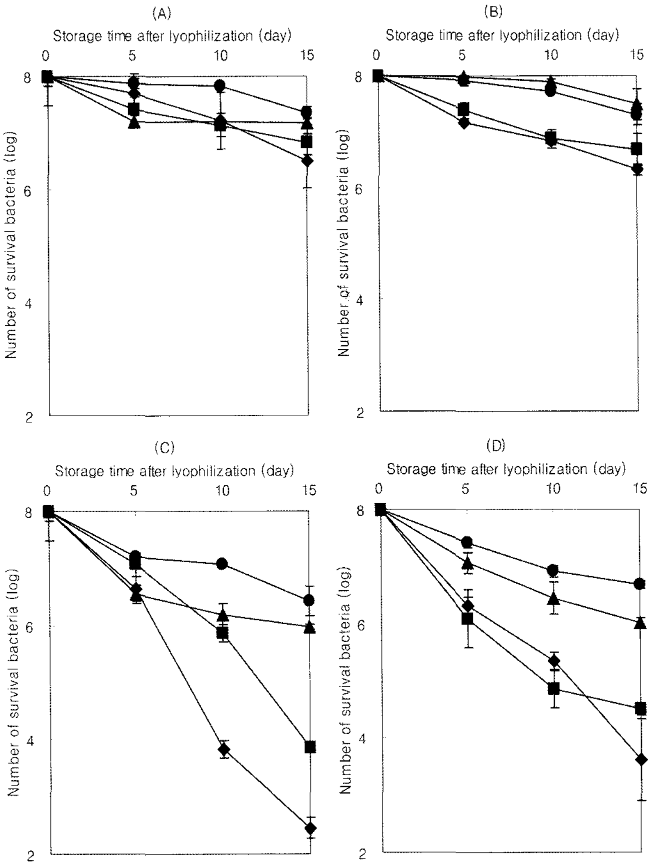

Fig. 2. The effect of skimmed milk on the stabilities of *L. acidophilus* and *B. breve*. A, Freeze-dried *L. acidophilus* stored at 4°C; B, Freeze-dried *L. acidophilus* stored at 25°C; C, Freeze-dried *B. breve* stored at 4°C; D, Freeze-dried *B. breve* stored at 25°C. Closed diamond, without skimmed milk; closed square, 2% skimmed milk; closed triangle, 5% skimmed milk; closed circle, 10% skimmed milk.

moisture content of freeze-dried LAB stored at 25°C was significantly lower than for those stored at 4°C.

The maintenance of highly viable lactic acid bacteria by adding appropriate additives during storage is an important practical issue. Initially, both *L. acidophilus* and *B. breve* were freeze dried with skimmed milk, at concentrations of 0, 2, 5 and 10%, and the effect of

Table I. Number of bacteria surviving and their water content during the storage of lactic acid bacteria freeze-dried with skimmed milk

	Store	Tem (°C)	Number of bacteria surviving and their water content during storage							
			0[a]		5 d		10 d		15 d	
			CFU (log)	Water (%)	CFU (log)	Water (%)	CFU (log)	Water (%)	CFU (log)	Water (%)
L. acidophilus	Open	4	8.0	1.1	7.97	15.3	7.92	18.2	7.41	20.3
	Closed	4	8.0	1.1	7.45	1.3	7.31	1.7	7.12	1.4
	Open	25	8.0	1.1	7.21	7.5	7.02	6.3	6.37	7.4
	Closed	25	8.0	1.1	6.82	1.5	6.10	1.5	5.38	1.8
B. breve	Open	4	8.0	1.7	7.90	19.3	7.71	19.3	7.30	21.0
	Closed	4	8.0	1.7	7.60	1.9	7.17	2.1	6.80	1.8
	Open	25	8.0	1.7	7.41	5.6	6.92	7.5	6.68	8.1
	Closed	25	8.0	1.7	7.40	1.6	6.82	2.3	6.27	2.1

Numbers of surviving bacteria cultured on GAM agar plates were counted as colony forming units (CFUs).
[a] Storage time after lyophilization.

