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Anticarcinogenic and Antigenotoxic Effects of *Bacillus polyfermenticus*

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Abstract The morbidity and mortality of colon cancer are increasing, because of the westernization of food habit. Probiotics such as lactic acid bacteria (LAB) have been known to play an important role in retarding colon carcinogenesis by possibly influencing metabolic, immunologic, and protective functions in the colon. In this study, we evaluated the effect of *B. polyfermenticus* SCD on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induced DNA damage in CHO-K_1 cells and human lymphocytes, and on proliferation of human colon cancer cell. Using the Comet assay to detect DNA damage, we found that *B. polyfermenticus* SCD protected cells from the DNA damage induced by MNNG in CHO-K_1 cells and in human lymphocytes. *B. polyfermenticus* SCD was also found to inhibit the growth of colon cancer cells in a dose-dependent manner, detected by the MTT assay. These results indicate that *B. polyfermenticus* SCD has the potential to inhibit not only DNA damage induced by a carcinogen, but also the proliferation of colon cancer cells.

Key words: *Bacillus polyfermenticus* SCD, DNA damage, comet assay, colon cancer, MTT assay, antigenotoxic, anticarcinogenic

Probiotics, defined as a viable microbial dietary supplement that beneficially affects the host through its effects on the intestinal tract, are rapidly gaining interest as functional foods in this era of self-care and complementary medicine [22]. Probiotics, which have been used for medical purposes, include strains of *Bifidobacterium, Lactobacillus, Enterococcus, Clostridium butyricum, Lactobacillus sporogenes, Bacillus subtilis,* and *Bacillus polyfermenticus* [17, 22]. In particular, strains of *Bacillus polyfermenticus*, commonly known as 'Bispan' strains, have been used for long-term intestinal disorders, since the live strains in the form of active endospores can reach the intestine, and produce a variety of enzymes, which lyse pathogenic strains such as typhoid bacillus, paratyphoid bacillus, shigella, and cholera [15].

Colon cancer is one of the leading causes of morbidity and mortality not only in Western industrialized countries, but also in developing Asian countries, suggesting a strong linkage between colon cancer and changes in Asian lifestyle, especially diet [12]. Much attention has been focused on reducing colon cancer risk through diet alterations, particularly in terms of increasing the intake of dietary fiber and the consumption of probiotics [6].

Cancer development is a multistage process that occurs when accumulation of mutations in certain protooncogenes and tumor suppressor genes leads to cancer initiation. DNA damage of these genes at the initiation stage could lead to mutations, and such mutations are known to be highly correlated with cancer development [14, 26]. Therefore, it appears reasonable in the study of anticarcinogenic mechanisms to search for substances that prevent DNA damage. DNA damage can be detected in single mammalian cells by the single-cell gel electrophoresis, also known as the comet assay, which was developed by Singh *et al.* [24] The oral administration of lactic acid bacteria (LAB) has been shown to effectively reduce the DNA damage induced by chemical carcinogens in gastric and colonic mucosa in rats [21]. Moreover, a number of studies in animal models and in the human population have demonstrated that the consumption of probiotic bacteria can reduce colon cancer risk. Most of these studies have focused on LAB, such as *Lactobacillus* spp. and *Bifidobacterium* spp. [5, 16, 19], whereas the anticarcinogenic effect of *B. polyfermenticus* strain has not yet been shown, despite of its other beneficial effect on health [15]. Therefore, we investigated the effects of *B. polyfermenticus* SCD on MNNG-induced DNA damage in CHO-K_1 cells and in human lymphocytes by using the comet assay and on the proliferation of human colon

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cancer cells by the MTT assay to elucidate the possible mechanism of the cancer preventing activities of *B. polyfermenticus* SCD.

MATERIALS AND METHODS

Bacterial Strain and Medium

B. polyfermenticus SCD was maintained at -70°C in a tryptic soy broth (TSB, Difco) to which 20% (v/v) glycerol was added. Working cultures were propagated in TSB with shaking at 37°C.

