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Anti-proliferative and Apoptosis Inducing Activity of *Lactobacillus brevis* OPK-3 Isolated from Kimchi on Leukemia Cell Lines

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Abstract: In the present work we investigated the effects of lactic acid bacteria (LAB) isolated from kimchi on proliferation and apoptosis of cancer cells. The cell-free supernatant concentrate of *Lactobacillus brevis* OPK-3 significantly retarded the proliferation of human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cell lines *in vitro* at concentrations over 2.25-9.0 mg/mL. The treatments of the concentrate leaded to the increased apoptosis and decreased mitochondrial transmembrane potential in cultured U937 leukemia cell lines. In addition, the treatments of the concentrate showed the increased expression of p53 gene in cultured U937 and HL60 leukemia cell lines. On the other hand, the cell-free supernatant concentrate of control *L. brevis* strain (KCCM 41028) showed a relatively little effect on the cancer cell proliferation, apoptosis, and mitochondrial transmembrane potential at the similar concentration ranges compared with the *L. brevis* OPK-3 samples. These results suggest that the consumption of *L. brevis* OPK-3 could be beneficial for the inhibitory action on leukemia cell proliferation and for the stimulatory action on the cancer cell apoptosis.

Keywords: Lactic acid bacteria, cancer cell, growth, inhibition, apoptosis

Introduction

Kimchi is a well-known Korean traditional fermented food. Cabbage kimchi is rich in lactic acid bacteria (LAB) which are the major group of bacteria that can grow in kimchi. *Lactobacillus plantarum, Lactobacillus brevis, Leuconostoc mesenteroides, Streptococcus faecakis, Weissella koreensis* and *Pediococcus pentosaceus* are the dominant species of LAB found in Cabbage kimchi (Kim, 2005; Kim *et al*., 2005; Lee *et al*., 2002; Park and Oh, 2004; Park and Oh, 2005; Yu *et al*., 2009). The anticarcinogenic, immunomodulatory, antimicrobial, antidiarrheal and anti-allergy activities of kimchi LAB have been reported by several authors so far (Hur *et al*., 2006; Kim, 2005; Kim *et al*., 2005). Kimchi LABs are beneficial inhabitants of the intestines of humans and animals (Aguirre and Collins, 1993; Kim, 2005; Park and Oh, 2004). Consumption of 300 g of *kimchi* a day, raises the colonic lactic acid bacteria 100 times more in Kimchi eater than the non eater. Thus, *kimchi* provides a suitable environment for the growth of LAB in the intestine where as it inhibits the flourishment of pathogenic bacteria and viruses in the gastrointestinal tract (Park and Oh, 2004). Kimchi LAB secretes two types of medicinal amino acids namely are γ-aminobutyric acid (GABA) and ornithine as secondary metabolites in the culture media (Oh *et al*., 2010; Park and Oh, 2005; Park and Oh, 2006; Seok *et al*., 2008; Yu *et al*., 2009).

GABA is produced primarily by the α-decarboxylation of L-glutamic acid (Glu) catalyzed by the enzyme glutamate decarboxylase (GAD) (Ueno, 2000). The role of GABA in animals has been well understood as an inhibitory neurotransmitter with hypotensive and analgesic properties (Krogsgaard-Larsen, 1989; Mody *et al*., 1994). It has also been reported that GABA has an improving effect of visual perception in older animals (Leventhal *et al*., 2003). Ornithine is frequently used in the U.S. as a food supplement as well as anti-obesity agent (Elam *et al*., 1988; Leventhal *et al*., 2003). L-ornithine is also widely used as an anabolic material for muscle development (Wernerman *et al*., 1987) and immunity improvement (Kawai *et al*., 1999; Kawai *et al*., 2000; Robinson *et al*., 1999) in the form of L-ornithine-α-Ketoglutaric Acid (OKG) that contains L-ornithine and α-ketoglutaric acid in the ratio of 2:1. Many LAB from fermented foods such as kimchi, wine and cheese produce ornithine by the trans-

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formation of arginine via the arginine deiminase (ADI) pathway or the arginase-urease pathway (Arena *et al*., 1999; Kuensch *et al*., 1974; Liu *et al*., 2003). Accumulating data suggested that the transformation of arginine by these enzymes are cytotoxic to cancer cells (Ensor *et al*., 2002; Kim *et al*., 2009; Rijn *et al*., 2003).

