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In Vitro Effects of Lactic Acid Bacteria on Cancer Cell Viability and Antioxidant Activity

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ABSTRACT

We used two local *Lactobacillus* strains (*L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102) and other 10 lactic acid bacteria (LAB) strains from the Biosource Collection and Research Center (BCRC) to evaluate the health promoting capability including inhibitory effects on cancer cell lines and antioxidant activities *in vitro*. The heat-killed cells (HKC) of *Lactobacillus acidophilus* BCRC14079 have the highest total antioxidative activity (82.3%). 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging effects were in the range of 14.8-32.9%, except for the HKC of *L. salivarius* ssp. *salivarius* BCRC 14759 and *Bifidobacterium breve* BCRC 11846. Twelve strains showed varying degrees of reducing activity. The reducing activity of cytoplasmic fraction (CF) of *L. acidophilus* BCRC 14079 was found to be significantly more pronounced (six-fold) than that of the HKC of *B. adolescentis* BCRC 14606, which was the lowest of all. In the cell viability, the inhibitory effects on cancer cells were very different which depended on various LAB strains. Flow cytometry study on human breast adenocarcinoma cells (MDA-MB-231) treated with local lactobacilli strains (*L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102) isolated in Taiwan showed the highest percentage of arrested at G0/G1 phase in the cell cycle. These results show that local lactobacilli strains have strong antioxidative and anticancer activities.

Key words: lactic acid bacteria, anticancer, antioxidative activity

INTRODUCTION

Lactic acid bacteria (LAB) are widely used in food industry and are listed as generally recognized as safe (GRAS). LAB are commonly known to have health-promoting attributes, including anti-mutagenic activity[1], anti-carcinogenic activity[2,3], hypocholesterolemic properties[4], antagonistic actions which can restrain the intestinal and food-borne pathogens[5] and have immunomodulation effects[6-8]. Many reports also suggested that LAB and their fermented products have anti-tumor effects[9,10]. The fermented products of some LAB, such as *Bifidobacterium infantis* and *Lactobacillus acidophilus,* are known to have antiproliferative effect against the growth of breast cancer cells[11]. Kefir (YK-1) has the ability to activate the immunosuppressive response of spleen cells of a mouse when treated with immunosuppressive substances against Ehrlich carcinoma[12]. In addition, certain cancer preventing LAB strains, such as *L. casei*[13], *L. rhamnosus*[14], *L. acidophilus*[15,16] and *B. longum*[17-19], all have the abilities of inhibiting tumor growth in rodents. Different fractions of LAB, such as the whole cells, heat-killed cells (HKC), cell wall, peptidoglycan and cytoplasmic fraction (CF), also have the antiproliferative effects against human cancer cell lines[20]. The CF of *Lactococcus lactis* ssp. *lactis* and *L. brevis* have strong arginine deiminase (ADI) activity, which can induce apoptosis of the stomach adenocarcinoma cells and human acute leukemia cells, respectively[21,22]. The HKC of *L. plantarum* L-137 can induce the interleukin-12 (IL-12) to inhibit tumor growth in mice *in vivo* as well as *in vitro*[23].

High level of reactive oxygen species (ROS) can cause damage to DNA, protein, lipid and carbohydrate. These species include the superoxide anion radical, hydrogen peroxide, hydroxyl radical and singlet oxygen. The accumulation of ROS in animal body may cause age-related disorders, such as cancer, atherosclerosis[24], hypertension and amyloidosis.

Our laboratory has screened two LAB strains, *L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102. These strains were isolated from human feces[25]

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and homemade Korean-style pickles cabbage[26]. They are resistant to gastric juice and bile salt *in vitro*[27]. They also have "probiotic" characteristics that are effective in reducing cholesterol in the blood and in the liver[27]. After feeding with *L. paracasei* ssp. *paracasei* NTU 101, the up-regulation of the antigen-presenting ability of dendritic cells, and expression of natural killer group-2 D (NKG2D) molecules capable of triggering natural killer cell-mediated cytotoxicity were observed[28]. Lymphocyte proliferation and antibody production were also significantly increased in mice after treatment[28]. Soy-skim milk fermented with *L. paracasei* ssp. *paracasei* NTU 101, and supplemented with or without *Momordica charantia* was effective in preventing and slowing hyperlipidemia-induced oxidative stress and atherosclerosis[29]. *L. plantarum* NTU 102 also induces higher superoxide dismutase (SOD) and phenol oxidase (PO) activities as immune response in *Litopenaeus vannamei*[30]. In 2009, soy-skim milk fermented with *L. paracasei* ssp. *paracasei* NTU 101 or *L. plantarum* NTU 102 is useful for the prevention of acute gastric ulcers induced by pylorus ligation and acidified ethanol treatment through PGE_2 and significantly enhanced the level of SOD activity[31]. In this study, we evaluated the local lactobacilli strains (*L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102) and other 10 LAB strains for their potential health promoting capability including inhibitory effects on cancer cell lines and antioxidant activities *in vitro*.

