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Antioxidative Effect of Lactic Acid Bacteria: Intact Cells vs. Intracellular Extracts

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ABSTRACT

The present study compared the anti-oxidative ability of intact cells and intracellular extracts of two lactic acid bacterial strains, *Bifidobacterium longum* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Results showed that both intact cells and intracellular extracts of 10^9 cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* had the ability to scavenge α-diphenyl-β-picrylhydrazyl (DPPH) free radical by 70.4-75.1%, to inhibit liposome peroxidation by 25-31%, and to decrease significantly the malondialdehyde (MDA) production in Intestine 407 cells. The effect of intact cells and intracellular extracts of these two bacterial strains on the oxidation of low density lipoprotein (LDL) isolated from cerebrovascular accident (CVA) patients and healthy subjects was also compared. Oxidation of LDL was monitored by measuring the lag time for the formation of conjugated dienes in isolated LDL particles. When LDL was treated respectively with 10^9 intact cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus,* the lag time of oxidation of LDL was prolonged significantly. The extent of inhibition was greater on LDL isolated from healthy subjects than from CVA patients. When LDL from either CVA patients or healthy subjects was treated with intracellular extracts of 10^9 cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus,* respectively, the copper-mediated oxidation was extensively inhibited with a lag time exceeding 180 min. Results from this study show a greater inhibitory effect on LDL oxidation exerted by the intracellular extract than the intact cells, suggesting the presence of effective inhibitory factors in the intracellular extract.

Key words: lactic acid bacteria, antioxidation, LDL oxidation, intact cells, intracellular extracts

INTRODUCTION

In last two decades, it has become clear that dietary strategy is effective in managing many chronic diseases, such as cardiovascular diseases. Lines of evidence suggested that intake of foods rich in natural antioxidants can reduce the risk of atherosclerosis[(1-4)]. Lactic acid bacteria, which have been traditionally used in various fermented foods, are important members of intestinal microflora. They play vital roles in the maintenance of the intestinal microbial ecosystem. The potential health attributes of lactic acid bacteria include the control of gastrointestinal infections, improvement of lactose intolerance, reduction of serum cholesterol, stimulation of immunomodulation, anticarcinogenicity, and longevity[(5-9)]. Recent studies have also shown that various strains of lactic acid bacteria exhibit antioxidative activity both *in vivo* and *in vitro*[(3,8,10-14)].

The objective of this study was to compare the antioxidative ability of two strains of lactic acid bacteria, *Bifidobacterium longum* and *Lactobacillus delbrueckii* ssp. *bulgaricus* as intact cells or the intracellular extracts. The antioxidative ability was measured based on the scavenging of α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals and the inhibition of lipid peroxidation of two model systems, a liposome system and a cell membrane system of Intestine 407 cells. The inhibitory effects of these two strains of lactic acid bacteria on the oxidation of LDL isolated respectively from cerebrovascular accident (CVA) patients and healthy subjects have also been evaluated.

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MATERIALS AND METHODS

I. *Lactic Acid Bacterial Strains*

Isolation and identification of *B. longum* MYL2 and *L. delbrueckii* ssp. *bulgaricus* MYL6 from new born infants were performed in the Microbiology Laboratory in Department of Food Science and Biotechnology of National Chung Hsing University, Taichung, Taiwan. These bacteria were grown at 37°C in MRS (Difco Laboratories, Detroit, MI, USA). Both strains were serially transferred at least three times prior to use.

II. *Preparation of Intact Cells and Intracellular Extracts*

Cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* were harvested by centrifugation at 6,000 *g* for 10 min after 18 h of incubation at 37°C. For the preparation of intact cells, cells were washed three times with phosphate buffer solution (PBS; 0.85% NaCl, 2.86 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH=7.4) and resuspended in PBS. For preparation of intracellular extracts, cell pellets were washed twice with deionized water and resuspended in deionized water followed by ultrasonic disruption in five 1-min intervals in an ice bath (setting = 4; Sonicator XL-2020; Heat System, Farmingdale, NY, USA). Cell debris was removed by centrifugation at 7,800 *g* for 10 min, and the resulting supernatant was used as the intracellular extract. The total cell numbers were adjusted to 10^9 cfu/mL for the preparation of both intact cells and intracellular extracts.