We isolated LAB species *L. brevis* OPK-3 with GABA and ornithine producing capacities (Oh *et al*., 2010; Park and Oh, 2006; Park and Oh, 2007) and *Lactobacillus sakei* OPK2-59 with GABA producing capacities (Bae *et al*., 2009; Seok *et al*., 2008; Yu and Oh, 2011) from fermented foods such as kimchi (Bae *et al*., 2009). Previously, we reported that *L. sakei* OPK2-59 culture lyophilized powder supplementation improves serum lipid and lipid catabolic enzyme profiles in rats administered with ethanol (Bae *et al*., 2009). Genetically engineered *Bacillus subtilis* with an insert of *L. brevis* OPK-3 derived GAD gene, improved the functional characteristics in *Bacillus subtilis* (Park and Oh, 2006). Several authors reported that GABA has anti-proliferative activities in cancer cells and also it significantly increases apoptosis in cancer cells (Fava *et al*., 2005; Joseph *et al*., 2002; Sun *et al*., 2003; Tatsuta *et al*., 1992).

Therefore, we were interested to investigate whether kimchi LAB with GABA and ornithine producing capacities can affect the proliferation and apoptosis of cancer cells.

Materials and Methods

Cell culture

Human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) cell lines were provided from Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2 until reaching approximately 70-80% confluence.

Bacterial strain and materials

L.brevis OPK-3 (KFCC 11330) with high GABA and ornithine producing capacityes (Oh *et al*., 2010; Park and Oh, 2007) and *L. brevis* (KCCM 41028) with low the capacities (Seok *et al*., 2008) were used for the present study. Difco MRS medium (Detroit, MI, USA) was used for bacterial cell cultures. The LAB were cultured in MRS media supplemented with 1% (w/v) monosodium glutamate (MSG) for 3 d at 37°C. Cells were collected by centrifugation at 1,250 *g* for 20 min at 4°C. The cell-free supernatants were filtered through 0.45 μM sterile filter (Millipore, Bedford, MA, USA) and freeze dried. The powders were dissolved in double distilled water, syringe filtered and used for the analyses of GABA and anti-cancer activities.

Analysis of GABA

GABA was extracted essentially as described by Baum *et al*. 1996. GABA content in samples was analyzed by HPLC (Waters, USA) as described Park and Oh, 2005, 2006 and was calculated using the Autochro WIN program (Young-Lin, Korea) and expressed as an average value of the triplicate analyses.

Effects of the LAB culture supernatants on the proliferation of leukemia cells

Human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cell lines were seeded at a density of 5×10^4 cells /well and were treated with the LAB culture cell-free supernatants at concentrations of 2.25, 4.5, and 9 mg/mL followed by incubation in CO_2 incubator at 37°C. After 48 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, final concentration, 0.5 mg/mL) was added, and then incubations were continued for further 4 h (Mosmann *et al*., 1983). To dissolve formazan, 100 μL of dimethyl sulforxide (DMSO) was added to each well and the OD of each well was read using ELISA reader (Molecular Devices, Sunnyville, CA).

Effect of the LAB culture supernatants on the apoptosis of U937 cells in vitro

U937 cells were treated with the LAB culture cell-free supernatants (4.5 and 9 mg/mL) and incubated for 24 h at 37°C. Detection of apoptosis by flow cytometry was performed using propidium iodide (PI). The staining was performed according to the producer's manual. Cells were analyzed Sub G1 peak using a flow cytometer (Coulter: Epics- XL, Miami, FL, USA).

Effect of the LAB culture supernatants on the mitochondrial transmembrane potential of U937 leukemia cells

U937 cell line was seeded at a density of 1×10^6 cells/well. LAB culture cell-free supernatants were added into the cultures at concentrations of 2.25, 4.5 and 9 mg/mL and cultured for 24 h at 37°C. The cells were collected and mitochondiral transmembrane potential was measured by

a flow cytometer staining with DiOC6 (final Conc. 40 nM) for 15 min (excitation: 488 nm, emission: 525 nm).

