FEMS Microbiology Letters 240 (2004) 131–136

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from *Bifidobacterium bifidum* BGN4

Hyun Ju You [a], Deok-Kun Oh [c], Geun Eog Ji [a,b,*]

[a] *Department of Food Science and Nutrition, Seoul National University, Seoul 151-742, Republic of Korea*
[b] *Research Center, BIFIDO Co., Ltd., Seoul 151-818, Republic of Korea*
[c] *Department of Bioscience and Biotechnology, Sejong University, Seoul 143-747, Republic of Korea*

Received 17 May 2004; received in revised form 17 September 2004; accepted 17 September 2004

First published online 28 September 2004

Edited by W. Kneifel

Abstract

Strains of bifidobacteria have many health-promotion effects. Whole cells or cytoplasm extracts of *Bifidobacterium bifidum* BGN4, isolated from human feces, inhibited the growth of several cancer cell lines. The polysaccharide fraction (BB-pol) extracted from *B. bifidum* BGN4 had a novel composition, comprising chiroinositol, rhamnose, glucose, galactose, and ribose. Three human colon cancer cell lines were treated with BB-pol: HT-29, HCT-116, and Caco-2. Trypan blue exclusion assay and BrdU incorporation assay showed that BB-pol inhibited the growth of HT-29 and HCT-116 cells but did not inhibit the growth of Caco-2 cells.

Keywords: *Bifidobacterium*; Chiroinositol-containing polysaccharide; Colon cancer; Growth inhibition; Probiotics

1. Introduction

Probiotics are traditionally defined as live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance [1]. In view of their perceived health-promotion effects [2,3], probiotic bacteria have been increasingly included in various types of food products (especially fermented milks) during the past two decades [4].

There is experimental evidence that probiotic microorganisms show an anticancer activity in vitro and in animal models. Pool-Zobel et al. reported that *Lactobacillus acidophilus*, *L. gasseri*, *L. confusus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, and *B. longum* were antigenotoxic toward *N*′-nitro-*N*-nitrosoguanidine- or 1,2-dimethylhydrazine-induced genotoxicities [5]. Other studies have shown that certain strains of lactic acid bacteria (LAB) prevent putative preneoplastic lesions or tumors induced by carcinogens such as 1,2-dimethylhydrazine or azoxymethane [6–9]. Many strains – such as *L. rhamnosus* GG [10], *L. acidophilus* [11], *L. casei*, *B. longum* [12,13], *B. infantis*, *B. adolescentis*, and *B. breve* – showed significant suppression of colon tumor incidence in this type of study. In addition, there is direct evidence for antitumor activities of LAB obtained in studies using preimplanted tumor cells in animal models. There are several reports [14,15] that the consumption of fermented milk and/or cultures containing LAB or the intralesional injection of live or dead *Bifidobacterium* cells inhibited the growth of tumor cells injected into mice.

The precise mechanisms by which LAB inhibit colon cancer are presently unknown. However, based on

* Corresponding author. Tel.: +82 2 880 8749; fax: +82 2 880 6282.
E-mail address: geji@bifido.com (G.E. Ji).

0378-1097/$22.00
doi:10.1016/j.femsle.2004.09.020

experimental and epidemiological studies [3,16,17], several mechanisms have been proposed, including (1) enhancing the host's immune response, (2) binding and degrading potential carcinogens, (3) qualitative alterations in the intestinal microflora incriminated in producing putative carcinogens and promoters (e.g., bile-acid-degrading bacteria), (4) production of antitumorigenic or antimutagenic compounds in the colon, and (5) alteration of metabolic activities of intestinal microflora.

Various types of LAB preparations showed antitumor activities. Sekine et al. [18] found antitumor activity in peptidoglycans isolated from *B. infantis* strain ATCC 15697, and Oda et al. [19] reported antitumor polysaccharide fractions originating from *Lactobacillus* cultures. Glycoproteins found in the supernatants of *Lactobacillus* cultures have also shown antitumor effects [20].

The present study analyzed the composition of polysaccharide (BB-pol) extracted from *B. bifidum* BGN4 and investigated the effects of BB-pol on human colon cancer cell lines. We examined the potential of BB-pol as an antiproliferation compound using a direct cell counting method and measuring the rate of DNA synthesis.

