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Hepatoprotective Effect of Lactic Acid Bacteria in the Attenuation of Oxidative Stress from *tert*-Butyl Hydroperoxide

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ABSTRACT

The pathogenesis and progression of liver disease are associated with free radical injury and oxidative stress, which can be partially attenuated by antioxidants and free radical scavengers. Lactic acid bacteria, which have been traditionally used in the production of various fermented foods, are important intestinal microflora and natural antioxidants. The hypothesis that lactic acid bacteria can prevent or decrease *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage in HepG2 cells was investigated. Intracellular extracts and heat-killed cells of *Lactobacillus acidophilus* La12, *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb23, *Bifidobacterium longum* Bl36 and *Streptococcus salivarius* ssp. *thermophilus* St28 were used in this study. Lactate dehydrogenase (LDH), alanine aminotransferase (ALT), reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were determined to explore the influence of lactic acid bacteria intervention on cell damage and antioxidative status. Toxic damage to hepatocytes by *t*-BHP was attenuated by lactic acid bacteria (which exerted protective effects by decreasing the risk of accumulated ROS and by reactivating antioxidant enzymes) in HepG2 cells treated with lactic acid bacteria before *t*-BHP exposure. The results of this study provide new insights into the mechanisms by which lactic acid bacteria with antioxidative properties can help to protect the liver.

Key words: lactic acid bacteria, *tert*-butyl hydroperoxide (*t*-BHP), oxidative damage, reactive oxygen species, lipid peroxidation, antioxidant enzymes

INTRODUCTION

Liver disease development begins with hepatitis followed by cirrhosis and progression to liver failure or hepatoma. The exact pathological mechanisms of chronic liver disease progression are still unclear. However, it has been demonstrated that oxidative damage plays a significant role in the initiation and progression of liver disease, including alcohol-induced liver disease[(1)], non-alcoholic steatohepatitis[(2)], viral hepatitis[(3)] and hepatic fibrosis[(4)]. In hepatocellular carcinoma patients, some markers of oxidative damage, such as 8-oxo-2′-deoxyguanosine (8-oxo-dG) and malondialdehyde concentration, are significantly increased in tumor tissue when compared with non-tumor tissue[(5)].

Under normal physiological conditions, oxygen-centered free radicals and other reactive oxygen species (ROS) are continuously generated in the human body. Excess ROS production can damage biological molecules. Antioxidants have been widely implicated in the protection against liver damage. Hepatoprotective effects of antioxidants from natural food sources include improved liver histology, decreased lipid peroxidation, elevated antioxidant enzyme activities and down-regulated expressions of inflammatory genes such as nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS)[(6,7)].

Given the importance of intestinal flora in liver disease, antibiotics or probiotics (to alter the intestinal flora) may influence the evolution of liver disease. VSL#3, a combination probiotic compound, modulates liver fibrosis but does not protect from inflammation or steatosis in non-alcoholic steatohepatitis (NASH)[(8)]. The oral administration of VSL#3 significantly improves the plasma levels of malondialdehyde

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(MDA) and 4-hydroxynonenal (4-HNE) in patients with non-alcoholic fatty liver disease (NAFLD) and alcoholic liver cirrhosis (AC)[9]. In addition, lactic acid bacteria can offer an effective dietary approach to improving hepatic encephalopathy[10] and to decreasing the risk of liver cancer[11]. From a survey of previous studies, lactic acid bacteria have various potential biological functions[12]. Several studies have provided evidence that certain lactic acid bacteria possess antioxidative activity[13-20]. Saide and Gilliland[21] suggested that some Lactobacilli strains are a source of dietary antioxidants. In our previous studies, we also demonstrated that lactic acid bacteria possess antioxidative ability through one or multiple mechanisms, including metal ion chelation, radical scavenging, inhibition of lipid peroxidation and activity reduction *in vitro*[16,17]. Among the studies conducted by other researchers, most have focused on the antioxidative activity of viable lactic acid bacteria. However, Kaizu *et al*.[18] demonstrated that intracellular cell-free extracts of two *Lactobacillus* strains have antioxidative activity *in vivo*. Furthermore, heat-killed *L. acidophilus* 606 exerts higher antioxidative activity than the soluble polysaccharide fraction[22].