MATERIALS AND METHODS

I. *Bacterial Strains*

All LAB strains used in this study are listed in Table 1. *L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102 were screened in our laboratory. Other LAB strains were obtained from the Biosource Collection and Research Center (BCRC), the Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan, R.O.C.

II. *Preparation of HKC*

Preparation of HKC was modified as described previously[32]. One percent of LAB was inoculated into 400 mL of MRS broth (Difco, Detroit, MI, USA) and cultured at 37°C for 20 hr under aerobic conditions. Cells were harvested by centrifugation for 5 min at 7,000 ×g (High Mac CR21, Hitachi Co., Tokyo, Japan) and the pellets were washed twice, resuspended in 20 mL of phosphate-buffered saline (PBS, pH 7.2) buffer, heated at 100°C for 30 min and lyophilized by Bench Tops3R (Virtis CO., Gardiner, NY, USA). The lyophilized cells were resuspended again to 10 mg dry cell/mL, autoclaved at 121°C for 15 min and stored in 4°C prior to use.

III. *Preparation of CF*

Preparation of CF was modified as described previously[20]. One percent of LAB was inoculated into 400 mL of MRS broth (Difco, Detroit, MI, USA) and cultured at 37°C for 20 hr under aerobic conditions. LAB cells were harvested by centrifugation for 5 min at 7,000 ×g (High Mac CR21, Hitachi Co.) and the pellet was washed twice and dissolved in 15 mL of PBS buffer. Cells were then passed through a French press Emulsi-Flex-C3 (Avestin, Ottawa, Canada) twice at 15,000 psi, centrifuged for 15 min at 36,000 ×g (L8-M ultracentrifuge, Beckman, Palo Alto, CA, USA). The supernatant were lyophilized using Tops3R. The lyophilized cells were resuspended 10 mg dry cell/mL and sterilized by filtration through a 0.22 μm filter and then stored in -80°C before being used.

IV. *Cell Culture*

Human cancer cell lines were tested in our study.

Table 1. Lactic acid bacteria used in this study

Strain	Characteristics	Source or reference
Lactobacillus paracasei ssp. *paracasei* NTU 101	Infant feces	27
Lactobacillus plantarum NTU 102	Pickled vegetables	28
Lactobacillus acidophilus BCRC 14079	Commercial yoghurt	FIRDI*
Lactobacillus plantarum BCRC 11697	-----	FIRDI
Bifidobacterium breve BCRC 11846	Infant intestine	FIRDI
Bifidobacterium infantis BCRC 14602	Infant intestine	FIRDI
Lactobacillus delbrueckii ssp. *bulgaricus* BCRC 10696	Bulgarian yogurt	FIRDI
Lactobacillus rhamnosus BCRC 16000	Human feces	FIRDI
Bifidobacterium adolescentis BCRC 14606	Adult intestine	FIRDI
Lactobacillus salivarius ssp. *salivarius* BCRC 14759	Saliva	FIRDI
Bifidobacterium longum BCRC 14634	Infant intestine	FIRDI
Lactobacillus plantarum BCRC 10069	Pickled cabbage	FIRDI

*FIRDI: Food Industry Research and Development Institute.

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The human breast adenocarcinoma cells (MDA-MB-231) were obtained from BCRC. The epithelial cell line Intestine 407 (Int 407) and colon adenocarcinoma cells (HT-29 and Caco-2) were obtained from American Type Culture Collections (ATCC, Rockville, MD, USA). MDA-MB-231 was cultured in L-15 medium (Gibco Life Technologies, Carlsbad, CA, USA). HT-29 was cultured in RPMI-1640 medium (Gibco); Intestine 407 and Caco-2 were cultured in DMEM medium (Gibco). All cell culture media were supplemented with 10-20% (v/v) fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin (Hyclone, Logan, UT, USA), 100 μg/mL streptomycin (Hyclone) and 250 ng/mL amphotericin (Hyclone) and cultured in an incubator with 5% CO_2 at 37°C except for MDA-MB-231 (without CO_2).