Preparation of *B. polyfermenticus* SCD

B. polyfermenticus SCD production was carried out as previously described [18]. Briefly, *B. polyfermenticus* SCD was inoculated into 60 ml sterile tryptic soy broth (TSB; Difco), and the seed culture (2%, v/v) was then transferred to a jar fermenter (3 l working volume; Korea Fermenter Co., Korea). Temperature and pH were maintained at 37°C and 7.0±0.1, respectively. *B. polyfermenticus* SCD was obtained by centrifugation (10,000 rpm, 30 min) of 500 ml of the resulting stationary phase cells after 16 h of incubation for vegetative cells and 72 h of incubation for spore cells. The pellets were then freeze-dried and stored at 4°C.

Cell Culture

Chinese hamster ovary cells (CHO-K_1) were purchased from the Korea Cell Line Banks (Seoul, Korea). CHO-K_1 cells were cultured at 37°C in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 292 mg/l L-glutamine, and 2,200 mg/l sodium bicarbonate (Sigma) in a 5% CO_2 atmosphere. CHO-K_1 cells were seeded into 6-well tissue culture plates at a density of 3×10^4 cells/well and grown in culture medium for 72 h.

Isolation and Cryoconservation of Human Peripheral Lymphocytes

Blood samples were obtained from two healthy male volunteers (non-smokers, 23 years old) and 5 ml of fresh whole blood was added to 5 ml of phosphorous buffered saline (PBS) and layered onto 5 ml of Histopaque 1077. After centrifugation for 30 min at 400 ×g and room temperature, the lymphocytes were collected from just above the boundary with the Histopaque 1077, washed with 5 ml PBS and, finally, resuspended in freezing medium (90% fetal calf serum, 10% dimethyl sulfoxide) at 6×10^6 cells/ml. The cells were frozen to -80°C using a Nalgene Cryofreezing container and stored in liquid nitrogen. Cells were thawed rapidly in a water bath at 37°C prior to each experiment.

Treatment of Cells with *B. polyfermenticus* SCD

Lyophilized *B. polyfermenticus* SCD vegetative cells or spore cells were suspended in Hank's balanced salt solution (HBSS) at concentrations of 0, 10, 25, 50, or 100 mg/ml. Each suspension was then preincubated with MNNG (Fluka) at a final concentration of 50 µg/ml for 30 min in a shaking incubator (37°C, 150 rpm). For the negative control, HBSS without bacteria suspension was treated with PBS instead of MNNG under the same conditions. Bacteria were then removed by centrifugation at 11,000 ×*g* for 10 min, and aliquots (10 µl) of the supernatants (preincubation mixture), containing unidentified metabolites of *B. polyfermenticus* SCD and MNNG (0.5 µg/ml), were used for cell treatments. The CHO-K_1 cells grown in 6-well plates were washed twice in PBS, pH 7.4, before being exposed to each preincubation mixture for 30 min at 37°C in a shaking incubator. The treated cells were then washed twice with PBS, recovered from the plate using trypsin/EDTA, and centrifuged at 2,000 rpm for 5 min. The cell pellets obtained were then resuspended in 300 µl of RPMI 1640 medium, and 20 µl of the cell suspension were mixed with 75 µl of 0.5% low melting agarose (LMA) for the comet assay.

For the treatment of lymphocytes with *B. polyfermenticus* SCD, 1 ml of lymphocyte suspension containing 2×10^4 cells/ml was also treated with aliquots (10 µl) of preincubation mixture for 30 min at 37°C in a shaking incubator. The lymphocytes were then washed with PBS, centrifuged at 2,000 rpm for 5 min, and resuspended in 75 µl of 0.5% LMA for the comet assay. Cells were checked for viability by the trypan blue exclusion method. Cell viabilities measured immediately after each treatment always exceeded 90%.