Numerous pharmacological benefits of lactic acid bacteria have been postulated, such as improved immune function, improved liver function, maintenance of intestinal microbial ecosystem, reduced bacterial translocation and decreased ammonia and endotoxin concentrations in blood[9, 23-26]. However, to date, little work has been done to explore the hepatoprotective effects of lactic acid bacteria in attenuating oxidative stress through antioxidative activity[27]. Thus, we used intracellular cell-free extracts and heat-killed lactic acid bacteria to clarify whether lactic acid bacteria cell components have an inhibitory effect on oxidative stress-induced liver injury. The hypothesis that lactic acid bacteria with antioxidative properties can prevent or decrease *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage in HepG2 cells was investigated in this study.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

t-BHP was purchased from Merck (Darmstadt, Germany). The diagnostic kit for alanine aminotransferase (ALT) was purchased from Randox Lab Ltd. (Crumilin Co., UK). 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT), L-glutathione, reduced (GSH) and lactose dehydrogenase assay kits were purchased from Sigma (St. Louis, MO, USA). Other chemicals used in this study were of analytical reagent grade.

II. *Preparation of Intracellular Extracts and Heat-Killed Cells of Lactic Acid Bacteria*

All strains used in this study were obtained from our frozen stock culture collection. Cells of *Lactobacillus acidophilus* La12, *Lactobacillus bulgaricus* Lb23 , *Bifidobacterium longum* Bl36 and *Streptococcus thermophilus* St28 were cultured in de Man, Rogosa and Sharpe (MRS) broth (Difco, Detroit, MI) at 37°C for 16 h and collected by centrifugation at 8000 ×g for 15 min. For the preparation of intracellular extracts, the cells were washed twice with sterile distilled water and suspended in phosphate-buffered saline (PBS; 0.85% NaCl, 2.68 mM of KCl, 10 mM of Na_2HPO_4 and 1.76 mM of KH_2PO_4 at pH 7.7). For cell disruption, a FRENCH® Pressure Cells Press (Thermo Electron, Waltham, MA, USA) was used. The pressure was set at 1,000 psi and the cell disruption cycle was set at 4. Cell debris was removed by centrifugation at 18,000 ×g for 25 min, and the resulting supernatant was used as the intracellular extract. The protein contents of intracellular extracts were adjusted to 100 μg/mL. For the preparation of heat-killed cells, the cells were washed twice with sterile distilled water, suspended in phosphate-buffered saline (PBS; 0.85% NaCl, 2.68 mM of KCl, 10 mM of Na_2HPO_4 and 1.76 mM of KH_2PO_4 at pH 7.7) and heat-killed at 100°C for 15 min. The total cell number was adjusted to 10^8 cfu/mL.

III. *Cell Culture*

The human hepatocellular carcinoma cell line HepG2 was obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). These cells have been shown to maintain many parenchymal cell functions[28]. HepG2 cells were routinely cultured in tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 mg/mL) at 37°C in a humidified (95%) atmosphere with 5% CO_2. The cell culture medium was replaced twice per week. After reaching confluence, the cells were trypsinized and split 1 : 3. Supernatant and cells were collected for various bioassays according to the corresponding experimental protocol.

IV. *MTT Assay*

Cell viability was detected on 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay[29], which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells. In order to determine hepatotoxicity concentration, HepG2 cells were treated with different concentrations of *t*-BHP. Briefly, approximately 2×10^4 cells were plated onto each well of a 96-well plate for 24 h, followed by treatment with different concentrations (1, 1.5 and 3 mM) of *t*-BHP for an additional 90 min. After incubation, 200 μL of MTT solution (0.5 mg/mL) were added to each well for 4 h. Finally, the supernatant was removed and 200 μL of dimethyl sulphoxide (DMSO) was added to each well to dissolve the dark blue formazan crystals. The plates were shaken for 10 min and the absorbance was measured by an ELISA plate reader (Jupiter, ASYS Hitech, Austria) at 570 nm. To compare the results, the relative cell viability was expressed as the mean

percentage of viable cells compared with untreated cells (100%). The protective effects of intracellular extracts or of heat-killed cells of lactic acid bacteria in HepG2 cells injured by *t*-BHP were also measured using MTT assay. HepG2 cells were seeded onto 96-well culture plates (2×10^4 cells/well) for 24 h. Then, the medium was discarded and fresh medium containing intracellular extracts or heat-killed cells of lactic acid bacteria were added to the cell culture for an additional 1 h. Afterwards, the culture media were replaced with media containing 3.0 mM of *t*-BHP, with further incubation for 1.5 h and rinsing with PBS. The supernatant was collected for LDH determination and the cells were used for MTT assay.

V. *Measurement of Lactate Dehydrogenase (LDH) Leakage*

The leakage of LDH into the media was taken as an indicator of cell injury and measured using commercial kits (St. Louis, MO, USA). After incubation, the 96-well plate was centrifuged and the culture supernatant was transferred to a new plate. The assay mixture was prepared and added to each well and the plate was incubated by wrapping in foil at room temperature for 30 min. The reaction was terminated by adding a stop solution to each well. The plate was read at 490 nm and at a reference wavelength of 690 nm. The extent of LDH leakage was expressed as the percentage of absorbance of the control.