Effect of the LAB culture supernatants on p53 gene expression in U937 and HL60 cells

U937 and HL60 cells were treated with LAB culture cell-free supernatants (9 mg/mL) and incubated in a CO_2 incubator at 37°C. After 24 h incubation, total RNA was isolated from the samples using Trizol (Invitrogen, Carlsbad, CA, USA). The RNA extraction was performed according to the producer's manual. cDNA synthesis was performed for 30 min at 50°C using oligo dT primer. The initial PCR step was activated by heating for 15 min at 95°C before PCR (30 s denaturation at 94°C, 30 s annealing at 57°C, 30 s extension at 72°C, 35 cycles) using a PCR Thermal cycler Dice (Takara, Japan). The PCR products were identified on a 1% (w/v) agarose gels. PCR reactions were performed using p53 primers: sense (5'-CCT CCT GGC CCC TGT CAT CT-3'), anti-sense (5'-ACA AAC ACG CAC CTC AAA GC -3').

Statistical analysis

Data from an individual experiment were described as a mean ± standard deviation. All statistical analyses were performed on a statistical analysis system (SAS) program, and significant difference between mean values was determined by using Student's t-test that $p<0.05$ was judged to be statistically significant.

Results

The cell-free supernatants concentrates (22.5-90.0 mg/mL) of LAB were prepared using *L. brevis* OPK-3 (KFCC 11330) and *L. brevis* (KCCM 41028) strains. The concentrates of *L. brevis* OPK-3 (KFCC 11330) and *L. brevis* (KCCM 41028) were standardized based on the contents of GABA determined using HPLC after 6-aminoquiolyl-N-hydroxysuccinimidyl carbonate (AQC) derivatization. GABA was found in the concentrates of *L. brevis* OPK-3 and *L. brevis* (KCCM 41028) at the levels of 10-40 μg/mL and 0.005-0.02 μg/mL, respectively.

To test the anti-cancer activities of the concentrates, human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cells were treated with the LAB concentrates at the concentrations of 2.25 mg/mL, 4.5 mg/mL, and 9.0 mg/mL, and incubated for 48 h, followed by proliferation and apoptosis of cancer cells were assayed. As shown in Fig. 1, the samples of *L. brevis* OPK-3 at concentrations over 2.25-9.0 mg/mL significantly retarded the proliferation rates of all tested leukemia cells compared with CON or control *L. brevis* sample. These results show that cell-free concentrates of the *L.brevis* OPK-3 culture inhibits cell pro-