III. *Determination of α,α-Diphenyl-β-Picrylhydrazyl (DPPH) Radical*

The ability to scavenge DPPH radicals by *B. longum* and *L. delbrueckii* ssp. *bulgaricus* was assessed by a modified method of Takahashi *et al.*[15]. Eight tenths of a milliliter of intact cells or intracellular extract and 1 mL of freshly prepared DPPH solution (0.2 mM in methanol) were mixed and allowed to react for 30 min at room temperature. Blank samples contained either PBS or deionized water. The level of scavenged DPPH was then monitored by the decrease in absorbance at 517 nm. The scavenging ability was defined as follows: [1-A_{517}(sample)/A_{517}(blank control)] × 100%.

IV. *Inhibition of Lipid Peroxidation in Liposomes*

Liposome suspension was prepared by following the protocol of Ansari *et al.*[16]. Phosphatidyl choline in ethanol was suspended in PBS (pH=7.4), followed by ultrasonic disruption (setting = 4; Sonicator XL-2020; Heat System, Farmingdale, NY, USA). Sonication was then performed on three 4-second intervals in an ice bath. One milliliter of liposomal suspension, 100 μL PBS buffer, 1mM $FeCl_3$, 200 μL ascorbic acid, and intact cells or intracellular extracts of lactic acid bacteria (or α-tocopherol for the control) were mixed and incubated at 37°C in a water bath. The lipid peroxidation was assessed by the thiobarbituric acid (TBA) assay as described by Decker and Faraji[17]. After 12 hr of incubation, the reaction solution was mixed with 1 mL of trichloroacetic acid (TCA, 2.8%), 1 mL of 1% TBA, and 1 mL of 0.5 mM butylated hydroxytoluene (BHT). The mixture was incubated at 100°C for 30 min. After cooling, methanol was added for extraction. The absorbance of the upper phase was measured at 532 nm. The level of inhibition (%) on lipid peroxidation was calculated as: [1-A_{532}(sample) /A_{532}(control)] × 100%.

V. *Cell Culture*

Intestine 407 (BCRC 60022) cells were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Cells were grown in basal Eagle medium (BEM) supplemented with Earle's BSS and 15% fetal bovine serum (Gibco BRL, Life Technologies, Grand Island, NY, USA) and incubated in a humidified atmosphere of air/CO_2 (95:5, v/v) at 37°C. Confluent cells were washed twice with PBS. Trypsin-EDTA (1 mL, 0.05% trypsin and 0.53 mM EDTA) was added and left at room temperature for 5 min. BEM (2 mL) was then added and cells were harvested by centrifugation at 600 g for 5 min.

VI. *Measurement of Malondialdehyde (MDA)*

MDA was chosen as the indicator for the secondary product of lipid peroxidation. The inhibitory effect of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* on lipid peroxidation in Intestine 407 cells was determined by thiobarbituric acid reactive substances (TBARS) assay[17]. The procedure for the quantitation of MDA was the same as TBA method mentioned above. For liposomal lipid peroxidation, BEM (2 mL) was first inoculated with 1×10^5 Intestine 407 viable cells. The intact cells or intracellular extracts of two bacterial strains were then added to the mixture and incubated at 37°C for 4 hr. Hydrogen peroxide (final conc. 0.8 mM) was added as a pro-oxidant. One milliliter of supernatant, 2.8% TCA, and 0.7% TBA were added and mixed. The mixture was allowed to react at 100°C for 10 min. The lipid peroxidation product was extracted with 1-butanol. The absorbance at 532 nm of the upper phase obtained by centrifugation was then determined.