2. Materials and methods

2.1. Bacterial culture and preparation of cytoplasm extract

B. bifidum BGN4 and other bifidobacteria which were isolated from the feces of healthy human subjects and previously reported by Park et al. [21] were used for the experiments. *B. bifidum* BGN4 was cultured and subcultured anaerobically in MRS broth (Criterion, USA) containing 0.5% L-cysteine (Sigma, USA) at 37 °C for 20 h. For the preparation of bacterial cytoplasm extracts, cultured bacterial cells were collected by centrifugation (Hanil, Korea) at 1390*g* for 20 min at 4 °C, and washed twice with autoclaved phosphate-buffered saline, followed by final washing with autoclaved double-distilled water. Washed whole cells were disintegrated using a cell disruptor, and the cell pellets were removed by centrifugation at 1390*g* for 20 min at 4 °C. The supernatant was centrifuged at 22,250*g* for 45 min at 4 °C for the separation of cell walls and cytoplasm extract. The sediment (cell-wall fraction) was removed, and the supernatant was used as the cytoplasm extract. This fraction was lyophilized (Ilshin, Korea) and stored at −70 °C until used.

2.2. Isolation of polysaccharide from bifidobacterial cytoplasm extract

The lyophilized cytoplasm extract was dissolved in autoclaved double-distilled water and heat-treated at 80 °C for 30 min in a water bath (Jeio Tech, Korea). Protein denaturing due to the heat treatment made the solution hazy. The sample was centrifuged until the supernatant turned clear, then phenol solution was added to the collected supernatant at a ratio of 1:1 (v/v) for protein denaturalization. After the end of the reaction with phenol, the solution was mixed vigorously and centrifuged again. The collected supernatant was treated with a phenol–chloroform solution at a ratio of 1:1 (v/v). Treatment of the collected supernatant with the phenol–chloroform solution was repeated. Lastly, chloroform solution was added at a ratio of 1:1 (v/v), and the collected supernatant was precipitated with cold ethanol, followed by freeze-drying. A phenol–sulfuric acid assay was used to determine whether this sample was the polysaccharide fraction.

2.3. Analysis of BB-pol composition

For analyzing the composition of the polysaccharide fraction, it was hydrolyzed by trifluoroacetic acid. The polysaccharide was reacted with trifluoroacetic acid at room temperature for 30 min and then reacted at 100 °C for 150 min. The composition of the hydrolyzed polysaccharide was determined by a Bio-LC with an electrochemical detector (Dionex ED-50, USA) using a Dionex CarboPac MA1 column with 600 mM sodium hydroxide at a flow rate of 0.4 $ml\,min^{-1}$. The composition of the polysaccharide was confirmed with standard samples of chiroinositol, rhamnose, glucose, galactose, and ribose (Sigma, USA).

2.4. Cell culture

This study used three human colon cancer cell lines (Table 1): HT-29 and HCT-116 cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea), and the Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, USA). They were grown in DMEM culture media supplemented with 10% (v/v)

Table 1
The characteristics of cell lines used in this study (KCLB, Korean Cell Line Bank; ATCC, American Type Culture Collection)

Cell line	Tissue	Species	Growth property	KCLB (ATCC) no.
HT-29	Colon or colorectal, adenocarcinoma	Human	Adherent	KCLB 30038
HCT-116	Colon or colorectal, carcinoma	Human	Adherent	KCLB 10247
Caco-2	Colon or colorectal carcinoma	Human	Adherent	HTB-37

fetal bovine serum (Gibco BRL, USA) and 1% antibiotic–antimycotic solution (Gibco BRL, USA). All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. After they were grown to confluence in sterile tissue culture plates (100 mm × 20 mm; Corning, USA), cells were detached and transferred to the new cell culture dishes using trypsin EDTA (Gibco BRL, USA) for each experiment. The detached cells were seeded to a 96-well cell culture cluster (at a density of 5×10^3 cells per well), 12 wells ($30–50 \times 10^3$ cells per well), or culture plates (60 mm × 15 mm; $100–200 \times 10^3$ cells per plate). They were then incubated for 48 h in the presence of 0, 20, 40, or 80 $\mu g\,ml^{-1}$ BB-pol.