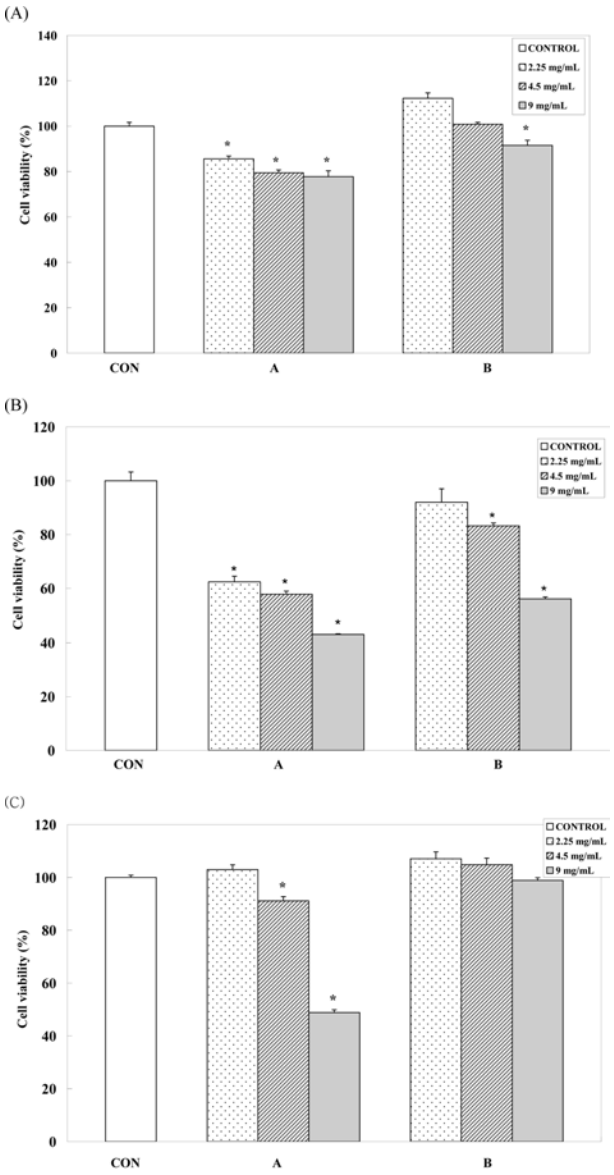

Fig. 1. Effect of the LAB samples on the proliferation of HL60 (human acute promyelocytic) (A), U937 (human histiocytic) (B), L1210 (mouse lymphocytic) (C) leukemia cell lines *in vitro*. The LAB samples were added to the various cultured leukemia cell lines at the indicated concentration and cultured for 48 h at 37°C. The proliferation of the cells was assayed by the MTT method. The O.D of each well was measured at 570 nm with a microplate reader. Each bar represents the mean ± SD of three experiments. CON; leukemia cell line without LAB sample treatment. A, *L. brevis* OPK-3 sample; B, *L. brevis* KCCM 41028 sample. *Significantly different from control group (*$p<0.05$).

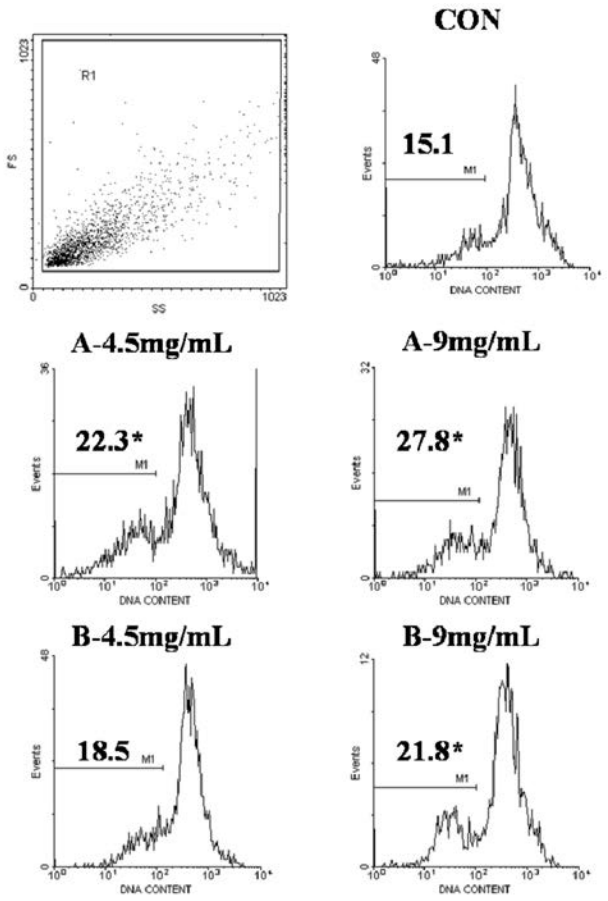

Fig. 2. Effect of the LAB samples on the Sub G1 peak of U937 cells *in vitro*. The LAB samples were added into the cultured U937 cells at the indicated concentration and cultured for 24 h at 37ºC. The apoptosis of the cells was assayed by flow cytometric analysis. Each values represent the mean of three experiments. CON; leukemia cell line without LAB sample treatment. A, *L. brevis* OPK-3 sample; B, *L. brevis* KCCM 41028 sample. *Significantly different from control group (*p<0.05).