V. *Total Antioxidant Assay*

The antioxidant capacity of the samples was measured by the method described[33] with some modifications[34]. Briefly, 0.25 mL of peroxidase (44 units/mL, Sigma, St. Louis, MO, USA), H_2O_2 (500 μM, Merck, Germany), 2,2-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid) [1000 μM, Sigma] and distilled water (1 mL) were mixed and kept in the dark for 1 hr. Two hundred and fifty microliters of LAB samples (10 mg/mL) were subsequently added and the absorbance at 734 nm was determined after 1 min. The antioxidant activity capacity was calculated as follows:

Total antioxidant activity capacity (%) = [1- (OD_{734}, sample/OD_{734}, blank)] × 100

VI. *1,1-diphenyl-2-picryl-hydrazyl (DPPH) Radical Scavenging Assay*

The capacity of LAB strains to clear DPPH (Sigma) has been described[35]. One hundred microliters of LAB samples (10 mg/mL) were added to 500 μL of freshly prepared 0.1 mM DPPH solution, mixed and reacted in the dark for 60 min. The absorbance at 517 nm was measured using a spectrophotometer (U-2001, Hitachi Co.). The blank was prepared by replacing the extract with methanol. The capability of the test material to scavenge DPPH radicals was calculated using the following equation:

DPPH radical scavenging ability (%) = [1- ($OD_{517, sample}$/$OD_{517, blank}$)] × 100

VII. *Reducing Power Assay*

The reducing powers of the HKC and CF of LAB were determined according to the method described[36]. One hundred microliters of LAB samples (10 mg/mL) were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide (w/v). The mixture was incubated at 50°C for 20 min, during which time ferricyanide was reduced to ferrocyanide. Half milliliter of 10% trichloroacetic acid (w/v) was added to the mixture and then centrifuged at 3,000 ×g for 10 min. The upper layer of the solution was mixed with deionized water and 0.1% $FeCl_3$ (w/v) at a ratio of 1:1:1 (v/v/v) and the absorbance at 700 nm was measured to determine the amount of ferric ferrocyanide (Prussian blue) formed. Increased absorbance of the reaction mixture indicated increased reducing power of the sample.

VIII. *MTT Assay*

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cell viability of LAB on cancer cells[37]. Cells were plated at a density of 2×10^5 per milliliter in a 24-well plate, then various concentrations (100-500 μg/mL) of HKC and CF of LAB strains were added and incubated at 37°C for 48 hr. The cells were washed once with PBS buffer and the MTT solution (0.5 mg/mL) was added to the cells to induce reaction for 4 hr. The purple MTT-formazan crystals were dissolved by adding dimethyl sulfoxide (DMSO). Absorbance at 550 nm was measured by ELISA reader (Thermo Labsystems, Vantaa, Finland) for cell viability calculation.

Cell viability = [(control OD_{550} - sample OD_{550})/ control OD_{550}] × 100.

IX. *Cell Cycle Analyses*

To evaluate the effect of the HKC and CF from LAB strains on the cell cycle, colon adenocarcinoma cells and breast adenocarcinoma cells were treated for 48 hr, washed twice with PBS and fixed with 70% ethanol. After incubation overnight at -20°C, the cells were washed with PBS, centrifugated (300 ×g, 5 min) and then suspended in staining buffer [10 μg/mL propidium iodide (PI) and 200 μg/mL RNase A in PBS] and reacted in the dark for 30 min. Cells (cell debris, cell doublets, and cell clumps) were removed through a 55 μm filter and analyzed by BD FACSCanto™ II flow cytometer (BD Bioscience, San Jose, CA, USA).

X. *Statistics Analysis*

All data were subjected to one-way analysis of variance in a statistical analysis system. Duncan's multiple range test was performed to compare significant differences ($p < 0.05$) in variables between groups. Each set of experiments was performed in triplicates.

RESULTS AND DISCUSSION

I. *Antioxidant Activity of LAB*

Antioxidative activities including total antioxidant activity, DPPH scavenging effects and reducing power

were determined. Total antioxidative activity of LAB depended on different fractions and strains used. CF of *L. rhamnosus* BCRC16000 showed the highest total antioxidative activity (84.78%) and this activity was undetectable in HKC of *L. plantarum* BCRC 11697 and *B. adolescentis* BCRC 14606 (Table 2). DPPH scavenging effects of HKC were in the range of 14.8-32.9%, except for *L. salivarius* ssp. *salivarius* BCRC 14759 and *B. breve* BCRC 11846. DPPH scavenging effects was also found in all CF of LAB and ranged between 8.7-29.0%. Twelve strains showed varying degrees of reducing activity. CF of *L. acidophilus* BCRC 14079 (0.45) showed the highest reducing activity. It was 6 times more than that of HKC of *B. infantis* BCRC 14602 (0.07), which was the lowest.

II. *Inhibitory Effects of LAB on Intestine 407 Cell Viability*

In our study, we used 12 different LAB strains as the source of the HKC and the CF. We also used Intestine 407 cell line as the control to evaluate the inhibitory effects of the HKC and the CF of LAB. With 500 μg/mL of HKC or CF, no inhibitory effects were seen on Intestine 407. In addition, no effects on the cell viability were seen after the Intestine 407 cells were incubated with the LAB samples for 48 hr as compared to the control group treated with PBS (data not shown).

