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Enhancement of Anti-tumorigenic Polysaccharide Production, Adhesion, and Branch Formation of *Bifidobacterium bifidum* BGN4 by Phytic Acid

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Abstract The polysaccharide (BB-pol) extracted from *Bifidobacterium bifidum* BGN4 showed growth inhibitory effects on several colon cancer cell lines such as HT-29 and HCT-116. To increase the yield of polysaccharide, *B. bifidum* BGN4 was cultured in various culture media with different compositions. When *B. bifidum* BGN4 was cultured in modified MRS broth containing phytic acid, the cells showed increased branch formation and enlarged morphology. The content of total carbohydrate and the ability of adhesion to intestinal epithelial cells were also increased by phytic acid. The polysaccharide obtained from the cells grown in the presence of phytic acid inhibited the proliferation of cancer cell lines such as HT-29 and MCF-7 cells but not normal colon cell line, FHC. Taken together, *Bifidobacterium* grown in the presence of phytic acid may confer enhanced beneficial function for the host.

Keywords: *Bifidobacterium*, polysaccharide, anti-tumorigenicity, phytic acid, cell adhesion

Introduction

Probiotics literally mean 'for life' and are defined as living microorganisms which exert beneficial functions for the host (1). It has been reported that the consumption of probiotics reduces numbers of fecal putrefactive bacteria such as coliforms, and increases numbers of commensal bacteria such as lactobacilli and bifidobacteria (2,3). Moreover, they are assumed to enhance the host's immune system, decrease the symptoms of lactose intolerance, and reduce the risk of certain cancers (4). The successful colonization of the probiotics in the gut is necessary for their beneficial health effects such as competitive exclusion against pathogens and modulation of immunological activities (5-8). Thus the ability of the probiotics to adhere to intestinal surfaces is thought to be crucial for their beneficial functions for the host. Hydrophobicity of the cells and the presence of specific receptors play a role in the adhesion of the probiotics to the intestinal epithelial cells (9). The anti-cancer activity of probiotics was reported to be related to the presence of various cell components such as peptidoglycans from *Bifidobacterium infantis* strain ATCC 15697 (10), polysaccharide fractions originated from *Lactobacillus* cultures (11), glycoproteins detected in the supernatant of *Lactobacillus* cultures, and polysaccharide fractions extracted from *Bifidobacterium bifidum* BGN4 (12). In our previous study, the anti-tumorigenic polysaccharide fraction (BB-pol) extracted from *B. bifidum* BGN4 was shown to have a novel composition consisting of chiroinositol, rhamnose, glucose, galactose, and ribose. The aim of this study was to enhance the probiotic abilities such as anti-tumorigenicity and adhesion to the epithelial cells by optimizing the medium composition and growth condition of the probiotic *Bifidobacterium*. The present study produced a new finding that the presence of phytic acid in the growth medium can enhance the production of anti-tumorigenic polysaccharide and adhesion of *Bifidobacterium* cells to the intestinal epithelial cells.

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Materials and Methods

Microorganism and growth conditions Ninety different media were prepared for the screening of optimal *Bifidobacterium bifidum* BGN4 culture conditions (Table 1). The description of *B. bifidum* BGN4 was previously reported (13,14). Two different basal media were used; nitrogen basal (NB) medium and de Man Rogosa and Sharpe (MRS) broth medium (Difco, Lawrence, KS, USA). NB medium contained yeast extract (0.5%, w/v), proteose peptone (1%, w/v), ammonium acetate (1%, w/v), manganese sulfate (0.01%, w/v), magnesium sulfate (0.005%, w/v), K_2HPO_4 (0.2%, w/v), and Tween 80 (0.1%, w/v). MRS medium contained proteose peptone (1%, w/v), beef extract (1%, w/v), yeast extract (0.5%, w/v), glucose (2%, w/v), polysorbate 80 (0.1%, w/v), ammonium citrate (0.2%, w/v), sodium acetate (0.5%, w/v), magnesium sulfate (0.1%, w/v), manganese sulfate (0.005%, w/v), and dipotassium phosphate (0.2%, w/v). Filter sterilized sugars were added as in Table 1. Final pH was adjusted to 6.8-7.0 by NaOH. Chiroinositol and pinitol were generously donated from Amicogen (Jinju, Korea). Phytic acid and the other sugar sources were purchased from Sigma-Aldrich (St. Louis, MO, USA). Yeast extract, proteose peptone, and beef extract were purchased from BD Biosciences (Sparks, MD, USA). The cultures of *B. bifidum* BGN4 were incubated anaerobically at 37°C for 24 hr in various media with 90 different compositions containing 0.05%(w/v) L-cysteine hydrochloride in common.