VII. *Preparation of Low-Density Lipoprotein (LDL) Fraction from Cerebrovascular Accident (CVA) Patients and Healthy Subjects*

CVA patients (19 women and 11 men, age ranging from 54 to 76 years old) were recruited from a special clinic in Chung Shan Medical University Hospital, Taichung, Taiwan. Twenty four healthy subjects (12 women

and 12 men, age ranging from 53 to 73 years old) recruited through advertisement were selected as the control of this study. All procedures were in accordance with the guidelines of the Human Subjects Review Committee of the Chung Shan Medical University. All participants have also provided the informed consent.

Overnight fasted blood sample (15 mL) from each subject was drawn into a test tube containing EDTA. Blood was centrifuged at 1,000 *g* for 15 min at 4°C to separate plasma and red blood cells. An aliquot of plasma was used to prepare LDL fraction, and the remaining plasma was stored at -80°C until analyzed.

LDL fraction from plasma was prepared by continuous density gradient ultracentrifugation at 180,000 *g* for 16 h at 4°C. One mL of LDL fraction was aspirated after twice ultracentrifugation and dialyzed in PBS (pH=7.4) at 4°C for over 16 h to eliminate salt, EDTA and albumin right prior to measurement of LDL oxidation. The protein concentration of LDL was determined by the method of Lowry *et al*.[18], using bovine serum albumin as the standard. In all experiments, the LDL fractions were diluted with PBS to give a final protein concentration of 200 μg/mL.

VIII. *Determination of Ascorbic Acid in Plasma*

The concentration of ascorbic acid in plasma was determined by the method of Kyaw[19]. Two milliliters of color reagent was added slowly to 2.0 mL fresh plasma in a centrifuge tube. The color reagent was prepared by mixing 0.75 M sodium tungstate, 0.75 M disodium hydrogen phosphate and 6.25% sulfuric acid (v/v). After thoroughly mixing, the reaction mixture was allowed to stand at room temperature for 30 min and then centrifuged at 6,000 *g* for 15 min. The blue color supernatant was transferred to a test tube with a pipette without disturbing the precipitate. Absorbance at 700 nm was measured against a reagent blank. For each set of analyses, a standard and a reagent blank were run in parallel throughout the procedure.

IX. *Determination of* α*-Tocopherol in Plasma*

The concentration of α-tocopherol in plasma was analyzed by HPLC method according to the protocol of Catignani and Bieri[20]. An aliquot of 50 μL of internal standards (52.5 mg/L α-tocopheryl acetate in ethanol) and 100 μL of plasma were mixed by vortexing for 1 min. Two-tenths mL of n-hexane was then added and mixed for another 1 min to extract the lipid. The organic and aqueous phases were separated by centrifugation at 800 g for 2 min. The n-hexane layer was withdrawn and evaporated under a nitrogen stream. The residue was re-dissolved in 50 μL of HPLC-grade methanol and an aliquot of 20 μL was injected into an HPLC system for chromatographic analysis of α-tocopherol at ambient temperature. The mobile phase was 98% HPLC grade methanol and the flow rate was 1.2 mL/min. Peak area ratios of the samples were converted to α-tocopherol (μg) by using the standard curves constructed with the internal standards mentioned previously. Total plasma lipid concentration was determined and used to adjust the value of α-tocopherol, which is expressed as μg/mg total lipid.

X. *Thiobarbituric Acid Reactive Substances (TBARS) Assay*

Lipid peroxidation in plasma and red blood cell was measured by TBARS by a modified method, as described by Fraga and co-workers[21]. Briefly, plasma and red blood cell (RBC) samples were homogenized in 50 mM potassium phosphate buffer (pH 7.4). To the plasma or RBC homogenate were added 0.5 mL of 3% sodium dodecyl sulfate, 2 mL of 0.1 N HCl, 0.3 mL of 10% phosphotungstic acid, and 1 mL of 0.7% TBA. The mixture was heated in a boiling water bath for 30 minutes, and TBARS were extracted into 5 mL of 1-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission in a fluorescence spectrophotometer (model F-4500, Hitachi, Tokyo, Japan). A malondialdehyde standard curve was also made using 1,1,3,3-tetramethoxypropane.