III. *Inhibitory Effects of LAB on Breast Adenocarcinoma Cell Viability*

The HKC of LAB can inhibit the cell growth of breast adenocarcinoma cells. HKC of *B. breve* BCRC 11846 had the strongest inhibitory effect on the MDA-MB-231 showing 46.3% viability of cancer cells (Table 3). HKC of *B. infantis* BCRC 14602, *L. rhamnosus* BCRC 16000, and *B. adolescentis* BCRC 14606 had no effect on the MDA-MB-231 even at a dose of 500 μg/mL. When 500 μg HKC/mL was used, the cell viabilities were between 46.3-91.5%. *L. plantarum* NTU 102, *L. plantarum* BCRC 11697, *L. plantarum* BCRC 10069, *B. breve* BCRC 11846 and *L. delbrueckii* subsp. *bulgaricus* BCRC 10696 showed inhibitory effects with 100 μg HKC/mL on the MDA-MB-231 cell. The cell viabilities were 71.5%, 74.0%, 74.4%, 86.1% and 76.8%, respectively.

The CF of LAB, *L. plantarum* BCRC 11697, *L. delbrueckii* ssp. *bulgaricus* BCRC 10696, *L. salivarius* ssp. *salivarius* BCRC 14759, and *L. plantarum* BCRC 10069 have no inhibitory effects on the MDA-MB-231. CF from other LAB strains have significantly inhibitory effect on MAD-MB-231 with 64.5-87.6% viability, with *B. adolescentis* BCRC 14606 showing the strongest inhibitory effect (64.5%) at 500 μg CF/mL. With the same amount of *L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102, the viability of cancer cells was 82.0% and 68.6%, respectively. These results suggest that compared to CF, HKC might have some heat-stable substances that were more effective than CF in inhibiting the growth of breast adenocarcinoma cell *in vitro*. However, three-fourth of HKCs and half of CFs could inhibit the growth of breast adenocarcinoma cell in this study.

IV. *Inhibitory Effects of LAB on Colon Adenocarcinoma Cell Viability*

In this study, we used HT-29 and Caco-2 as the cell model of colon adenocarcinoma to evaluate the inhibitory effects of HKC and CF of LAB *in vitro*. CF of *L. rhamnosus* BCRC 16000 has no inhibitory effects on the cell viability of HT-29. The HKC of other LAB strains reduced the cancer cell viabilities to a range of 29.7-79.6%. *L. plantarum* NTU 102, *L. delbrueckii* ssp. *bulgaricus* BCRC 10696, *L. salivarius* ssp. *salivarius* BCRC 14759, *B. longum* BCRC 14634 and *L. plantarum* BCRC 10069 could reduce the cell viability to less than 50%, especially the HKC (500 μg/mL) of *L. plantarum* NTU 102 (29.7%).

The CF of LAB strains were also evaluated in this study. At 500 μg/mL, only *L. plantarum* NTU 102, *L. acidophilus* BCRC 14079 and *B. longum* BCRC 14634 had significant inhibitory effects on the cell viability of HT-29.

The HKC of *B. infantis* BCRC 14602 had the strongest inhibitory effect on Caco-2 cells (24.8%) (Table 3), *B. adolescentis* BCRC 14606 and *L. plantarum* BCRC 10069 had no inhibitory effects on Caco-2. Other HKC of LAB strains could decrease the cell viability to a range of 24.8-87.0%. *L. plantarum* NTU 102, *L. acidophilus* BCRC 14079, *L. salivarius* ssp. *salivarius* BCRC 14759, *B. longum* BCRC 14634 and *B. infantis* BCRC 14602 showed the inhibitory effect of more than 50%.

The CF of *B. longum* BCRC 14634 had the strongest inhibitory effect on the Caco-2 cells (68.6%). *L. rhamnosus* BCRC 16000 and *B. breve* BCRC 11846 had no growth inhibitory effects on Caco-2. Ten out of twelve LAB strains can inhibit the cancer cell viability (68.6-86.6%).

V. *Flow Cytometric Analysis of the Cell Cycle Phase of Cancer Cells*

To explore the antiproliferative properties of the HKC and CF, we performed cell cycle analysis on colon (HT29 and Caco-2) and breast (MDA-MB-231) adenocarcinoma cells treated with HKC and CF of LAB strains. Effect of HKC and CF of LAB on the cancer cell lines cell cycle phase distribution is shown in Table 4. Compared with the untreated control cells, the percentages of MDA-MB-231 in the G0/G1 phase increased by 20.7% and 22.5% after treatment with CF of *L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102, respectively; the percentages of Caco-2 and HT-29 in the G0/G1 phase increased by 8.6% after treatment with HKC of *L. plantarum* NTU 102. These results show that