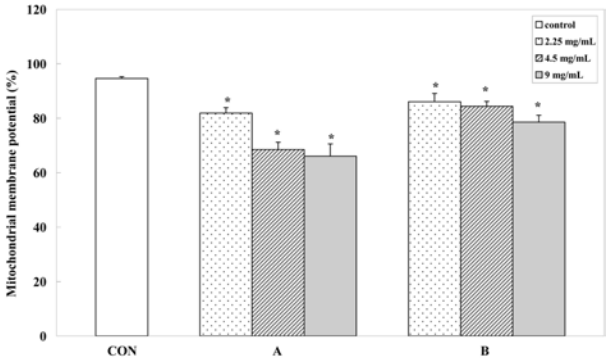

Fig. 3. Effect of the LAB samples on mitochondrial transmembrane potential in U937 leukemia cells. The suspension of cells was prepared at 1×10^6 cells/well. The LAB samples were added into the cultures at the indicated concentration and incubated for 24 h at 37ºC. The cells were collected and MTP was measured by a flow cytometer staining with DiOC6 (40nM). The each bar represents the mean ± standard deviation of three experiments. CON; leukemia cell line without LAB sample treatment. A; *L. brevis* OPK-3 sample; B, *L. brevis* KCCM 41028 sample. *Significantly different from control group (*p<0.05).

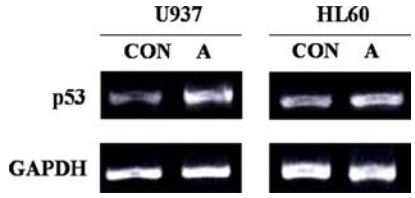

Fig. 4. Effect of the LAB samples on p53 gene expression in U937 and HL60 cells. The LAB samples were added to the various cultured leukemia cells for 24 h at 37ºC. CON; cell lines without sample treatment. A; cell lines treated with the *L. brevis* OPK-3 samples (9 mg/mL). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

liferation in wide range of leukemia cell lines.

To further investigate the effects of the LAB concentrates on anti-cancer activity, we treated U937 leukemia cells with the concentrates and measured Sub G1 peak and mitochondrial transmembrane potential (MMP) in these cells. As shown in Figs. 2 and 3, U937 cells treated with the concentrates of *L. brevis* OPK-3 culture demonstrated a significantly increased in Sub G1 peak and decreased MMP levels comparing with untreated cells and cells treated with control concentrates. L1210 cells showed a similar pattern in the Sub G1 peak and MMP levels (data not shown). These data suggests that the cell-free supernatant concentrates of *L. brevis* OPK-3 culture effectively induce apoptosis in U937 leukemia cells. The enhanced apoptosis of the leukemia cells could be one of the underlying reasons for the anticancer activities of the concentrates.

To investigate the effects of the cell-free supernatant concentrates on p53 gene expression, we treated U937 and HL60 cells with the concentrates and measured the expression of p53 gene by RT-PCR analysis. As shown in Fig. 4, the treatment of cell lines with the concentrates of *L. brevis* OPK-3 culture showed an enhanced expression of p53 gene compared with untreated cells.

Discussion

In this present study, we have investigated the effects of the cell-free supernatant concentrate of *L. brevis* OPK-3

culture on proliferation and apoptosis of cancer cells. The concentrates from *L. brevis* OPK-3 culture showed anti-proliferation and apoptosis activities on the cancer cell lines; human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cells. On the other hand, control concentrates of *L. brevis* (KCCM 41028) culture showed a relatively little effects on the cancer cell lines.

It has been reported that both the culture medium and live whole cells from probiotic strains can have anticancer effects. For example, culture medium and live whole cells of *Enterococcus faecium* RM11 and *Enterococcus fermentum* RM28 isolated from fermented dairy milks triggered anti-proliferation of colon cancer cells at the rates of 21-29% and 22-29%, respectively (Thirabunyanon *et al*., 2009). The cell-free supernatants of *Lactobacillus acidophilus* 74-2 upregulated cyclo-oxygenase (COX)-1 in gastric cancer cells showing an anticancer potential (Mahkonen *et al*., 2008). Probiotic effects of kimchi LAB has also been reported (Hur *et al*., 2006; Kim, 2002; Kim, 2005; Kim *et al*., 2005). For example, *Lactobacillus plantarum* showed immunopotentiating and antimutagenic activities (Hur *et al*., 2006; Rhee and Park, 1999). However, little is known on the mechanism and the products underlying the probiotic effects of kimchi LAB.