Table 1. Ninety different microbial media tested in this experiment[1)]

NB Basal media																	
Lac 1%+Gal 1%						Glc 1%+Gal 1%						Glc 2%					
Rham1%		Ribo1%		Rham1%+Ribo1%		Rham1%		Ribo1%		Rham1%+Ribo1%		Rham1%		Ribo1%		Rham1%+Ribo1%	
1	Chi	6	Chi	11	Chi	16	Chi	21	Chi	26	Chi	31	Chi	36	Chi	41	Chi
2	Myo	7	Myo	12	Myo	17	Myo	22	Myo	27	Myo	32	Myo	37	Myo	42	Myo
3	Man	8	Man	13	Man	18	Man	23	Man	28	Man	33	Man	38	Man	43	Man
4	Phy	9	Phy	14	Phy	19	Phy	24	Phy	29	Phy	34	Phy	39	Phy	44	Phy
5	Pini	10	Pini	15	Pini	20	Pini	25	Pini	30	Pini	35	Pini	40	Pini	45	Pini
NB Basal media												MRS Basal media					
Lac 2%+Glc 2%						Lac 4%											
Rham1%		Ribo1%		Rham1%+Ribo1%		Rham1%		Ribo1%		Rham1%+Ribo1%		Rham1%		Ribo1%		Rham1%+Ribo1%	
46	Chi	51	Chi	56	Chi	61	Chi	66	Chi	71	Chi	76	Chi	81	Chi	86	Chi
47	Myo	52	Myo	57	Myo	62	Myo	67	Myo	72	Myo	77	Myo	82	Myo	87	Myo
48	Man	53	Man	58	Man	63	Man	68	Man	73	Man	78	Man	83	Man	88	Man
49	Phy	54	Phy	59	Phy	64	Phy	69	Phy	74	Phy	79	Phy	84	Phy	89	Phy
50	Pini	55	Pini	60	Pini	65	Pini	70	Pini	75	Pini	80	Pini	85	Pini	90	Pini

[1)]Various concentrations of glucose, galactose, lactose, rhamnose, and ribose were added to the 2 basal media, NB and MRS medium. Phytic acid and several sugar alcohols such as chiroinositol, myoinositol, mannitol, and pinitol were also added at 1%(w/v). Lac, lactose; Gal, galactose; Glc, glucose; Rham, rhamnose; Ribo, ribose; Chi, chiroinositol; Myo, myoinositol; Man, mannitol; Phy, phytic acid; Pini, pinitol.