Determination of DNA Damage (Comet Assay)

The alkaline comet assay was conducted as described by Singh *et al.* [24] with slight modification (Fig. 1). Briefly, cell suspension mixed with LMA was added to slides precoated with 1.0% normal melting agarose (NMA). After the agarose had solidified, the slides were covered with another 75 µl of 0.5% LMA and then immersed in

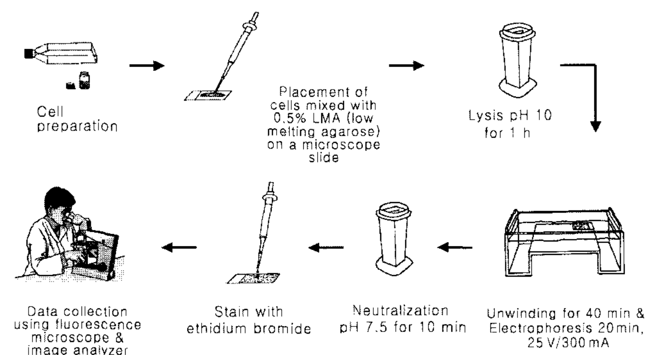

Fig. 1. Comet assay procedure.

lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4°C. The slides were then placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min, to allow the DNA to unwind. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4°C. The slides were then washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C, and treated with ethanol for another 5 min before being stained with 50 µl of ethidium bromide (20 µg/ml). Fifty cells from each of two replicate slides were processed for each treatment group. The cells were scored for the parameter "% fluorescence in tail," the intensity of fluorescence in the comet tail (Fig. 4), using image analysis (Kinetic Imaging, Komet 5.0, U.K.) and fluorescence microscopy (LEICA DMLB, Germany).

Determination of Cell Proliferation (MTT assay)

Caco-2 cells, which originated from a human colon adenocarcinoma, were purchased from the Korea Cell Line Bank. Cells were cultured in Eagle's minimal essential medium (Gibco) supplemented with 20% fetal bovine serum (Gibco), 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 292 mg/l L-glutamine, and 2,200 mg/l sodium bicarbonate (Sigma), and maintained at 37°C in a 5% CO_2 atmosphere.

Caco-2 cells were seeded at a density of 2.5×10^4 cells/100 µl in 96-well plates. The cells were allowed to adhere for 24 h, and were then treated with *B. polyfermenticus* SCD vegetative cells or spore cells suspended in culture medium at concentrations of 100, 500, 1,000, and 2,000 µg/ml for 72 h in an incubator (37°C, 5% CO_2).

After incubation, the culture media containing *B. polyfermenticus* SCD were removed, and 50 µl of 2 mg/ml solution of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in PBS were added to each well, and incubation was continued for a further 4 h at 37°C. The dark blue formazan crystals formed in intact cells were solubilized with 150 µl of DMSO, and absorbance at 540 nm was measured using a microplate reader (ELx800UV, Biorad). The percentage of viable cells was calculated using the following formula:

$$\text{Viability (\% of control)} = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100$$

where A_{sample} is the absorbance of the cells treated with *B. polyfermenticus* SCD, A_{blank} the absorbance of the solution used, and $A_{control}$ the absorbance of the cells not treated with *B. polyfermenticus* SCD.

Statistical Analysis

Data were analyzed using the SPSS package for Windows (Version 10). Values are expressed as mean±standard error (SE). The mean values of the tail intensities of each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P*-values of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Effect of *B. polyfermenticus* SCD on MNNG-Induced DNA Damage in $CHO\text{-}K_1$ Cells and Human Lymphocytes

Figure 2 shows the antigenotoxic effect of *B. polyfermenticus* SCD on MNNG-treated $CHO\text{-}K_1$ cells, determined by the