2.5. Trypan blue staining

Cell numbers and their viability were assessed by the trypan blue dye-exclusion method [22]. Cancer cells were seeded onto 12 multiwell plates at a density of $30–50 \times 10^3$ cells per well in serum-containing medium. All cells were allowed to attach for 12–24 h, and each well was treated with BB-pol at a concentration of 20, 40, or 80 $\mu g\,ml^{-1}$. At two days after BB-pol treatment, cultured cells were trypsinized and mixed with an equal volume of trypan blue solution (Sigma–Aldrich, UK). The mixture was loaded on a hemacytometer (Superior, Neubauer, Germany) and the stained/unstained cells were observed using an inverted microscope (BX-40, Olympus, Japan) at 100× magnification.

2.6. BrdU (5-bromo-2′-deoxyuridine) incorporation assay for measurement of DNA synthesis rate

As a microplate format, cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates, and also incubated for 48 h in the presence of 0, 20, 40, or 80 $\mu g\,ml^{-1}$ BB-pol.

In this study, a nonradioactive alternative to the [^{3}H]-thymidine incorporation assay was performed using a commercial cell proliferation ELISA kit (BrdU, colorimetric; Roche, Germany) according to the manufacturer's instructions. Briefly, at the time for assay (two days after BB-pol treatment), 10 μl of BrdU labeling solution was added to control and BB-pol-treated cells, and the plates were reincubated for 3 h for labeling. The cells were then denatured and fixed using Fix/Denat solution for 30 min at 37 °C, and anti-BrdU-POD was added to the plates and incubated for 90 min at 15–25 °C. Lastly, 100 μl of substrate solution was added to each well and the plates were incubated at 15–25 °C until color development was sufficient for photometric detection. Twenty-five microliters of 1 M H_2SO_4 was added as a stopping solution to each well, and absorbance was measured with an ELISA reader (Bio-Rad Laboratories, USA) at 450 nm.

2.7. Statistical analysis

Data were analyzed by a one-way ANOVA procedure of SAS software (SAS Institute Inc.,1999). The differences between mean values were detected by the Duncan's multiple range test.

3. Results and discussion

3.1. Selection of the best Bifidobacterium strain for inhibiting the growth of cancer cell lines

In order to determine if *Bifidobacterium* cells have an inhibitory effect on the growth of colon cancer cell lines, HT-29 cells were treated with heat-treated cells of 30 different bifidobacterial strains and trypan blue assay was performed. In this preliminary assay, *B. bifidum* BGN4 showed the greatest effect (data not shown). To further characterize the functional substances of *B. bifidum* BGN4, HT-29 cells were treated with different types of cell fraction. Among the whole-cell, cell-wall, and cytoplasm extracts, the cytoplasm extract showed the highest inhibitory effect.

3.2. Composition of BB-pol

The composition of the polysaccharide was determined by a Bio-LC using a Dionex CarboPac MA1 or PA1 column. The detected peaks were compared with various carbohydrate standards: chiroinositol, myoinositol, inositol, rhamnose, glucose, galactose, ribose, fructose, adonitol (ribitol), glycerol, mannose, arabinose, fucose, xylose, sorbose, tagatose, mannitol, sorbitol, dulcitol, xylitol, allose, psicose, glucuronic acid, glucaric acid, galacturonic acid, galactosamine, mannosamine, and glucosamine. The polysaccharide was found to consist of 26.4% chiroinositol, 3.9% rhamnose, 31.5% glucose, 11.0% galactose, and 23.8% ribose (Fig. 1). We think that the BB-pol fraction consisted of one polysaccharide because of a peak profilefor the BB-pol fraction in GPC. The molecular weight ofthe BB-pol fraction was about 1,500,000 by extrapolation using dextran standards (M_W, 48,500, 273,000, 830,000). It is interesting that BB-pol contained chiroinositol as one of the major sugar units, because a chiroinositol-based polysaccharide from microbial sources has not previously been reported.