Determination of total carbohydrate and isolation of polysaccharides After incubation, the cultured cells were harvested by centrifugation at 3,000×g for 30 min at 4°C. The collected pellets were washed twice with phosphate-buffered saline (PBS) and resuspended with same buffer, followed by disruption using ultrasonicator (Sonics and Materials, Inc., Newtown, CT, USA) for 5 min. The disrupted cells were heat treated at 95°C for 30 min to denature proteins, and the aggregated proteins were removed by centrifugation at 13,000×g for 30 min. The supernatant was filtered through 0.22-μm syringe filter (Pall, Ann Arbor, MI, USA) to remove residual protein aggregates. The total carbohydrate content of the filtrate was determined by phenol-sulfuric assay as described before (15). For the extraction of polysaccharide from *B. bifidum* BGN4, the heat-treated cell lysate was centrifuged until the supernatant turned clear, then phenol solution was added to the collected supernatant at a ratio of 1:1 (v/v) in order to denature proteins (12). After vigorous mixing and centrifugation, the upper layer was collected and treated with phenol-chloroform solution and chloroform sequentially at a ratio of 1:1 (v/v). Finally, the upper layer was mixed with cold ethanol to precipitate polysaccharide, and then the pellet was freeze-dried and stored at −80°C before use.

Culture of cancer and normal colon cell lines Three epithelial adenocarcinoma cell lines, MCF-7 (breast), HT-29 (colon), and Caco-2 (colon) cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). MCF-7 and HT-29 cells were cultured in Dulbecco's modified essential medium (DMEM) media, and Caco-2 cells was cultured in RPMI 1640 media containing 10%(v/v) fetal bovine serum (FBS), and 1%(v/v) antibiotic-antimycotic solution in common. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C and subcultured every 5 days. The normal colonic epithelial cell line, FHC cells was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM/F12 media containing 25 mM HEPES, 10 ng/mL cholera toxin, 5 μg/mL insulin, 5 μg/mL transferrin, 100 ng/mL hydrocortisone, 10%(v/v) FBS, and 1%(v/v) antibiotic-antimycotic solution (Invitrogen, Calsbad, CA, USA). The medium was changed with fresh medium every 3 to 4 days and subcultured every 10 to 15 days. When cells were in the early plateau phase, culture medium was removed and washed with PBS. After treatment with 0.25% trypsin, 0.03% ethylenediamine tetraacetic acid (EDTA) solution for 5 min, fresh culture medium containing 10%(v/v) FBS was added. Then single cell suspensions were prepared, and cell quantities were counted. The cell suspensions at 5×10^3 cells/well were transferred to the 96 wells, and final volumes were adjusted to 100 μL by adding fresh culture media.

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay For the measurement of changes in DNA synthesis and growth after treatment of polysaccharide to cells, the BrdU incorporation assay using cell proliferation enzyme-linked immunosorbent assay (ELISA, Roche Applied Science, Penzberg, Germany) was performed according to instructor's manual. FHC cells and 3 cancer cell lines were cultured in 96 wells at a concentration of 5×10^3 cells/well with different concentrations of polysaccharide extracted from *B. bifidum* BGN4. After 48 hr, the cells were labeled with 10 μM of BrdU labeling reagent which can be incorporated into DNA. After 3 hr of labeling period, the cells were fixed and denatured, and the incorporated BrdU was captured by reaction with anti-BrdU-peroxidase antibodies, followed by colorimetric immunoassay procedures. The absorbance of the samples was measured by ELISA reader at 450 nm.

Hydrophobicity of *B. bifidum* BGN4 cells The hydrophobicity of bacterial cell surface was assayed by modified method of Perez *et al.* (16). After washing with PBS, *B. bifidum* BGN4 cells cultured in MRS and MRS broth containing phytic acid (MRS-PCM) were resuspended in PBS. Bacterial suspensions were vortexed with 1/4 volumes of xylene for 1 min and allowed for phase separation for 20 min. The hydrophobicity was measured by using absorbance values of the aqueous phase before and after mixing with xylene and calculated using the following equation.

Cell surface hydrophobicity (%)= $(A_i-A_f)/A_i \times 100$

A_i: initial absorbance, A_f: final absorbance

Bacterial cell adhesion to epithelial cells After culturing of Caco-2 cells in RPMI medium for 3 days, cell suspension of *B. bifidum* BGN4 in PBS were added. After 1 hr incubation with Caco-2 cells at 37°C in a 5% CO_2/95% air atmosphere, non-adherent bacterial cells in the culture medium were removed by washing 10 times with PBS. *B. bifidum* BGN4 cells attached to the Caco-2 cell layers were fixed with methanol for 10 min. Remaining cells were examined microscopically (1,000×) under oil immersion after staining by crystal violet solution. The numbers of adherent bacteria were counted in 10 random microscopic areas.