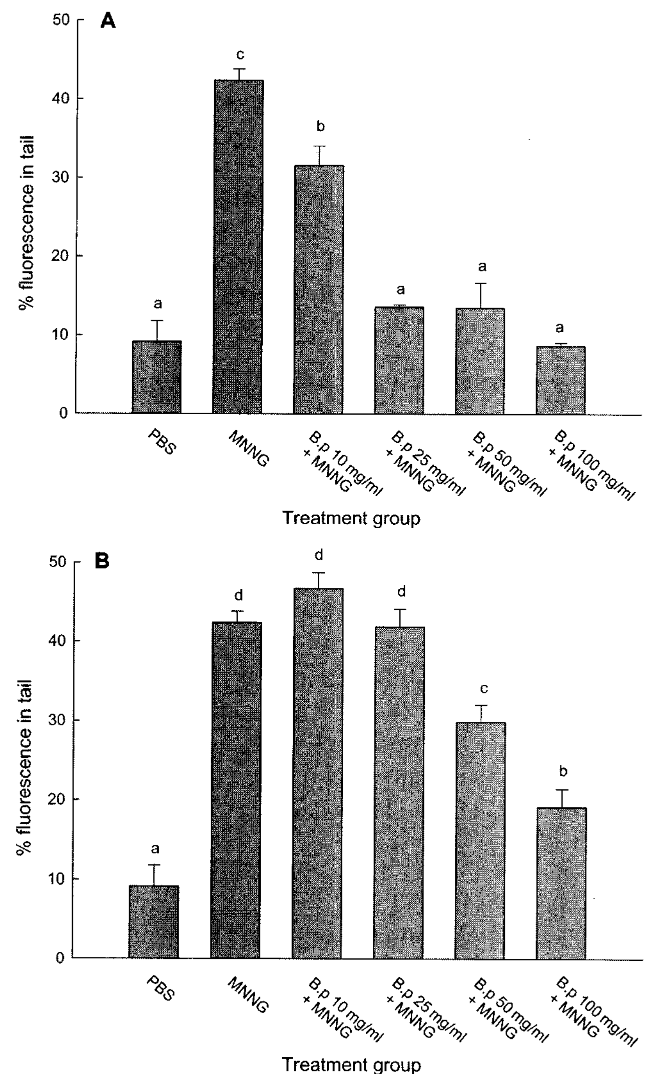

Fig. 2. Antigenotoxic effect of *B. polyfermenticus* SCD against MNNG in $CHO\text{-}K_1$ cells, determined by the comet assay.

% Fluorescence tail DNA as a parameter of DNA damage was determined in cells treated for 30 min with negative control, positive control (MNNG, 0.5 µg/ml), or with the preincubation mixture of MNNG together with vegetative cells (A) or spore cells (B) of *B. polyfermenticus* SCD in HBSS at concentrations of 10, 25, 50, 100 mg/ml. Values are means and standard errors of triplicate experiments on $CHO\text{-}K_1$ cells. Different letters above bars indicate significant differences at α=0.05 by Duncan's multiple range test.

comet assay. The % fluorescence tail DNA intensity of CHO-K_1 cells treated for 30 min with negative control was significantly different from the positive control (MNNG preincubated in HBSS, 0.5 µg/ml). This increase of DNA damage induced by MNNG was significantly inhibited in a dose-response manner by preincubating MNNG together with the vegetative cells of *B. polyfermenticus* SCD at concentrations of 10, 25, 50, and 100 mg/ml in HBSS (Fig. 2A). The tail intensity began to significantly decrease at the lowest concentration (10 mg/ml) by 32.1% of the positive control. The maximum inhibition was 79.3% at 100 mg/ml concentration, showing no statistical difference from the PBS-treated negative control. In the case of pretreatment with *B. polyfermenticus* SCD of the spore cell type and MNNG, the % inhibition of MNNG-induced DNA strand breaks were by 35.9% and 61.1% of the positive control at concentrations of 50 and 100 mg/ml, respectively, whereas no protective effects were evident at the low concentrations of 10 and 25 mg/ml (Fig. 2B).