Table 2. *In vitro* antioxidant activity of LAB

	Reducing activity		DPPH cleaving (%)		Total antioxidant activity (%)	
	HKC	CF	HKC	CF	HKC	CF
NTU 101	0.24 ± 0.03^{bc}	0.24 ± 0.03^{b}	27.67 ± 4.98^{de}	28.02 ± 1.86^{c}	68.80 ± 1.72^{e}	48.64 ± 3.06 ^{a}
NTU 102	0.18 ± 0.01^{b}	0.17 ± 0.02^{a}	27.87 ± 2.40^{de}	25.96 ± 3.69^{c}	78.78 ± 1.84^{f}	79.98 ± 1.89 ^{fg}
BCRC 14602	0.07 ± 0.02^{a}	0.28 ± 0.02^{b}	28.14 ± 1.72^{de}	27.20 ± 4.83^{c}	2.76 ± 2.04^{a}	83.18 ± 1.16 ^{gh}
BCRC 16000	0.22 ± 0.02^{bc}	0.23 ± 0.02^{b}	20.64 ± 6.28^{bc}	22.63 ± 5.54^{bc}	40.38 ± 3.07^{c}	84.78 ± 1.54 ^{h}
BCRC 14079	0.20 ± 0.04^{b}	0.45 ± 0.03^{c}	24.83 ± 3.05^{cd}	25.47 ± 4.49^{c}	82.30 ± 5.01^{f}	62.85 ± 2.82 ^{c}
BCRC 14759	0.09 ± 0.03^{a}	0.24 ± 0.03^{b}	0.00^{a}	16.48 ± 6.51^{b}	41.06 ± 3.36^{c}	75.11 ± 1.62 ^{de}
BCRC 14634	0.20 ± 0.02^{b}	0.14 ± 0.02^{a}	32.92 ± 2.74^{e}	8.66 ± 4.17^{a}	18.36 ± 2.87^{b}	73.23 ± 2.26 ^{d}
BCRC10696	0.23 ± 0.03^{bc}	0.25 ± 0.02^{b}	28.27 ± 2.62^{de}	21.14 ± 6.16^{bc}	59.20 ± 3.01^{d}	73.65 ± 1.76 ^{de}
BCRC 11697	0.26 ± 0.02^{cd}	0.18 ± 0.01^{a}	28.92 ± 3.35^{de}	16.22 ± 1.98^{b}	0.00^{a}	77.34 ± 2.66 ^{ef}
BCRC 11846	0.31 ± 0.02^{d}	0.15 ± 0.02^{a}	0.00^{a}	28.95 ± 4.63^{c}	14.33 ± 3.52^{b}	74.91 ± 1.51 ^{de}
BCRC 14606	0.08 ± 0.01^{a}	0.27 ± 0.02^{b}	20.88 ± 2.93^{bc}	21.62 ± 3.65^{bc}	0.00^{a}	75.01 ± 1.76 ^{de}
BCRC 10069	0.18 ± 0.01^{b}	0.28 ± 0.03^{b}	14.81 ± 7.79^{bc}	20.95 ± 5.14^{bc}	43.53 ± 2.96^{c}	54.59 ± 3.03 ^{b}
BHA	1.83 ± 0.07^{e}	1.83 ± 0.07^{d}	100.00 ± 2.12^{f}	100.00 ± 2.12^{d}	100.00 ± 3.56^{g}	100.00 ± 3.56 ^{i}

1. Each value is an average of 3 replications.
2. The concentration of BHA was 1 mg/mL.
3. Data are presented as means ± SD. Mean values within each column with different superscripts are significantly different ($p < 0.05$).
4. See Table 1 for LAB strains information.

Table 3. Inhibitory effects of heat-killed cells (HKC) and cytoplasmic fraction (CF) of LAB strains on the viability of various cancer cells