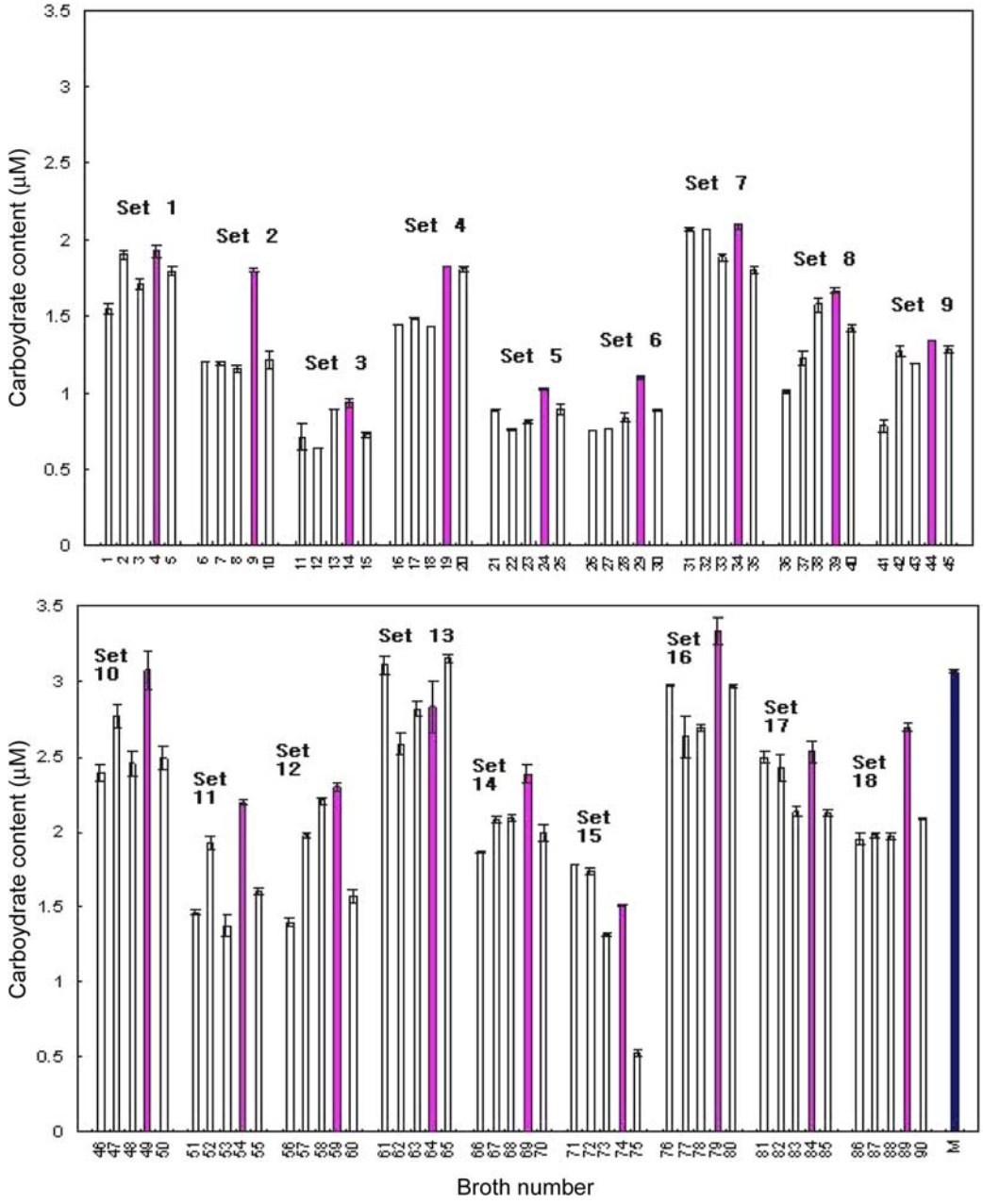

Fig. 1. Contents of total carbohydrate of *B. bifidum* BGN4 cultured in various media. The numbers of broth corresponded to the respective bacterial cultures grown in the different medium compositions shown in Table 1. M denotes MRS control medium.

Results and Discussion

Assessment of total carbohydrate and morphology of *Bifidobacterium* cells Total polysaccharide contents of *B. bifium* BGN4 grown in 90 different media compositions measured by phenol-sulfuric acid are assessed (Fig. 1). Five different media were grouped for comparison in each set. Among 18 sets, the greatest contents of polysaccharide were obtained in phytic acid containing medium in sets 2, 5, 6, 8, 10, 11, 12, 14, 16, and 18. The degrees of growth were not noticeably affected by phytic acid (data not shown). Microscopic examination showed that the presence of phytic acid in growth medium induced marked branch formation of *B. bifium* BGN4. As an example, the morphological changes of *B. bifium* BGN4 grown in the presence of phytic acid are shown in Fig. 2. No other media except phytic acid containing medium showed marked branch formation (data not shown). To further characterize the effect of phytic acid, its concentration was varied from 0 to 5%(w/v). In consequence, the degree of branch formation increased as the concentration of phytic acid increased up to 3%, at which concentration phytic acid might have chelated most of the calcium ions present in the medium (Fig. 3).