VI. *Assay of Enzyme Activity*

Alanine aminotransferase (ALT) is an enzyme that is found in the highest amount in the liver. ALT in medium was analyzed by a diagnostic kit according to the manufacturer's instructions (Randox Lab Ltd., Crumilin Co., UK).

VII. *ROS Measurement*

Cellular oxidative stress was quantified using the cell permeable fluorescent dye 2',7'-dichlorofluoresceindiacetate (DCFH-DA), as described by Donato et al.[30] Upon entering the cell, the diacetate bond of the fluoroprobe is cleaved by the intracellular esterase, leaving DCFH, which is oxidized to dichlorofluorescein (DCF). The fluorescence of DCF is taken as an indicator of oxidant production in the cell. Briefly, cells were seeded onto 30-mm plates at a rate of 2×10^6 cells per plate. Five μM of dichlorofluorescein (DCFH) were added in the dark at 37°C for 30 min. Then, the cells were washed twice with serum-free medium, scraped and collected in brown eppendorff. After being oxidized by intracellular oxidants, DCFH became DCF and emitted fluorescence. The fluorescence intensity was measured using a flow cytometer (Cytomics FC500, Beckman Coulter, Britain) at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

VIII. *Determination of Lipid Peroxidation*

The end product, malondialdehyde (MDA), formed due to membrane lipid peroxidation was measured. Briefly, 0.2 mL of medium were added to 0.2 mL of 8.1% sodium dodecylsulphate (SDS), 1.5 mL of 20% acetic acid, 0.8 mL of double distilled water (DDW) and 1.5 mL of 0.8% thiobarbituric acid (TBA). The mixture was incubated at 100°C for 60 min and then kept on ice for 15 min. The colored complex was extracted into *n*-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission using a fluorescence spectrophotometer (Model F-4500, Hitachi, Tokyo, Japan). A malondialdehyde standard curve was constructed using 1,1,3,3-tetramethoxypropane[31]. Lipid peroxidation was expressed as TBARS nmol per 10^6 cells.

IX. *Preparation of Cell Homogenate*

For the measurements of GSH level and antioxidant enzyme activities, the cells were treated as described previously. At the end of each treatment, the cells in each well were washed twice with 1 mL of ice-cold PBS and harvested by centrifugation at 100 ×g for 5 min. The pellets were suspended in PBS and then subjected to ultrasonic disruption over five 1-min intervals in an ice bath (Setting = 4; Sonicator XL-2020; Heat System, Farmingdale, NY). The cell homogenate was then centrifuged at 3000 ×g at 4°C for 10 min (Eppendorf centrifuge 1-15K, Sigma, Germany) to remove debris and nuclei. The resulting supernatant was stored at -80°C for subsequent GSH and antioxidant enzyme activity assays.

X. *Determination of Intracellular Reduced Glutathione (GSH)*

The GSH content was measured by the method of Tietze[32] with slight modification. The protein in sample was precipitated with 50% TCA and then centrifuged at 1000 ×g for 5 min. The reaction mixture containing 50 μL of supernatant, 200 μL of 0.2 M Tris–EDTA buffer (pH 8.9) and 10 μL of 0.01 M 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) was kept at room temperature for 5 min, and the yellow color developed was measured spectrophotometrically at 412 nm. Results were calculated from a standard curve and expressed as nanomoles per milligram of protein.

XI. *Determination of Antioxidant and Detoxification Enzyme Activity*

For the assay of antioxidant and detoxification enzyme activities, cells were suspended in PBS, sonicated and centrifuged. All enzyme activities were measured in the supernatants. The level of superoxide dismutase (SOD) activity was determined using the method of Winterbourn, in which the light-triggered release of superoxide radicals from riboflavin leads to the formation of a blue complex through reaction with nitroblue tetrazolium[33]. The supernatant fraction was incubated in solution containing 0.067 M of phosphate buffer (pH 7.8), 0.1 M of EDTA, 1.5 mM

of NBT and 0.12 mM of riboflavin for 10 min in an illuminated chamber with an 18-W fluorescent lamp. Absorbance was recorded at 560 nm and SOD activity was expressed as units/mg protein. Catalase (CAT) activity was determined by following the decomposition of H_2O_2 measured as a decrease in absorbance at 240 nm[34]. With H_2O_2 as a substrate, Glutathione peroxidase (GPx) activity was measured using GRd and NADPH in a coupled reaction. The disappearance of NADPH by GRd was monitored at 340 nm[35]. Glutathione reductase (GRd) activity was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADPH utilized in the reduction of oxidized glutathione[35]. Glutathione-S-transferase (GST) activity was measured according to the method of Habig *et al.*[36], which is based on the GST-catalyzed reaction between GSH and 1-chloro-2,4-dinitrobenzene, an electrophilic substrate for GST.