Cell line	Conc. (μg/mL)	Viability (%)											
		Heat-killed cell (HKC)											
		NTU 101	NTU 102	BCRC strain									
				14079	14606	14759	14634	10069	11697	11846	14602	10696	16000
HT29	100	100.7 ± 20.9	51.1 ± 5.9*	94.4 ± 14	90.8 ± 3.2	82.1 ± 5.5	66.8 ± 4.4*	57.0 ± 3.5*	117.1 ± 14	78.8 ± 4.9*	70.6 ± 3.1*	40.1 ± 2.6*	104.2 ± 7.4
	500	52.5 ± 9.0*	29.7 ± 1.1*	80.7 ± 8.0	70.6 ± 12*	49.7 ± 1.4*	34.8 ± 2.7*	48.8 ± 3.3*	79.6 ± 24*	66.7 ± 6.1*	56.1 ± 12*	35.6 ± 3.6*	108.3 ± 11
Caco-2	100	80.1 ± 7.3*	72.0 ± 4.0*	76.4 ± 8.2*	106.7 ± 5.1	118.6 ± 8.4	97.2 ± 4.2	120.9 ± 13	86.2 ± 2.3*	82.4 ± 7.5*	54.1 ± 9.0*	118.6 ± 22	101.3 ± 14
	500	64.3 ± 4.6*	44.5 ± 2.3*	34.3 ± 5.6*	100.0 ± 7.8	42.7 ± 14*	42.7 ± 14*	109.2 ± 12	75.7 ± 3.8*	43.7 ± 15.3*	24.8 ± 12*	87.0 ± 7.3	74.3 ± 8.2*
MDA-MB-231	100	106.4 ± 4.2	71.5 ± 6.3*	108.6 ± 5.4	95.0 ± 12	98.0 ± 7.4	93.4 ± 8.5	74.4 ± 12*	74.0 ± 4.0*	86.1 ± 9.1*	97.0 ± 8.5	76.8 ± 17*	116.3 ± 15
	500	79.6 ± 8.5*	66.8 ± 7.4*	82.3 ± 4.7*	85.8 ± 13	83.6 ± 15*	56.5 ± 8.3*	64.0 ± 4.0*	70.9 ± 5.6*	46.3 ± 17.6*	91.5 ± 9.3	63.8 ± 10*	85.3 ± 15
		Cytoplasmic fraction (CF)											
HT29	100	114.8 ± 16	109.0 ± 10	91.7 ± 4.7	109.5 ± 3.3	99.8 ± 6.4	91.7 ± 2.1*	94.7 ± 4.5	105.1 ± 5.8	102.1 ± 0.7	108.2 ± 2.3	103.6 ± 3.9	98.0 ± 7.5
	500	98.3 ± 11	83.2 ± 10*	85.3 ± 2.9*	107.8 ± 3.9	100.5 ± 2.4	89.1 ± 0.2*	95.4 ± 4.2	107.1 ± 2.1	105.6 ± 4.7	100.7 ± 16	96.0 ± 4.6	95.6 ± 6.2
Caco-2	100	81.6 ± 1.6*	95.1 ± 6.5	84.1 ± 2.3*	88.8 ± 1.8*	85.9 ± 3.0*	70.0 ± 6.7*	87.5 ± 4.7	86.7 ± 3.1*	103.1 ± 3.2	94.1 ± 5.4	89.9 ± 1.5*	98.7 ± 5.9
	500	79.4 ± 6.5*	76.1 ± 7.0*	71.9 ± 5.1*	80.3 ± 2.5*	76.9 ± 6.4*	68.6 ± 4.9*	86.6 ± 2.2*	76.4 ± 7.5*	99.9 ± 0.9	85.5 ± 4.8*	76.3 ± 8.6*	96.4 ± 3.3
MDA-MB-231	100	88.9 ± 3.1	93.0 ± 10	88.1 ± 6.1*	77.7 ± 1.6*	103.6 ± 2.5	99.8 ± 15.6	96.7 ± 4.7	101.3 ± 5.1	103.6 ± 9.1	105.6 ± 3.6	100.4 ± 2.2	102.5 ± 2.8
	500	82.0 ± 17	68.6 ± 11*	76.1 ± 5.7*	64.5 ± 4.9*	93.8 ± 3.6	93.8 ± 3.6	94.6 ± 5.3	95.7 ± 3.9	67.2 ± 7.8*	87.6 ± 6.9*	98.7 ± 5.3	69.2 ± 4.8*

1. Cell viability (%) = [(control OD_{550} - sample OD_{550}) / control OD_{550}] × 100.
2. The control was treated with PBS buffer and as 100.
3. Data are presented as means ± SD (n =3).
4. Value with "*" was significantly different from control ($p < 0.05$).
5. See Table 1 for LAB strains information.

Table 4. Flow cytometric analysis of cell cycle phase of breast and colon adenocarcinoma cells treated with various LAB strains

	HT29						MDA-MB-231						Caco-2					
	HKC			CF			HKC			CF			HKC			CF		
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Control	74.8±1.8	12.9±3.6	9.5±1.7	74.8±1.8	12.9±3.6	9.5±1.7	46.6±2.4	14.9±1.9	36.4±3.4	46.6±2.4	14.9±1.9	36.4±3.4	53.3±1.8	20.1±2.1	26.1±2.4	53.3±1.8	20.1±2.1	26.1±2.4
10696	80.7±1.3	8.8±1.5	10.1±0.4	74.5±3.0	10.6±4.5	12.6±2.5	52.5±0.8*	19.9±4.0*	20.7±3.0*	50.7±2.4	17.0±2.3	28.9±2.2*	48.4±3.4*	22.9±2.6	18.5±3.4	44.7±4.1*	27.5±3.0	18.4±1.0*
11697	70.4±5.0	12.1±2.0	16.2±4.2*	77.3±2.8	13.5±2.4	6.9±1.4	56.7±2.2*	11.5±2.1	24.1±3.5*	60.4±1.9*	13.3±1.1	14.3±0.5*	51.8±2.5	22.5±1.9	19.1±4.3	45.3±2.9*	24.3±3.4	21.2±3.2*
11846	76.6±4.5	9.1±3.2	12.9±2.6	81.0±3.2*	9.1±2.1	8.0±3.3	49.3±3.1	16.6±1.8	30.8±2.0*	56.7±2.7*	16.4±1.3	24.7±4.2*	51.8±1.1	23.8±2.6	17.6±3.9	49.8±4.8	21.1±1.9	21.3±2.1*