Effects of isolated polysaccharides on the growth of cancer cells and normal cells Based on the observation that the production of polysaccharide was enhanced in the presence of phytic acid, the inhibitory effects of polysaccharide obtained from phytic acid containing medium on the proliferation of tumor cells were assessed by BrdU incorporation assay in 3 cancer cell lines (MCF-7, HT-29, and Caco2 cells), and 1 normal colon cells, FHC cells for comparison. The polysaccharide showed marked growth inhibitory effects on HT-29 cells and MCF-7 cells, whereas it stimulated proliferation of FHC and Caco-2 cells at 50 μg/mL concentration (Fig. 4). Considering Caco-2 cells resemble normal cells compared with HT-29 cells, the polysaccharide showed selective inhibition on more tumorigenic cell lines.

Hydrophobicity and cell adhesion of *Bifidobacterium* to Caco-2 cells Adhesion of the probiotics to the intestinal mucosa is regarded as one of the most important criteria

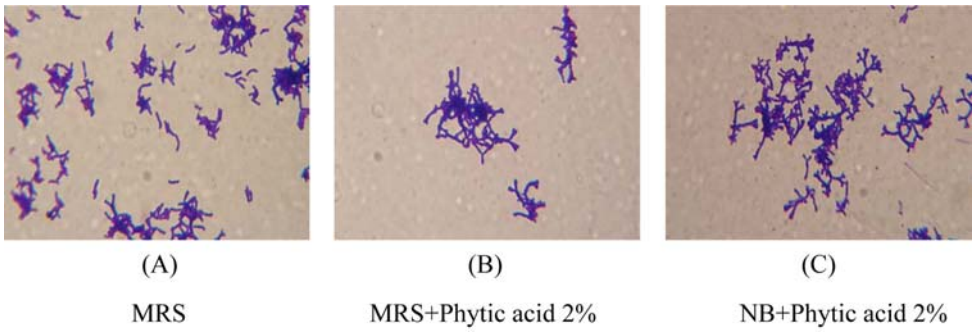

Fig. 2. Bacterial morphology (at 1,000× magnification) of *B. bifidum* BGN4 cultured in media containing 2%(w/v) phytic acid. (A) Bacilloid form of BGN4 cultured in MRS control medium. (B) Branched form of *B. bifidum* BGN4 cultured in MRS medium containing 2% of phytic acid. (C) Branched form of *B. bifidum* BGN4 cultured in NB medium containing 2% of phytic acid.