One of the underlying possible reason for inhibitory effects could be the presence of GABA. It has been reported that GABA has inhibitory effects on the growth of colon carcinoma (Joseph *et al*., 2002), gastric cancer (Tatsuta *et al*., 1992), hepatocarcinoma (Sun *et al*., 2003) and cholangiocarcinoma (Fava *et al*., 2005). For example, GABA decreased *in vitro* cholangiocarcinoma growth by both cyclic AMP-dependent regulation of the protein kinase A and *D-myo*-inositol-1,4,5-triphosphate/Ca^{2+}-dependent pathways (Fava *et al*., 2005). The decrease in the growth was significantly correlated with the increase in GABA concentrations of 10 and 100 μmol/L. GABA (100 μmol/L) also inhibited the migration of cholangiocarcinoma cell lines such as Mz-ChA-1 whereas increasing apoptosis compared with controls (Fava *et al*., 2005). The GABA concentrations in the *L. brevis* OPK-3 sample treated cells were calculated to be the ranges of 9.5-38.5 μmol/L. However, authentic GABA showed no inhibitory effects at the concentration rages on the proliferation of the cancer cells (data not shown). Therefore, these data suggested that the *L. brevis* OPK-3 sample contain other factors contributing the anti-proliferative and apoptosis inducing activity.

Short-chain fatty acids such as butyrate produced by the fermentation process with LAB could also be key factors for the anti-cancer activities. In fact, butyrate induced cyclooxygenase (COX)-1 expression in the metastatic gastric cancer cells, and thereby COX-1/COX-2 ratio, suggesting a protective role of butyrate in gastric cancer (Mahkonen *et al*., 2008). In addition, it has been shown that the sodium salt of butyric acid, sodium butyrate, inhibits cell growth and induces apoptosis in a number of cancer cells (Barnard and Warwick 1993; Bernhard *et al*., 1999; Chopin *et al*., 2002). It has also been shown that butyrate inhibited the growth of MCF-7 human breast cancer cells in a P53-indpendent manner (Chopin *et al*., 2002).

In this present study, it is not clear how the treatment of the LAB concentrates could induce p53 gene expression. Many studies showed that the expression of p53 gene can be altered by exogenously added compounds or enzymes in many cancer cells (Atten *et al*., 2005; Beniston *et al*., 2001; Kim *et al*., 2009). For example, recombinant arginine deiminase originating from *Lactococcus lactis* ssp. *Lactis* (LADI) increased the expression of p53 protein in SNU-1 stomach adenocarcinoma cells (Kim *et al*., 2009). Kim *et al*. (2009) also showed that arginine deiminase from *Lactococcus lactis* ssp. *Lactis* induces G_1-phase cell-cycle arrest and apoptosis in SNU-1 stomach adenocarcinoma cells. Furthermore, the inhibition of SNU-1 cell proliferation by the LADI was dose-dependently recovered by adding extra arginine to the cell culture medium (Kim *et al*., 2009). It has been shown that the arginine deiminase (ADI; *EC* 3.5.3.6) killed melanomas and hepatocellular carcinomas *in vitro* and *in vivo* (Ensor *et al*., 2002). Rijn *et al*. (2003) showed that the treatment of arginase inhibitor L-norvaline completely prevented the development of cytotoxicity in the X-ray irradiated H35 hepatoma cells. Thus, candidate products for the inhibitory effects could be ornithine cycle compounds and enzymes such as ornithine, citrulline, ammonia, ADI and arginase. In this regard it is of interest to note that *L. brevis* OPK-3 has potential to produce ornithine (Oh *et al*., 2010) and citrulline (unpublished data) by depleting arginine, indicating that the strain has the ADI pathways (Kuensch *et al*., 1974; Liu *et al*., 2003). Recently, we showed that the kimchi LAB used as a starter for kimchi preparation can grow and produce GABA and ornithine by depleting glutamate and arginine during the fermentation of kimchi (Oh *et al*., 2010; Seok *et al*., 2008). Thus, the consumption of kimchi with the kimchi LAB could be beneficial for the anticancer effects. Also, studies to elucidate the underlying

active compounds and mechanism by which the kimchi LAB with GABA and ornithine producing capacities mediate cancer cell growth inhibition, may reveal great insight into the approach to using the LAB and culture concentrates as pharmaceutical materials.

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