Table 4. Continued

	HT29						MDA-MB-231						Caco-2					
	HKC			CF			HKC			CF			HKC			CF		
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
14602	75.8±1.5	10.9±3.7	13.1±1.5	83.4±3.5*	7.8±2.0*	7.7±2.8	53.9±2.5*	15.2±1.5	28.6±1.5*	54.9±1.8*	19.2±2.5	25.2±3.4*	49.4±2.9	21.4±3.6	12.6±4.0	47.7±0.7	19.8±0.8	32.4±2.5*
14634	78.7±4.6	7.0±1.0*	10.9±2.2	72.8±3.6	12.1±1.4	13.4±2.0	58.6±2.8*	11.6±1.1	23.1±2.8*	52.7±3.8*	12.7±2.9	21.4±1.7*	48.1±3.2*	25.0±3.7	19.7±4.5	48.5±1.9	23.0±4.3	20.8±0.7*
14759	82.9±4.1	7.1±1.9*	8.7±1.4	73.2±4.8	12.4±1.0	13.4±3.5	47.6±2.4	15.7±1.7	32.9±3.3	52.3±3.9*	16.3±2.9	30.4±3.1*	46.5±2.4*	26.0±2.3	18.9±2.2	46.8±5.2*	24.0±4.2	19.5±3.0*
16000	77.4±7.5	6.0±1.4*	14.1±3.1*	74.8±3.1	12.9±2.0	9.5±2.1	62.4±3.1*	10.4±2.2	26.1±3.9*	45.3±2.6	20.4±3.0*	31.7±3.4	53.2±1.2	20.3±3.1	19.6±2.6	51.1±2.0	21.3±5.8	28.4±4.4*
NTU101	82.6±6.4	9.5±1.1	7.5±0.6	83.0±2.7*	7.7±1.6*	8.5±1.1	47.4±4.3	14.9±1.4	34.7±2.5	67.3±2.4*	16.0±3.6	15.7±1.6*	47.2±1.6*	23.5±3.6	19.5±3.0	49.3±1.3	20.6±3.6	30.2±3.0*
NTU102	83.4±3.6	8.7±1.0	7.7±2.8	78.0±1.5	10.7±2.0	8.7±0.8	52.9±3.4*	29.1±3.0*	16.2±1.7*	69.1±3.4*	13.0±1.4	16.4±2.9*	61.9±2.6*	26.5±5.0*	11.9±3.1	49.4±2.8	19.5±1.9	31.5±2.2*

1. The control was treated with PBS buffer.
2. Data are presented as means ± SD (n =3).
3. Value with "*" was significantly different from control ($p < 0.05$).
4. Cells were treated with 500 μg/mL HKC and CF of LAB for 48 hr. After the cells were stained with propidium iodide (PI), the DNA content was measured by flow cytometry. The proportion of nuclei at each phase of the cell cycle was obtained using DNA analysis software of FACSCanto II flow cytometer.
5. See Table 1 for LAB strains information.

local lactobacilli strains could efficiently inhibit the cell proliferation of cancer cells via G0/G1 phase arrest in the cell cycle.

High level of reactive oxygen species[23] will cause host's DNA, protein, lipid and carbohydrate damage. Current research in antioxidant ability of LAB has shown that some LAB strains are not only able to decrease the risk of ROS accumulation through food ingestion but can also degrade the superoxide anion and hydrogen peroxide. In this study, antioxidant activity was found in the HKC and CF of LAB strains *in vitro*. In 1993, Kaizu *et al.* first reported on antioxidant activity and demonstrated that hemolysis of red blood cells was inhibited in rats after treatment with the intracellular cell-free extract of *Lactobacillus* sp. SBT 2028 and can potentially decrease the risk of oxygen stress[38]. Lin and Yen indicated the intact cells and intracellular cell-free extracts of the intestinal bacteria *B. longum* ATCC 15708 and *L. acidophilus* ATCC 4356 have antioxidant activity including chelating metal ions, scavenging reactive oxygen species, or possessing reducing activity[39]. Kullisaar *et al.* isolated two probiotic strains, *L. fermentum* E3 and *L. fermentum* E18, and found that intact cell and cell lysates had some antioxidant properties and could overcome the oxidative stress[40]. Some reports indicated that CF from *L. delbrueckii* ssp. *lactis*, *L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. casei* had strong antioxidant activity[41]. Our result shows that almost all the HKC and CF from these strains have antioxidant activity.