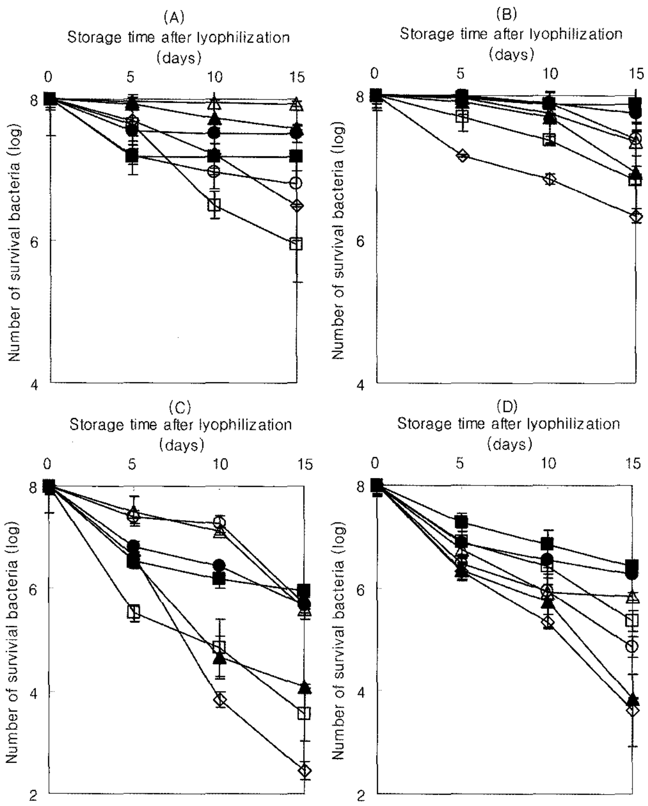

Fig. 3. The effects of some polysaccharides on the stabilities of *L. acidophilus* and *B. breve* toward storage. A, Freeze-dried *L. acidophilus* stored at 4°C; B, Freeze-dried *L. acidophilus* stored at 25°C; C, Freeze-dried *B. breve* stored at 4°C; D, Freeze-dried *B. breve* stored at 25°C. Open diamond, without an additive; open square, 10% apple pectin; open triangle, 10% fucoidan; open circle, 10% chitosan; closed square, 10% skimmed milk; closed triangle, 10% inulin; closed circle, 10% RGP.

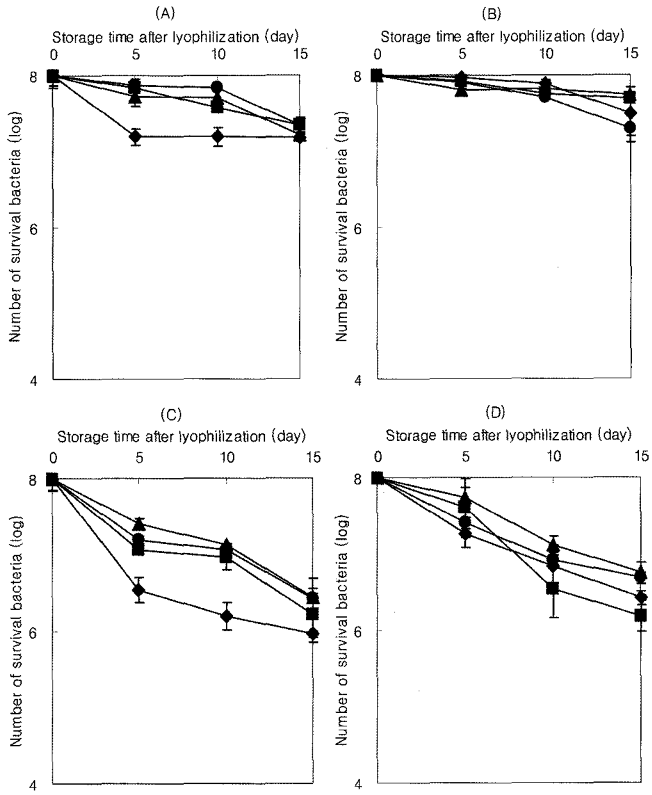

Fig. 4. The effect of skimmed milk plus red ginseng polysaccharide on the stabilities of *L. acidophilus* and *B. breve*. A, Freeze-dried *L. acidophilus* stored at 4°C; B, Freeze-dried *L. acidophilus* stored at 25°C; C, Freeze-dried *B. breve* stored at 4°C; D, Freeze-dried *B. breve* stored at 25°C. Closed diamond, 5% skimmed milk treated; closed square, 10% red ginseng polysaccharide (RGP) treated; closed triangle, 5% skimmed milk & 5% RGP treated; closed circle, 10% skimmed milk alone.

skimmed milk on the storage stability investigated. As shown in Fig. 2, skimmed milk potently inhibited the cell death of both types of LAB, and had the greatest effect on inhibition at a concentration of 10%.