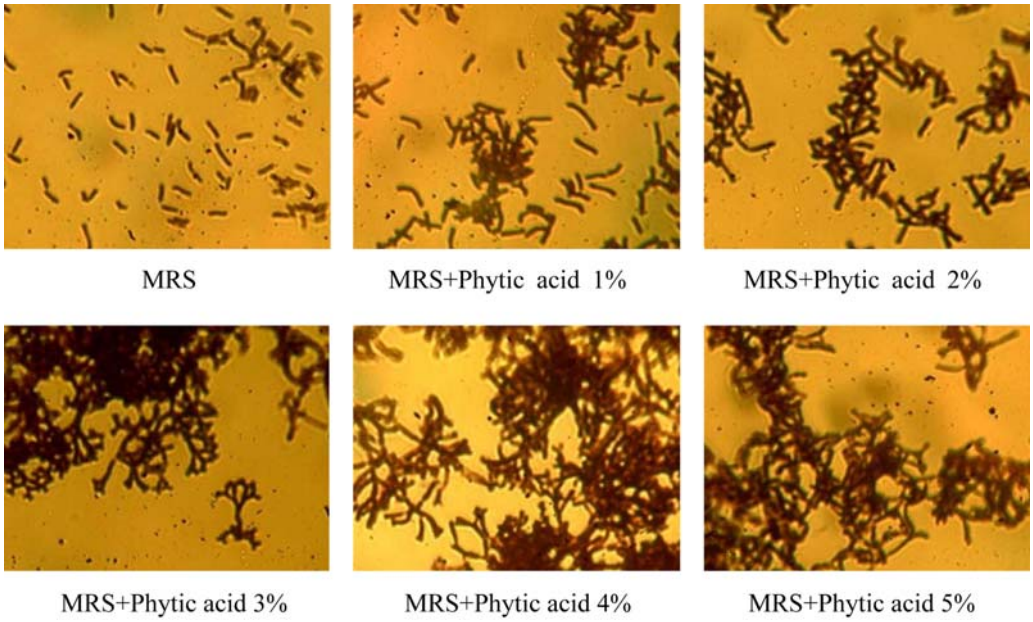

Fig. 3. Effect of phytic acid at various concentrations from 0 to 5%(w/v) on the morphological change of *B. bifidum* BGN4.