3.3. Effect of BB-pol on the growth inhibition of HT-29 and HCT-116 cells

This study examined the effects of BB-pol on the growth of three types of human colorectal adenocarcinoma cell lines using a trypan blue exclusion assay (Fig. 2). BB-pol tended to decrease the numbers of

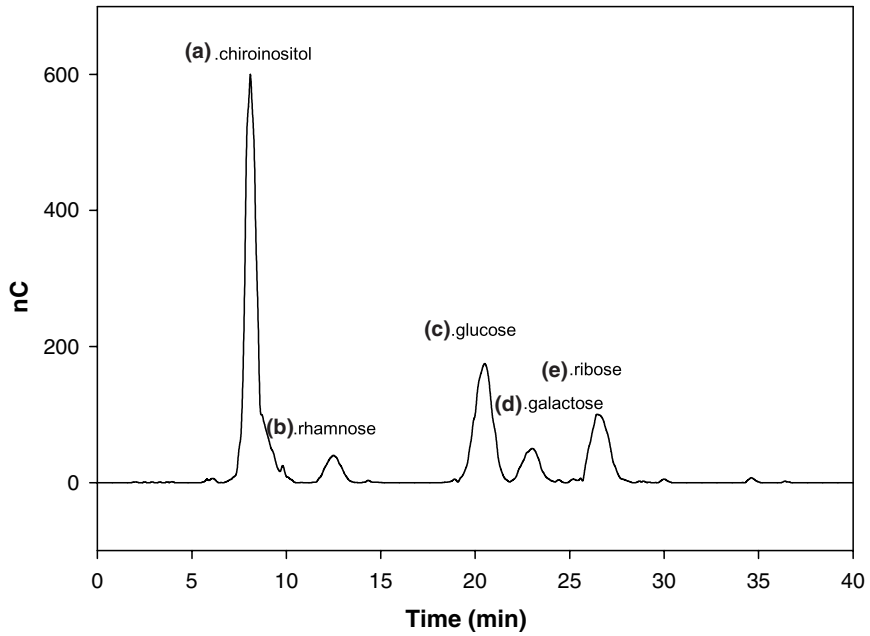

Fig. 1. Bio-LC analysis of the composition of BB-pol. (a) chiroinositol (26.4%); (b) rhamnose (3.9%); (c) glucose (31.5%); (d) galactose (11.0%); (e) ribose (23.8%).

HT-29 cells even at a concentration of 10 $\mu g\,ml^{-1}$. However, Caco-2 cells were not noticeably inhibited at concentrations up to 100 $\mu g\,ml^{-1}$ (data not shown). Although all of the three cell lines originated from human adenocarcinomas, Caco-2 is known to resemble the physiology of normal cells [23–25] more than the other two cell lines used in this study. This suggests that BB-pol had a greater effect on tumor cell lines with higher malignancy. Fig. 2 shows the growth inhibition caused by BB-pol in HT-29 and HCT-116 cells. BB-pol at 20 $\mu g\,ml^{-1}$ inhibited the growth of HT-29 cells by 50.5 ± 3.6% (mean ± SD). The growth inhibition of HCT-116 cells increased with the BB-pol concentration, but these cells were slightly less sensitive to BB-pol than HT-29 cells. Few, if any, cells became nonviable during 48 h of incubation, which suggests that the inhibition of tumor cell growth by BB-pol was due to the retardation of cell growth rather than cytotoxicity. This was further supported by an lactate dehydrogenase (LDH) release assay in which no significant difference was evident between the experimental groups (data not shown).

3.4. Effect of BB-pol on the growth inhibition of HT-29 and HCT-116 cells measured by BrdU incorporation assay

The trypan blue exclusion assay is a direct method for measuring cell growth or cytotoxicity, but it involves many steps that may introduce experimental errors. Therefore, a BrdU incorporation assay was performed to confirm the trypan blue staining data. The rationale of the BrdU incorporation assay was to measure the changed DNA synthesis of treated cells as for [^{3}H]-thymidine incorporation assay.

Data from BrdU incorporation assays in HT-29 and HCT-116 cells showed results similar to those of growth inhibition from the trypan blue exclusion assay (Fig. 3). Similar to the result of trypan blue staining, BB-pol had no effect on the DNA synthesis rate measured by the BrdU incorporation assay in Caco-2 cells (data not shown). In HT-29 cell lines, the DNA synthesis rate decreased after BB-pol treatment in a dose-dependent manner. When treated with 80 $\mu g\,ml^{-1}$ BB-pol, the adjusted DNA synthesis rate was 52.6 ± 0.9%. Treatment with the same concentration of BB-pol produced a smaller decrease in the DNA synthesis of HCT-116 cells: the adjusted DNA synthesis rate was 64.3 ± 1.9%. However, HT-29 and HCT-116 cells treated with 20 $\mu g\,ml^{-1}$ BB-pol showed similar reductions in DNA synthesis rates.