The effects of 10% natural polysaccharides on the storage stability of LAB were then investigated (Fig. 3). Of the polysaccharides tested, fucoidan or RGP most potently inhibited the cell death of LAB, followed by chitosan, inulin and apple pectin. The stabilities conferred by RGP and fucoidan were comparable to that of skimmed milk. However, WGP did not promote any storage stability (data not shown).

Milk-based formulae are commercially used to increase storage stability. Therefore, the effect of milk-based RGP on the stability of freeze-dried LAB was also investigated (Fig. 4). When LAB was freeze-dried with 5% RGP/5% skimmed milk and then stored, its viability was higher than that of LAB treated with either 5 or 10% RGP. The effect of 5% RGP/5% skimmed milk was comparable to that of 10% skimmed milk.

DISCUSSION

Many compounds have been tested for the improvement of LAB survival during freeze-drying and storage, e.g., polyols, polysaccharides, disaccharides, amino acids, proteins, vitamins and salts (Champagne *et al.*, 1991 and 1996), with several of these agents found to have protective effects. Nevertheless, few have further improved the effects of skimmed milk or milk-based formulations. However, milk is not an ideal stabilizer, because it causes lactose intolerance. Therefore, agents that improve the stability of LAB during freeze-drying and storage, which allow their full biological effects to be exhibited are still required. Natural medicines-derived polysaccharides can be considered as alternative agents. Natural medicine polysaccharides, such as RGP and lentinan (Kim *et al.*, 1990; Mizuno *et al.*, 1995), may synergistically increase the biological activities of LAB, such as their antitumor and immunomodulating activities.

In addition, ginseng is the best known and most representative herbal medicine. Three kinds of ginseng are known commercially: white, red and heat-processed ginseng, which vary according to the temperature profiles used by different ginseng manufactures. These three ginsengs contain different saponins and polysaccharides in variable amounts (Kim *et al.*, 2000; Lee *et al.*, 2001), and the effects of processing temperatures on the natures and biological activities of these materials remain to be studied. Therefore, we isolated polysaccharides from WG and RG, and measured the molecular weights of those obtained by gel filtration. The average molecular weights of WGP and RGP were determined to be >100-70 and 90-30 kDaltons, respectively, using Sephadex G-75 column chromatography (data not shown).

The effects of some natural polysaccharides (WGP, RGP, chitosan, apple pectin, inulin, and fucoidan) on the viability of LAB after freeze-drying were also investigated. Of the polysaccharides tested, RGP exhibited a strong stabilizing effect on LAB during freeze-drying and storage, whereas WGP showed no increase in the viability of the LAB. Therefore, the effect of milk-based RGP was also investigated on the stability of freeze-dried LAB, because skimmed milk is a commonly used stabilizer, and RGP has various biological effects, such as, immune stimulation, antirotaviral and anti-*Helicobacter pylori* activities (Belogortseva *et al.*, 2002; Kim *et al.*, 1990; Lee *et al.*, 2001). When 5% skimmed milk/5% RGP treated LAB were freeze-dried and stored, their viability was found to be significantly higher than those treated with either 5 or 10% RGAP. The effect of 5% RGP/5% skimmed milk on the freeze-drying and storage stability of LAB was comparable to that of LAB treated with 10% skimmed milk. In addition, RGP also showed a bifidogenic effect (data not shown).

Based on these findings, we believe that RGP beneficially improves the stability during the freeze-dry process and storage of LAB.

REFERENCES

Adachi, S., Lactic acid bacteria and the control of tumors, In Wood, B.J.B. (ed) The Lactic acid bacteria, vol. 1, Elsevier Science Publishers, ltd., Essex, pp. 233-261 (1992).

Ahn, J. Y., Choi, I. S., Shim, J. Y., Yun, E. K., Yun, Y. S., Jeong, G., and Song, J. Y., The immunomodulator ginsan induces resistance to experimental sepsis by inhibiting Toll-like receptor-mediated inflammatory signals. *Eur. J. Immunol.*, 36, 37-45 (2006).