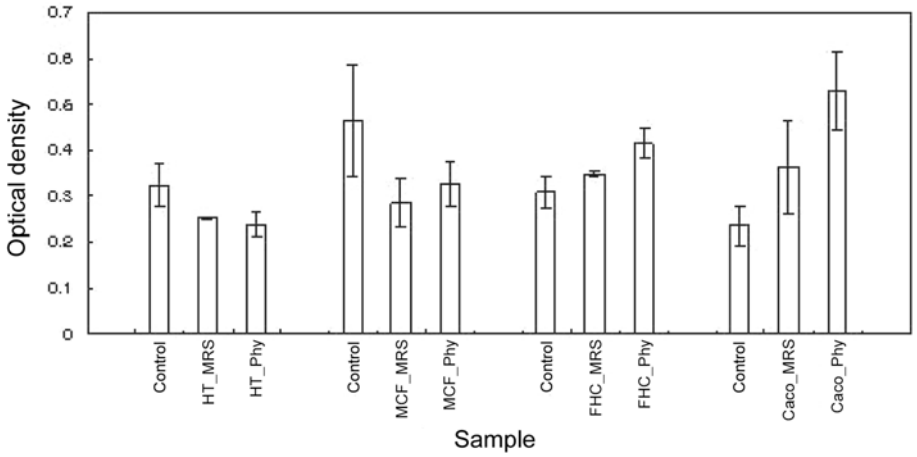

Fig. 4. Effect of *B. bifidum* BGN4 polysaccharide on the growth of different cell lines. Cell lines were reacted with polysaccharides (50 μg/mL) from *B. bifidum* BGN4 grown in MRS and MRS-PCM PCM media. The inhibitory effects of polysaccharides were assessed by BrdU incorporation assay. Control, PBS vehicle; HT, HT-29 colon cancer cell line; MCF, MCF-7 breast cancer cell line; FHC, normal colon cell line; Caco, Caco-2 colon cancer cell line; MRS and Phy, polysaccharides from *B. bifidum* BGN4 grown in MRS and MRS-PCM (2%, w/v of phytic acid) medium.

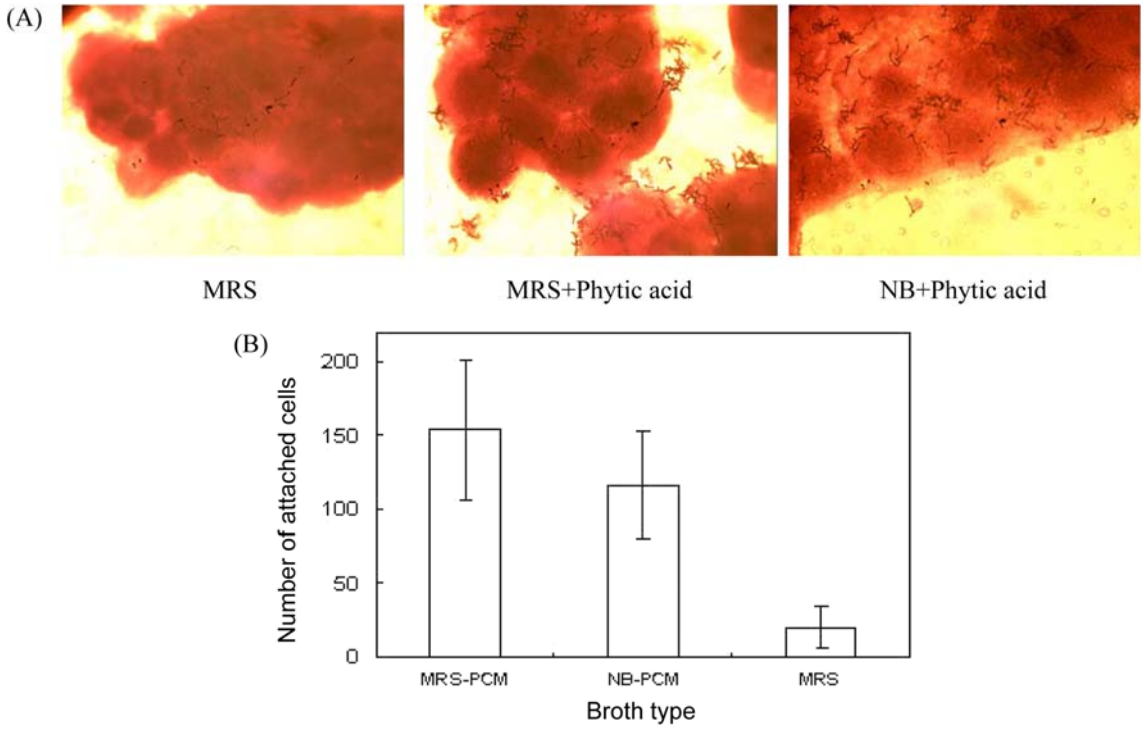

Fig. 5. Adhesion ability of *B. bifidum* BGN4 cultured in 3 different media. After treatment of *B. bifidum* BGN4 on Caco-2 cells, samples were stained by crystal violet (A) (1,000×). (B) MRS-PCM, MRS medium with phytic acid at 2%(w/v); NB-PCM, NB medium with glucose and phytic acid at 2%(w/v).