The HKC from LAB strains have some antioxidant activity, which is most probably related to Maillard reaction, a chemical *reaction* between an amino acid and a reducing sugar, usually requiring heat. The Maillard reaction products (MRPs) may have some properties such as reduction of lipid peroxidation[42], clearance of hydroxyl radicals[42,43], clearance of hydrogen peroxide[42] and inhibition of the oxidant activity of LDL protein in human[44]. We speculate that the source of antioxidant activity may be a result of reaction between sugar and amino acid in LAB after heating (100°C, 30 min). However, further investigation is needed.

Recently, numerous research studies have focused on anticancer property as protective adjuncts against a host of diseases. LAB and its fermented milk have shown some anticancer effects. Data from epidemiological and experimental studies have also indicated that the ingestion of certain LAB strains or its fermented dairy products might alleviate the risk of certain type of cancers and inhibit the growth of tumors. Anticancer effects of LAB include immunogically and non-immunogically-mediated cytotoxicity. LAB strains have been shown to affect selected aspects of immune function that include one or several components of the humoral, cellular or nonspecific immunity. HKC of *L. casei* strain Shirota (LcS) stimulated macrophages to secrete IL-12. It shifted the cytokine production pattern from Th2 predominance

to Th1 predominance. Such cytokine had the ability of suppressing the production of IgE[45]. Peptidoglycan from *Lactobacillus* sp. could highly activate the TLR-NF-kB and Jak-STAT signal pathways; it also appeared to inhibit the colon tumor cells *in vitro* and induced a significant antitumor effect *in vivo*[46]. Fichera and Giese reported that peptidoglycan from *L. casei* could inhibit the viability of various murine (Yac-1, P815, Ehrlich ascites tumor and mammary carcinoma) and human (K562 and KB) tumor cell lines through primary cytotoxic activity[47]. Its activity was also strongly stimulated in normal cells and could decrease the cell viability to 25-30%. Kim *et al*. screened the cytotoxicity of HKC, CF and peptidoglycan of 10 LAB on 11 types of cancer cell lines using ^{3}H thymidine uptake assay[48]. They exerted significant antiproliferative activities against several cancer cell lines; the CF had particularly strong inhibitory effects on colon and gastric cancer cell lines.We screened the HKC and CF of 12 LAB strains for antiproliferative effect on the colon and breast adenocarcinoma cells. In particular, the HKC had strong growth inhibitory effects on HT29 and Caco-2 cells, whereas the CF exhibited marked direct antiproliferative activities against MDA-MB-231. Many reports indicated that HKC has less growth inhibitory effects than other fractions of LAB strains, such as the CF of *L. lactis* ssp. *lactis* have strong antiproliferative activities both *in vitro* and *in vivo*[20,49]. However, in our study, there are nine LAB strains (*Lactobacillus* and *Bifdobacterium*) that can effectively inhibit the proliferation of breast adenocarcinoma cell (500 μg HKC/mL). These LAB strains may have some heat-stable substances which can inhibit the growth of tumor cells. However, the precise mechanism by which LAB fractions exert anticancer effects remains unknown.

Most LAB strains currently in use exert their antiproliferative effects via the induction of apoptosis and necrosis. The CF of *L. lactis* ssp. *lactis* has been demonstrated to induce apoptosis in SNU-1 stomach adenocarcinoma cells by nuclear staining assay[22]. The soluble polysaccharide from *L. acidophilus* 606 was observed to induce apoptosis in the HT-29 cell by PI staining assay[32].

Some of LAB such as *L. acidophilus, L. plantarum*, and *B. adolescentis* had been demonstrated which can inhibit the colon and breast adencarcinoma cells *in vitro*[48]. In this study, we did not use these LAB strains to analyze the cell cycle phase. Nine other strains of LAB were used to treat the colon and breast adenocarcinoma cells and analyzed the cell cycle phase by flow cytometry. We found that most HKC and CF of *L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102 inhibited the proliferation of cancer cells via cell cycle arrest at G0/G1 phase. The mechanism of cancer cell growth inhibition by LAB is poorly understood. Some reports stated that it is related to modulation of apoptotic signaling-regulated proteins. Kim *et al*. has already demonstrated CF of *L. lactis* ssp. *lactis* inhibited the proliferation of the SNUC2A human colon cancer cell line by down-regulation of cyclin-dependent kinase 2 and overexpression of cyclinA[20]. Further study can focus on isolating the active components from HKC of LAB and determining the specific apoptosis inducing pathway as cell cycle blockade to confirm the mechanism of cell cycle arrest using animal models.

CONCLUSIONS

In conclusion, *L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102 give a strong evidence of HKC and CF possessing significant antioxidant activities and antiproliferative activities against breast and colon cancer cell lines *in vitro*. These results suggest that local lactobacilli strains can be used as adjuncts in fermentation of food and are potential candidates for cancer prevention. In order to understand the mechanisms of inhibitory effects, we will continue the research and investigation from the molecular perspective.

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