Belogortseva, N. I., Yoon, J. Y., and Kim, K. H., Inhibition of *Helicobacter pylori* hemagglutination by polysaccharide fractions from roots of *Panax ginseng*. *Planta Med.*, 66, 217-220 (2002).

Campieri, M. and Gionchetti, P., Probiotics in inflammatory bowel disease: New insight to pathogenesis or a possible therapeutic alternative. *Gastroenterology*, 116, 1246-1260 (1999).

Champagne, C. P., Gardner, N., Brochu, E., and Y. Beaulieu, Y., The freeze-drying of lactic acid bacteria. A review. *Can. Inst. Food Sci. Technol. J.*, 24, 118-128 (1991).

Champagne, C. P., Mondou, F., Raymond, Y., and Roy, D., Effect of polymers and storage temperature on the stability of freeze-dried lactic acid bacteria. *Food Res. International*, 29, 555-562 (1996).

Collins, M. P. and Gibson, G. R., Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nutr.*, 69, s1052-s1057 (1999).

Font de Valdez, G., DeGiori, G. S., de Ruiz Holgado A. P., and Oliver, G., Comparative study of the efficiency of some additives in protecting lactic acid bacteria against freeze-drying. *Criobiology*, 23, 574-578 (1983).

Gilliland, S. E., Nelson, C. R., and Maxwell, C., Assimilation of cholesterol by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.*, 49, 377-381 (1985).

Kim, Y. S., Kang, K. S., and Kim, S. I., Study on antitumor and immunomodulating activities of polysaccharide fractions from *Panax ginseng*: Comparison of effects of neutral and acidic polysaccharide fraction. *Arch. Pharm. Res.*, 13, 330-337 (1990).

Kim, H. M., Han, S. B., Oh, G. T., Kim, Y. H., Hong, N. D., and Yoo, I. D., Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom Phellinus linteus. *Int. J. Immunopharmacol.*, 18, 295-303 (1996).

Kim, W. Y., Kim, J. M., Han, S. B., Lee, S. K., Kim, N. D., Park, M. K., Kim, J. K., and Park, J. H., Steaming of ginseng at high temperature enhances biological activity. *J. Nat. Prod.*, 63, 1702-1704 (2000).

Lee, J.-C, Na, K., Yun, J.-M., and Hwang, J.-K., *In vitro* Bifidogenic effect of nondigestible oligosaccharides isolated from red ginseng Marc. *J. Microbiol. Biotechnol.*, 11, 858-862 (2001).

Lim, D. S., Bae, K. G., Jung, I. S., Kim, C. H., Yun, Y. S., and Song, J. Y., Anti-septicaemic effect of polysaccharide from *Panax ginseng* by macrophage activation. *J. Infect.*, 45, 32-38 (2002).

Mizuno, T., Saito. H., Nishitoba, T., and Kawagashi, H., Anti-tumor-active substances from mushrooms. *Food Rev. Int.*, 11, 23-61 (1995).

Perdigon, G., de Jorrat, W. E. B., de Petrino, S. F., and Valerde de Budeguer, M., Effect of oral administration of *Lactobacillus casei* on various biological functions of the host. *Food Agric. Immunol.*, 3, 93-102 (1991).

Rodas, B. Z., Gilliland, S. E., and Maxwell, C. V., Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC43121 and calcium in swine with hypercholesterolemia induced by diet. *J. Dairy Sci.*, 79, 2121-2128 (1996).

Salminen, S., Deighton, L., and Gorbach, S., Lactic acid bacteria in health and disease, In Salminen, S. and von Wright, A. (Eds.), Lactic acid bacteria., Marcel Dekker, Inc., New York, pp. 199-226 (1974).

Tabuchi, M., Ozaki, M., Tamura, A., Yamada, N., Ishida, T., Hosoda, M., and Hosono, A., Antidiabetic effect of *Lactobacillus* GG in streptozotocin-induced diabetic rats. *Biosci. Biotechnol. Biochem.*, 67, 1421-1424 (2003).

Taranto, M. P., Medici, M., Perdigon, G., Ruiz Holgado, A. P., and Valdez, G. F., Evidence for hypoglycemic effect of *Lactobacillus reuteri* in hypercholesterolemic mice. *J. Dairy Sci.*, 81, 2336-2340 (1998).