As shown in the former two assays, the decrease in the number of counted cells in the BB-pol-treated group was concordant with the reduced DNA synthesis rate. These experimental data allude to a growth-retarding effect of BB-pol on HT-29 and HCT-116 cell lines rather than cytotoxic effects.

In contrast, most previously reported polysaccharides that exhibit antitumor activities did not directly inhibit the growth of tumor cells in vitro, but instead exerted antitumor activity by stimulating macrophages and various immune systems. Such polysaccharides included α- and β-glucan from various mushrooms [26], SPR-901 α-glucan from rice bran [27], α-glucan from *Mycobacterium bovis*, Bacille Calmette–Gurin [28], and water-soluble polysaccharide containing glucose and galactose as

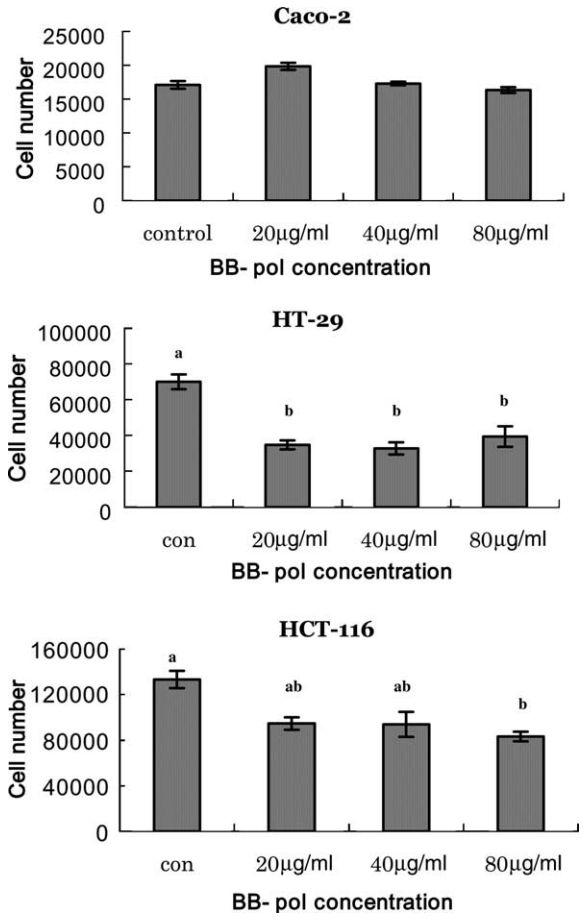

Fig. 2. Effect of BB-pol on the growth of human colon cancer cell lines. The cell lines were cultured for 48 h in the presence of BB-pol at various concentrations: 0 (control), 20, 40, and 80 $\mu g ml^{-1}$ in 12-well plates. The cell numbers were determined by trypan blue exclusion assay. Data values are mean ± SE of triplicates in a representative assay ($P < 0.05$).

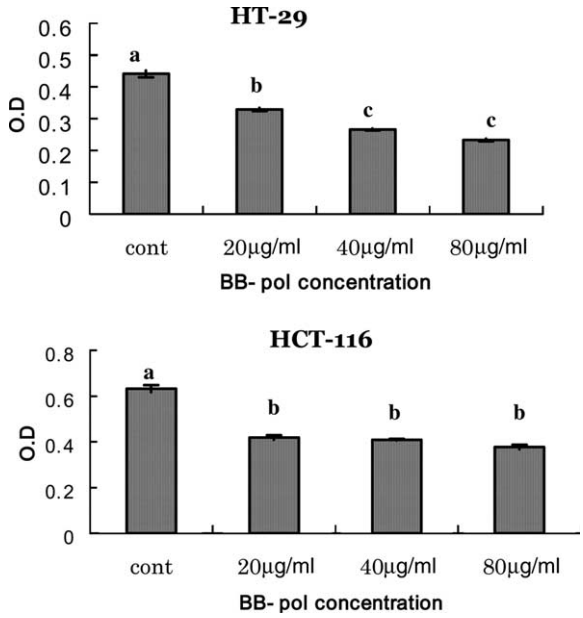

Fig. 3. Effect of BB-pol on the growth inhibition of HT-29 and HCT-116 cell lines measured by BrdU incorporation assay. The cells were cultured for 48 h in the presence of BB-pol at various concentrations in 96-well plates: 0 (control), 20, 40, and 80 $\mu g ml^{-1}$. The DNA synthesis rates were determined by BrdU incorporation assay. Data values are mean ± SE of triplicates in a representative assay ($P < 0.0001$).