XI. *Measurement of LDL Oxidation*

LDL oxidation was measured by a modified method of Abuja and Albertini[22]. Oxidation was initiated by adding 0.5 mM $CuSO_4$ to a spectrophotometer cuvette containing 1.2 mL LDL (200 μg/mL protein) in PBS buffer with or without either intact cells or intracellular extracts of lactic acid bacteria. The kinetics of LDL oxidation was monitored by reading the absorbance (234 nm) every 5 min for 3 hr at 37°C in a Hitachi U-3000 spectrophotometer. The start of the propagation phase was determined by the intersection of tangential lines drawn through the flat lag or induction phase and the steeply sloping propagation phase.

XII. *Statistical Analysis*

The experiment was conducted using a completely random design. Data were analyzed using analysis of variance (ANOVA). Student's t test was applied to test differences between healthy and CVA patients. *Ducan's* test was applied to test differences between treatments. A significant level was detected at 0.05 probability level. All statistical analyses of data were performed using SAS software.

RESULTS

I. *Scavenging of α,α-Diphenyl-β-Picrylhydrazyl (DPPH) Radical*

Results of scavenging of DPPH by intact cells and intracellular extracts of 10^9 cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* are shown in Table 1. Both *B.*

longum and *L. delbrueckii* subsp. *bulgaricus* had excellent DPPH scavenging ability. Intact cells and intracellular extracts of 10^9 cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* showed the ability to scavenge 70.4-75.1% of DPPH free radical. There was no significant difference between the two bacterial strains and between the intact cells and the intracellular extracts.

II. *Inhibition of Liposome Peroxidation*

The effect of 10^9 cells of *B. longum* and *L. delbrueckii* subsp. *bulgaricus* (as intact cells or intracellular extracts) on liposome lipid peroxidation is presented in Table 2. Results showed that both strains were able to protect liposome lipid from oxidation. The inhibition rate ranged from 25 to 31%, which was equivalent to approximately 4 mg (1 mg = 1.49 I.U.) of α-tocopherol. There was no significant difference between two bacterial strains and between the intact cells and the intracellular extracts.

III. *Inhibition of Cell Membrane Lipid Peroxidation*

In this study, the cell membrane of Intestine 407 cells was used as the source of biological lipid and H_2O_2 was added as the pro-oxidant. Results in Table 3 show that the level of MDA, a lipid peroxidation product used as an index of lipid peroxidation, significantly decreased when cell membrane was treated with intact cells of either lactic acid bacterial strain. There was no difference between two strains. The MDA levels were also suppressed by the intracellular extracts of either lactic acid bacterial strain. However, the inhibitory effect was significantly enhanced when Intestine 407 cells were treated with the intracellular extract from the *B. longum* or *L. delbrueckii* ssp. *bulgaricus* ($p < 0.05$).

IV. *Antioxidant Vitamins and TBARS*

As shown in Table 4, although the levels of lipid-soluble antioxidant α-tocopherol were not different between two groups, the levels of the water-soluble anti-

Table 1. Scavenging effect of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* on DPPH free radicals*

Strain	DPPH scavenged (%)
B. longum	
Intact cells	71.3 ± 3.4
Intracellular extract	72.6 ± 3.8
L. delbrueckii ssp. *bulgaricus*	
Intact cells	70.4 ± 5.2
Intracellular extract	75.1 ± 4.9

* Scavenging ability of 1 mL of intact cells or intracellular extracts of *B. longum* and *L. delbrueckii* ssp. *bulgaricus*. Total cell numbers were adjusted to 10^9 CFU/mL for the preparation of intact cells and intracellular extracts. Scavenging ability is expressed as the percentage of DPPH scavenged, which was defined as: $[1\text{-}A_{517}(\text{sample})/A_{517}(\text{blank})] \times 100\%$. Data are means ± SD of triplicate experiments.