for selecting probiotics (5,6,17,). Previously, *B. bifidum* BGN4 showed the greatest adherence to Caco-2 cells among the various experimental strains of lactic acid bacteria (18). Adhesion of *B. bifidum* BGN4 cultured in MRS and MRS-PCM were compared in the present study. As a result, cell adhesion ability of *B. bifidum* BGN4 cultured in MRS-PCM was higher than normal *B. bifidum* BGN4 cultured in MRS about 8 folds (Fig. 5). Probiotics are living microorganisms that affect the host in a beneficial manner. Interestingly, the polysaccharide fraction of *B. bifidum* BGN4 was previously shown to exert anti-tumor effect on colon cancer cell lines (12). The anti-tumor activity of lactic acid bacteria may be exerted by multiple mechanisms including enhancement of host's immune system, degradation of potential carcinogens, production of anti-tumorigenic or anti-mutagenic compounds, and alteration of metabolic activities of intestinal microflora in the intestine. The anti-tumorigenic polysaccharide fraction (BB-pol) extracted from *B. bifidum* BGN4 grown in MRS had a novel composition consisting chiroinositol, rhamnose,

glucose, galactose, and ribose (12). In this study, the content of polysaccharide was significantly enhanced by phytic acid, but the composition of polysaccharide obtained from phytic acid containing medium were not yet verified. When 3 human colon cancer cell lines (HT-29, HCT-116, and Caco-2) were treated with BB-pol, BB-pol inhibited the growth of HT-29 and HCT-116 cells but did not inhibit the growth of Caco-2 cells. Consistent with the finding of You *et al*. (12), polysaccharide obtained from phytic acid containing medium showed marked inhibition of proliferation in HT-29 cells and MCF-7 cells but not Caco-2 and FHC cells. Considering Caco-2 cells possess characters of normal cell compared with HT-29 cells, the polysaccharide showed selective growth inhibitory effect on more tumorigenic cell lines. In the present study, we found that phytic acid enhanced not only the production of anti-tumorigenic polysaccharide but also adhesion and branch formation of *B. bfidum* BGN4. Adherence to epithelial cells and mucosal surfaces is an important property of *Bifidobacterium* as probiotics, because adhesion to the intestinal epithelium prevents them from being eliminated by peristalsis, thus providing a competitive advantage in the intestinal ecosystem (5). In addition, the high adhesive ability of *B. bifidum* BGN4 cells may facilitate the cell-bound cytotoxic polysaccharide to gain access to target tumor cells (19). However, some strains, in spite of their hydrophobic surface properties, were not capable of adhering to the intestinal epithelium, which suggested that multiple mechanisms are involved in the adhesion process. The strong hydrophobicity of *B. bifidum* cells was previously reported (18) and confirmed again in the present study. Considering phytic acid did not change the hydrophobicity of *B. bifidum* BGN4 cells, the enhanced adhesion of the phytic acid-grown cells might be related to other adhesion factor than cellular hydrophobicity. It is well known that *Bifidobacterium* species often shows branch forms, but the mechanism of branch formation has not been elucidated. Kojima *et al.* (20-22) reported that branched form of *Bifidobacterium* was induced in medium in which calcium ions were deficient or not available by the calcium chelating agents. Consequently, it was suggested that calcium ions were needed for the formation of cross wall formation in *Bifidobacterium*. The enhancement of branch formation by phytic acid might have been due to chelating action of phytic acid with calcium ions, and further supported from the observation that dephosphorylated forms of phytic acid such as myoinositol, pinitol, and chiroinositol did not induce branch formation in *B. bifidum* BGN4. Taken together, the *Bifidobacterium* grown in the presence of phytic acid showed enhanced anti-tumorigenic ability and adhesion to intestinal epithelial cells, which may confer enhanced beneficial function for the host.

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