major sugar constituents from *B. adolescentis* M101-4 [29] and from *L. helveticus* var. *jugurti* [19]. Also, it is well known that various bacterial peptidoglycans induce antitumor activity through the modulation of immune systems. Tumor necrosis factor α and reactive nitrogen intermediates played a major role in the in vitro antitumor activity of mouse peritoneal exudates cells from mice stimulated with wall peptidoglycan from *B. infantis* [18]. Therefore, the direct inhibitory effect of BB-pol on tumor cell growth observed in the present study is rather exceptional for polysaccharide biomaterials extracted from various organisms. A partially purified cytotoxic substance from the culture supernatants of *L. casei* D-34 against three tumor cell lines – HeLa, HEP-2, and HFS-9 – was found to be proteinaceous in nature [20]. Recently, the cytoplasmic fraction from *Lactococcus lactis* ssp. *lactis* was reported to exert direct antiproliferation activity against the SNUC2A human colon cancer cell line by inducing S phase accumulation in SNUC2A cells [30], but the active components remain to be elucidated.

The present study shows that BB-pol is a novel polysaccharide with glucose, chiroinositol, and ribose as its major constituents, which inhibits the growth of colon cancer cell lines in vitro. We are currently analyzing general transcriptional responses of tumor cells following treatment with BB-pol to better understand the growth-inhibition mechanism of BB-pol.

Acknowledgement

This work was supported by the Korean Ministry of Science and Technology (Grant no. M1-0302-00-0098).

References

[1] Fuller, R. (1989) Probiotics in man and animals. J. Appl. Bacteriol. 66, 365–378.
[2] Goldin, B.R. (1998) Health benefits of probiotics. Br. J. Nutr. 80, S203–S207.
[3] Malhotra, S.L. (1977) Dietary factors in a study of colon cancer from Cancer Registry, with special reference to the role of saliva, milk and fermented milk products and vegetable fibre. Med. Hypotheses 3, 122–126.
[4] Sanders, M.E. (1998) Development of consumer probiotics for the US market. Br. J. Nutr. 80, S213–S218.
[5] Pool-Zobel, B.L., Neudecker, C., Domizlaff, I., Ji, S., Schillinger, U., Rumney, C., Moretti, M., Vilarini, I., Scassellati-Sforzolini, R. and Rowland, I. (1996) *Lactobacillus*- and *Bifidobacterium*-mediated antigenotoxicity in the colon of rats. Nutr. Cancer 26, 365–380.