Table 2. Inhibition of liposome lipid peroxidation by *B. longum* and *L. delbrueckii* ssp. *Bulgaricus**

Treatment	Inhibition rate (%)
B. longum	
Intact cells	30.8 ± 3.2 c
Intracellular extract	30.2 ± 2.9 c
L. delbrueckii ssp. *bulgaricus*	
Intact cells	26.5 ± 4.9 c
Intracellular extract	25.4 ± 4.5 c
Vitamin E	
4 mg	34.4 ± 3.6 c
8 mg	44.2 ± 4.1 b
16 mg	53.2 ± 2.8 a

* Lipid peroxidation inhibition of 1 mL of intact cells or intracellular cell-free extracts of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* or vitamin E. Total cell numbers were adjusted to 10^9 CFU/mL for the preparation of intact cells and intracellular extracts.
For vitamin E, the recommended dietary allowance is 15 mg (1 mg = 1.49 I.U.). Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.

Table 3. Inhibitory effect of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* on lipid peroxidation of Intestine 407 cells[a]

	Malondialdehyde (μM) produced	% inhibition
Control[b]	4.9 ± 0.5 a	—
Strain		
B. longum		
Intact cells	3.5 ± 0.5 b	28.6
Intracellular extract	2.5 ± 0.5 c	49.0
L. delbrueckii ssp. *bulgaricus*		
Intact cells	3.7 ± 0.2 b	24.5
Intracellular extract	2.9 ± 0.5 c	40.8

[a] Lipid peroxidation inhibition of 1 mL of intact cells or intracellular cell-free extracts of *B. longum* and *L. delbrueckii* ssp. *bulgaricus*. Total cell numbers were adjusted to 10^9 CFU/mL for the preparation of intact cells and intracellular extracts. Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.
[b] Control was 0.8 mM H_2O_2 without treating with lactic acid bacteria.

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oxidant ascorbic acid were significantly lower in patients with CVA. The levels of TBARS in both red blood cells and plasma were significantly higher in CVA patients when compared to those from healthy subjects ($p < 0.05$).

V. *LDL Oxidation*

Table 5 shows the mean oxidation lag time (min) of LDL isolated from CVA patients and healthy subjects were 58.3 ± 19.6 min and 62.3 ± 22.9 min, respectively. No significant difference was found between these two groups. When treated respectively with intact cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus*, the oxidation lag time of LDL isolated from CVA patients was increased by 44% and 56%, respectively, whereas that from healthy subjects was increased by 53% and 65%, respectively. When LDL was treated respectively with intracellular extracts of 10^9 cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus*, the copper-mediated oxidation was significantly suppressed with the mean oxidation lag time exceeding 180 min for both CVA patients and healthy subjects (Figure 1).

DISCUSSIONS

Lactic acid bacteria have long been known to be probiotic for maintenance of intestinal microbial ecosystem and promotion of host health. *B. longum* and *L. delbrueckii* ssp. *bulgaricus* make up a significant part of the natural microflora of the human intestinal tract. They create a healthy balance between beneficial and potentially harmful microorganisms in the gut ecosystem when they are present in sufficient numbers. The use of these cultures in fermented foods or as dietary supplements is desirable for their potential probiotic effects. These cultures may or may not survive in the intestine after they are consumed. The major factors determining survival of these bacteria include characteristics of the strains (e.g., acid and bile tolerance), composition of food ingested, and competition of microflora in the intestine. Strains isolated from human sources are likely to have a better chance to survive and even colonize in the intestine. However, certain numbers of these bacteria are lysed and their intracellular extracts released into the gut. Therefore, it is of interest to compare the antioxidative ability of intact cells and intracellular extracts of *B. longum* and *L. delbrueckii* ssp. *bulgaricus*.

Table 4. Plasma antioxidant vitamins and lipid peroxidation products in healthy subjects and CVA patients[a]

	Healthy subjects (n = 24)	CVA patients (n = 30)
Ascorbic acid (mg/dL)	2.3 ± 1.0 a	1.1 ± 0.6 b
Vitamin E (μg/mg lipid)	2.6 ± 1.1 a	2.4 ± 1.3 a
LDL-vitamin E (μM/mg LDL protein)	6.6 ± 1.7 a	7.2 ± 1.9 a
Red blood cell-TBARS[b] (nM/mL)	9.5 ± 1.4 b	12.2 ± 1.3 a
Plasma-TBARS (nM/mL)	5.7 ± 0.9 b	8.6 ± 0.8 a

[a] Student's t test was applied to test differences between healthy and CVA patients. Data in the same row with different letters are significantly different at $p < 0.05$.
[b] TBARS: thiobarbituric acid reactive substances.