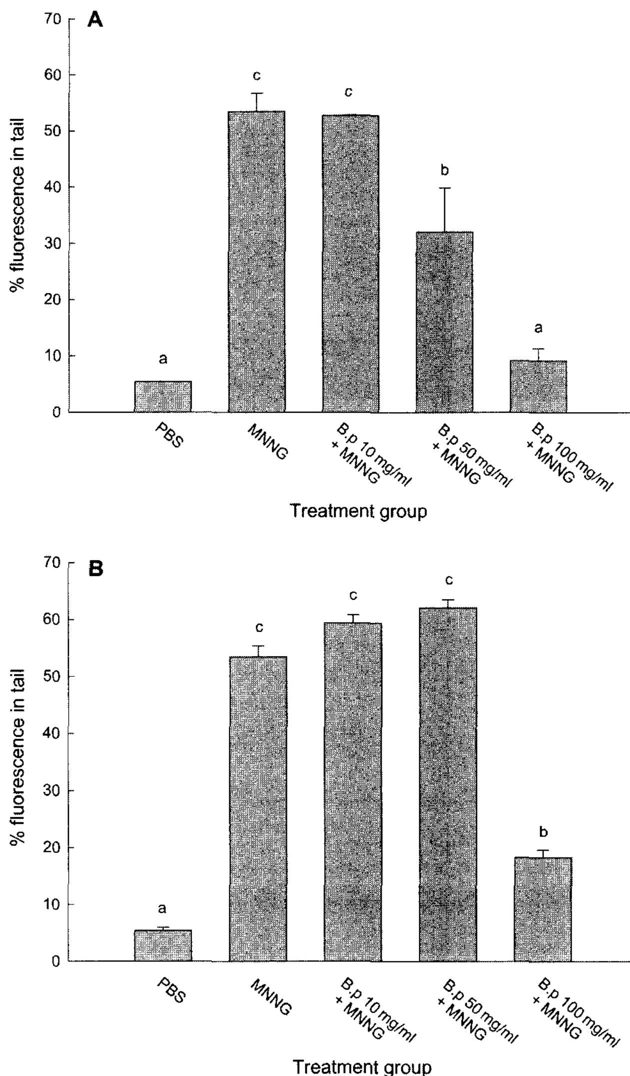

Fig. 3. Antigenotoxic effect of *B. polyfermenticus* SCD against MNNG in human lymphocytes, determined by the comet assay.
% Fluorescence tail DNA as a parameter of DNA damage was determined in cells treated for 30 min with negative control, positive control (MNNG, 0.5 µg/ml), or with the preincubation mixture of MNNG together with vegetative cells (A) or spore cells (B) of *B. polyfermenticus* SCD in HBSS at concentrations of 10, 50, 100 mg/ml. Values are means and standard errors of triplicate experiments using lymphocytes from two different donors. Different letters above the bars indicate significant differences at α=0.05 by Duncan's multiple range test.

We also tested the antigenotoxic effect of *B. polyfermenticus* SCD on human lymphocytes (Fig. 3). The preventive effect of the vegetative cells began to appear at 50 mg/ml concentration and reached a maximum inhibition at 100 mg/ml concentration by 86.8% of the positive control, which was almost the same tail intensity as that of the PBS-treated negative control (TI (%): 7.05±0.76 for 100 mg/ml vs. 5.36±0.55 for negative control) (Fig. 3A). For spore cells type (Fig. 3B), cells treated at 100 mg/ml showed significant protection by 65.9% of the positive control, whereas an increase of DNA damage was observed at low concentrations (10 and 50 mg/ml). These results are shown visually in Fig. 4, which represents the comet image of the results of each treatment in human lymphocytes.

MNNG, a direct-acting carcinogen with alkylating properties, was chosen as a representative of compounds that endogenously form N-nitroso compounds, and it induces DNA base substitution mutation by attacking the nucleophilic sites of DNA bases [10]. Bingham *et al.* [4] showed that high meat diet could induce threefold increase in endogenous N-nitroso compounds in humans. In this study, we found that preincubation of *B. polyfermenticus* SCD reduced the mutagenicity of MNNG and prevented MNNG-induced DNA damage in CHO-K_1 cells and human lymphocytes. The antigenotoxic action of *B. polyfermenticus* SCD may be due to binding and degradation of MNNG before it reaches the DNA in CHO-K_1 cells and human lymphocytes during the 30 min preincubation. Previously, Hosoda *et al.* [10] and Hosono *et al.* [11] reported that cultured milk could inactivate MNNG by binding it to various LAB in the sample. Moreover, the vegetative cells of *B. polyfermenticus* SCD showed more effective antigenotoxic activity than the spore cells, indicating that the viability of *B. polyfermenticus* SCD seems to play a significant role in antimutagenesis. Although the spore cells were less protective, the significance of the spore cells should not be underestimated, because of the resistant nature of the endospore form, which is capable of reaching the intestine [15]. The effect of the vegetative cells of *B. polyfermenticus* SCD was found to be dose dependent. Overall, 100 mg/ml ($\approx 1\times10^8$ CFU/ml) of *B. polyfermenticus* SCD was able to protect against the MNNG-induced DNA damage in CHO-K_1 cells or in human lymphocytes. Abdelali *et al.* [1] found that the antimutagenic effect of *Bifidobacterium* sp. in the Ames test was only