[6] Arimochi, H., Kinouchi, T., Kataoka, K., Kuwahara, T. and Ohnishi, Y. (1997) Effect of intestinal bacteria on formation of azoxymethane-induced aberrant crypt foci in the rat colon. Biochem. Biophys. Res. Commun. 238, 753–757.
[7] Gallaher, D.D., Stallings, W.H., Blessing, L.L., Busta, F.F. and Brady, L.J. (1996) Probiotics, cecal microflora, and aberrant crypts in the rat colon. J. Nutr. 126, 1362–1371.
[8] Abdelali, H., Cassand, P., Soussotte, V., Daubeze, M., Bouley, C. and Narbonne, J.F. (1995) Effect of dairy products on initiation of precursor lesions of colon cancer in rats. Nutr. Cancer 24, 121–132.
[9] Onoue, M., Kado, S., Sakaitani, Y., Uchida, K. and Morotomi, M. (1997) Specific species of intestinal bacteria influence the induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats. Cancer Lett. 113, 179–186.
[10] Goldin, B.R., Gualtieri, L.J. and Moore, R.P. (1996) The effect of *Lactobacillus* GG on the initiation and promotion of DMH-induced intestinal tumors in the rat. Nutr. Cancer 25, 197–204.
[11] Goldin, B.R. and Gorbach, S.L. (1980) Effect of *Lactobacillus acidophilus* dietary supplements in 1, 2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats. J. Natl. Cancer Inst. 64, 263–265.
[12] Rowland, I.R., Rumney, C.J., Coutts, J.T. and Lievense, L.C. (1998) Effect of *Bifidobacterium longum* and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. Carcinogenesis 19, 281–285.
[13] Singh, J., Rivenson, A., Tomita, M., Shimamura, S., Ishibashi, N. and Reddy, B.S. (1997) *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. Carcinogenesis 18, 833–841.
[14] Kohwi, T., Imai, K., Tamura, A. and Hashimoto, Y. (1978) Antitumor effect of *Bifidobacterium infantis* in mice. Gann 69, 613–618.
[15] Kato, I., Kobayashi, S., Yokokura, T. and Mutai, M. (1981) Antitumor activity of *Lactobacillus casei* in mice. Gann 72, 517–523.
[16] Shahani, K.M. and Ayebo, A.D. (1980) Role of dietary lactobacilli in gastrointestinal microecology. Am. J. Clin. Nutr. 33, 2448–2457.
[17] Maclennan, R. and Jensen, O.M. (1977) Dietary fibre, transit time, fecal bacteria, steroids, and colon cancer in two Scandinavian populations. Lancet 30, 207–211.
[18] Sekine, K., Ohta, J., Onishi, M., Tatsuki, T., Shimokawa, Y., Toida, T., Kawashima, T. and Hashimoto, Y. (1995) Analysis of antitumor properties of effector cells stimulated with a cell wall preparation (WPG) of *Bifidobacterium infantis*. Biol. Pharm. Bull. 18, 148–153.
[19] Oda, M., Hasegawa, H., Komatsu, S., Kambe, M. and Tsuchiya, F. (1983) Anti-tumor polysaccharide from *Lactobacillus* sp. Agric. Biol. Chem. 47, 1623–1625.
[20] Manjunath, N. and Ranganathan, B. (1989) A cytotoxic substance produced by a wild culture of *Lactobacillus casei* D-34 against tumor cells. Indian J. Exp. Biol. 27, 141–145.
[21] Park, S.Y., Ji, G.E., Ko, Y.T., Jung, H.K., Ustunol, Z. and Pestka, J.J. (1999) Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. Int. J. Food Microbiol. 46, 231–241.
[22] Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M. and Strober, W. (1990) Trypan blue exclusion test of cell viability. In: Current Protocols in Immunology (John Wiley and Sons, eds.) vol. 2, p. A.3.3., Greene Publishing Associates and Wiley-Interscience, New York.
[23] Coconnier, M.-H., Bernet, M.F., Kerneis, S., Chauviere, G., Fourniat, J. and Servin, A.L. (1993) Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol. Lett. 110, 299–305.
[24] Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell 47, 323–330.
[25] Kim, I.H., Park, M.S. and Ji, G.E. (2003) Characterization of adhesion of *Bifidobacterium* sp. BGN-4 to human enterocyte-like Caco-2 cells. J. Microbiol. Biotechnol. 13, 276–281.
[26] Ebina, T. (2003) Activation of antitumor immunity by intratumor injection of biological preparations. Gan. To. Kagaku Ryoho 30, 1555–1558.
[27] Kado, H., Yoneta, Y., Takeo, S., Mitsui, M. and Watanabe, N. (1991) Studies on an enzymatically synthesized antitumor polysaccharide SPR-901. Chem. Pharm. Bull. 39, 1078–1079.
[28] Dinadayala, P., Lemassu, A., Granovski, P., Cerantola, S., Winter, N. and Daffe, M. (2004) Revisiting the structure of the anti-neoplastic glucans of *Mycobacterium bovis* bacille calmette-guomicron rin analysis of the different types of glucans produed by the vaccine substrains. J. Biol. Chem. 279, 12369–12378.
[29] Hosono, A., Lee, J., Ametani, A., Natsume, M., Hirayama, M., Adachi, T. and Kaminogawa, S. (1997) Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101-4. Biosci. Biotechnol. Biochem. 61, 312–316.
[30] Kim, J.Y., Woo, H.J., Kim, Y.S., Kim, K.H. and Lee, H.J. (2003) Cell cycle dysregulation induced by cytoplasm of *Lactococcus lactis* ssp. *lactis* in SNUC2A, a colon cancer cell line. Nutr. Cancer 46, 197–201.