Table 5. Effect of intact cells of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* on the oxidation lag time (min) of LDL from healthy subjects and CVA patients[a]

	Healthy subjects (n = 24)	CVA patients (n = 30)
Placebo	62.3 ± 22.9 a	58.3 ± 19.6 a
Intact cells of *B. longum*	95.6 ± 24.3 bc (↑53%)[b]	83.9 ± 27.3 bc (↑44%)
Intact cells of *L. delbrueckii* ssp. *bulgaricus*	102.8 ± 19.9 c (↑65%)	91.0 ± 28.1 bc (↑56%)

[a] Data with different letters are significantly different at $p < 0.05$.
[b] Percentage increase of the oxidation lag time of LDL

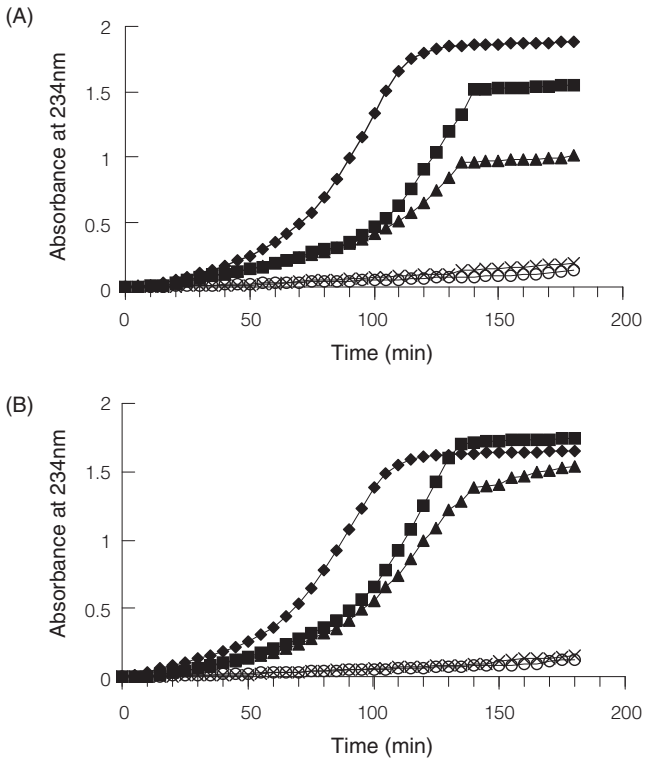

Figure 1. The inhibitory effect of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* on oxidation of LDL isolated from the blood of healthy subjects (A) and CVA patients (B) (◆, control; ■, treated with intact cells of *B. longum*; ▲, treated with intact cells of *L. delbrueckii* ssp. *bulgaricus*; ×, treated with intracellular extract of *B. longum*; ○, treated with intracellular extract of *L. delbrueckii* ssp. *bulgaricus*).

In this study, the free radical scavenging ability of two bacterial strains was first compared. As shown in Table 1, no significant differences in scavenging ability were found between two bacterial strains either as intact cells or intracellular extracts. This finding suggests that levels of the antioxidative factor contributed by either strain were comparable.

In our second study, the antioxidative effect of two bacterial strains was examined using the liposomal lipid system, as peroxidation of liposomal lipids is often utilized as a simple model system to evaluate the lipid peroxidation in biological cell membrane[16,23]. Results in Table 2 indicate that both bacterial strains either as intact cells or as intracellular extracts inhibited significantly the Fe^{+2}/H_2O_2-induced peroxidation in liposomes. It again suggests that level of the antioxidant factor contributed by either strain was comparable.