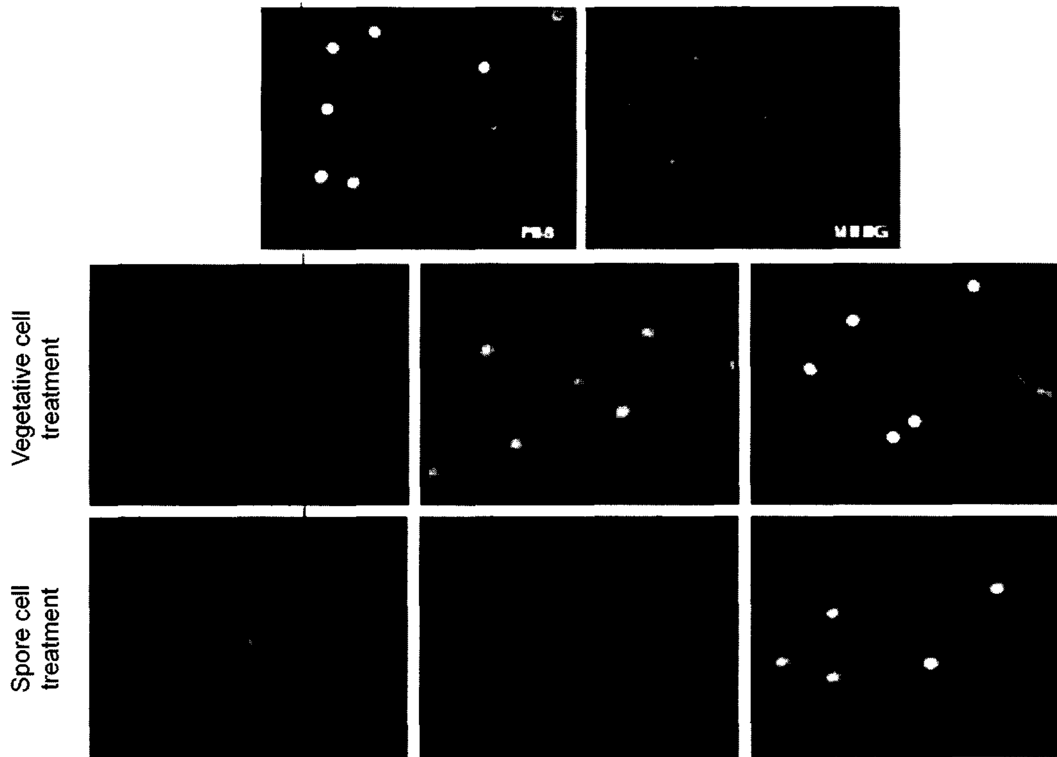

Fig. 4. Comet images of each human lymphocyte treatment, by fluorescence microscopy (×40).

significant, when >5×10⁹ CFU/ml were present. *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* 191R, and *Streptococcus thermophilus* CH3 showed significant antigenotoxic activities against MNNG, when bacteria at 1×10⁹ CFU/ml were applied to the primary colon cells of rats, determined by the comet assay [13].