In the third study, Intestinal 407 cells were used as the intestinal cell membrane model system to investigate the inhibitory effect exerted by these two strains on the H_2O_2-induced oxidative damage. Results in Table 3 show that the lipid peroxidation (measured by the formation of MDA) decreased in the presence of intact cells and intracellular extracts of these two strains, indicating that both intact cells and intracellular cell-free extracts of *B. longum* and *L. delbrueckii* ssp. *bulgaricus* were effective in inhibiting the H_2O_2-induced oxidative damage. However, the intracellular extracts, in comparison with the intact cells, exhibited a greater inhibitory effect. It suggests that the level of antioxidant factor in the intracellular extracts was greater than that released in the medium by the intact cells.

The inhibitory effect of these two strains was also examined, either as intact cells or intracellular extracts on oxidation of LDL isolated from patients with cardiovascular disease and from healthy subjects. Results in the preliminary study have demonstrated a similar lag time of LDL oxidation between LDL isolated from CVA and those from the healthy subjects. However, the lag time of LDL oxidation was significantly prolonged in the presence of these two strains of bacteria, more so in LDL isolated from the healthy subjects than those from the CVA. Since the LDL-α-tocopherol contents were not significantly different between CVA and healthy subjects, the difference in inhibitory capability could not be attributed to different α-tocopherol level in LDL. Results in Figure 1 also show when LDL was treated with the intracellular extracts, nearly all the oxidation of LDL was inhibited. The findings suggest that the availability of the antioxidative factor was greater when cells were disrupted than intact. A similar finding has previously been reported by Saide and Gilliland[12] that intracellular extracts of lactobacilli possessed markedly increased antioxidative activity than intact cells. Since it has been indicated that *Lactococcus* expresses activity of antioxidative enzyme superoxide dismutase (SOD)[24], it is possible that the significant increase in inhibitory activity of the intracellular extracts could be due to the greater accessibility of antioxidative enzymes to the oxidant substractes.

The antioxidative effects were found for the intact cells and intracellular extracts of lactic acid bacteria *in vitro*. According to our previous study[25], the intracellular extracts of lactic acid bacteria have metal ion chelating ability, reactive oxygen species scavenging ability and reduction activity. Although conditions in the gastrointestinal tract are very complicated, the study of Kaizu *et al.*[10] demonstrated that the intracellular extract is also antioxidative *in vivo*. In addition, Kaizu *et al.* have demonstrated that hemolysis of red blood cells was inhibited in rats which were administered with the intracellular extracts of *Lactobacillus* sp. SBT 2028. Rats deficient in α-tocopherol, a well-known natural antioxidant, were used for the experiments. The results provided evidence that the intracellular extract is antioxidative and, therefore, improved the α-tocopherol deficiency status. It is reasonable to expect a significant number of lactic acid bacteria are lysed and release their intracellular antioxidative constituents during transit through the gastrointestinal tract[26,27]. We strongly recommend that consumers consume lactic acid bacteria fermented products containing live probiotic bacteria. However, these

probiotic bacteria do not necessarily have to be acid or bile resistant to stay alive in gastrointestinal tract to be probiotic, i.e. antioxidative in this study. Intact cells of lactic acid bacteria were indeed found possessing antioxidative ability *in vitro*[28]. Nevertheless, using the intact cells as the delivery vehicles passing through the gastrointestinal tract, the intracellular constituents released from the lactic acid bacteria in gastrointestinal tract can be also antioxidative. Consumption of lactic acid bacteria containing foods or supplements may be recommended as healthy. It is well established that a wide variety of oxygen-centered free radicals and other reactive oxygen species are continuously produced in the human body and in food systems[29]. Antioxidants are needed for practical applications. Although various antioxidants have been reported, the safety and long-term effects on health of synthetic antioxidants are to be confirmed. Antioxidants from natural sources are likely to be more desirable. Besides the long history of consumption, which proves the safety of consuming lactic acid bacteria, they have been reported to have health-promoting characteristics that make these microorganisms desirable for use in the production of various health and functional foods.

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