Effect of *B. polyfermenticus* SCD on the Proliferation of Caco-2 Cells

MTT is a tetrazolium salt, which is reduced to formazan (1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan) by living cells via the 'succinate-tetrazolium reductase' system [9, 25]. The formazan produced by the cellular suspension is directly correlated with the number of metabolically active cells, and the colorimetric MTT assay is used to assay cell proliferation. Therefore, we conducted the MTT assay to evaluate the growth inhibitory effect of *B. polyfermenticus* SCD on human colon adenocarcinoma cells. As shown in Fig. 5, *B. polyfermenticus* SCD was found to inhibit the growth of colon cancer cells in a dose-dependent manner. Cell growth was suppressed by 24.6, 20.3, 37.1, and 42.2% after 72 h of treatment with the vegetative cells of *B. polyfermenticus* SCD at 100, 500, 1,000, and 2,000 μg/ml, respectively (Fig. 5A). The spore cells showed weaker cytotoxic effect even at the high concentrations of 1,000 and 2,000 μg/ml, which inhibited the growth by 21.7% and 24.0% (Fig. 5B). Previously, Baricault *et al.* [2] reported that LAB significantly reduced the growth and viability of the HT-29 human colon cancer cells in culture, and that dipeptidyl peptidase IV and brush border enzyme levels were significantly upregulated, suggesting that these cells may have entered a differentiation process. Although the anticarcinogenic action of *B. polyfermenticus* SCD or LAB is far from clearly being elucidated, the increased apoptosis of colon cancer cell, as observed in an animal study, may play a role [7]. Another possible mechanism for the anticarcinogenic effect of *B. polyfermenticus* SCD arises from the results of a recent human intervention study. Recently, Park *et al.* [20] showed that 2 weeks of *B. polyfermenticus* SCD administration to healthy adults (3.334×10⁷ CFU/day) improved fecal microflora by increasing the numbers of total aerobic bacteria, LAB, and bifidobacteria, and by reducing *Clostridium*, *Clostridium perfringens* and coliform. In addition, it lowered the pH of fecal samples. These changes are likely to provide a protection mechanism against colon cancer [3, 8, 23].

In conclusion, the present work showed that *B. polyfermenticus* SCD has the potential to inhibit not only the DNA damage induced by a carcinogen (i.e., the initial stage of carcinogenesis), but also to inhibit the proliferation of colon cancer cells. These results provide new insights into the mechanism of the anticancer properties of *B. polyfermenticus* SCD. *In vivo* studies using an animal

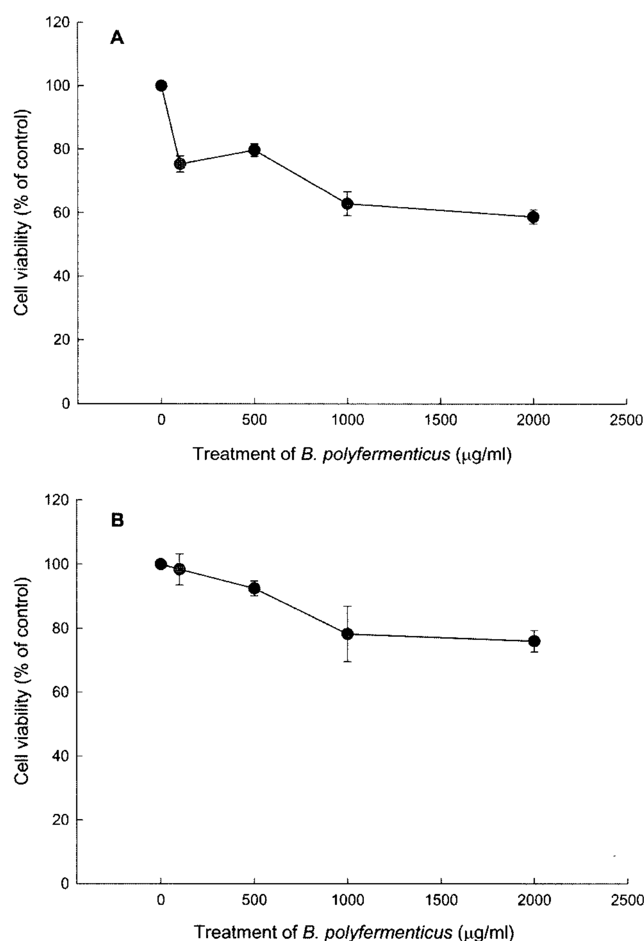

Fig. 5. Cytotoxic effect of *B. polyfermenticus* SCD in Caco-2 cells.

Caco-2 cells were treated with the indicated concentrations of *B. polyfermenticus* SCD vegetative cells (A) or spore cells (B) for 72 h. The cells were then incubated with MTT solution for another 4 h. Each point represents mean±SE values of three separate experiments.

model are in progress to provide more information on this possibility.

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