XII. *Protein Assay*

The protein content in cell lysates was measured by Bradford's method using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

XIII. *Statistical Analysis*

All values are expressed as mean ± standard deviation (SD). Each value is the mean of at least three separate experiments in each group. Statistical comparisons were carried out by ANOVA, followed by Tukey's test. $p < 0.05$ was considered significantly different.

RESULTS

I. *Cytotoxicity of t-BHP*

To determine the hepatotoxicity of *t*-BHP, HepG2 was treated with 3 different concentrations of *t*-BHP (1, 1.5 and 3 mM). On MTT assay, the oxidative stress induced by 3 mM of *t*-BHP caused over 50% cell death after 1.5 h, when compared with the untreated control cells (Figure 1). Thus, the concentration of 3 mM of *t*-BHP was chosen for subsequent experiments on the hepatoprotective effect of lactic acid bacteria.

II. *Effect of Lactic Acid Bacteria on t-BHP-Induced Cytotoxicity in HepG2 Cells*

To evaluate the hepatoprotective effects of lactic acid bacteria on *t*-BHP-induced cytotoxic injury in cultured HepG2 cells, cell viability was determined using MTT assay (Figure 2). When HepG2 cells alone were exposed to *t*-BHP, cell viability significantly decreased in comparison with untreated control cells. All lactic acid bacteria strains used in this study significantly abrogated the toxicity induced in the cells by *t*-BHP ($p < 0.05$) except for *L. acidophilus* La12 (HK), which reduced the toxicity but not significantly. The results demonstrated that lactic acid bacteria have significant protective effects against *t*-BHP-induced cytotoxicity in HepG2 cells.

III. *Effect of Lactic Acid Bacteria on Liver Enzyme LDH and ALT Leakage in HepG2 Cells*

LDH and ALT leakages in culture supernatant were used as indicators of oxidative damage to HepG2 cells by *t*-BHP, as shown in Table 1. The results were consistent with

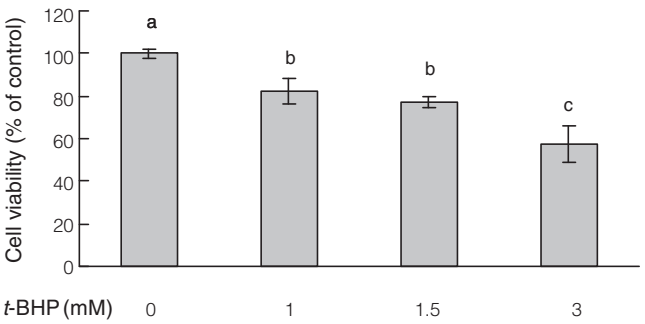

Figure 1. HepG2 cellular damage caused by exposure to different doses of *t*-BHP (1, 1.5 and 3 mM) for 1.5 h. The cell viability was detected by MTT assay as described in Materials and Methods. Untreated cells served as the control. Each bar represents the mean ± SD (n = 3). Bars with different letters represent significant differences among different treatments ($p < 0.05$).

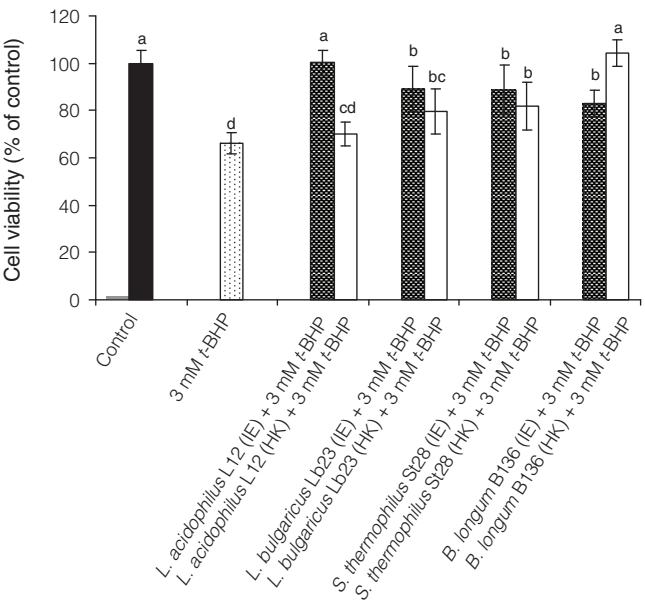

Figure 2. Effects of pre-treatment with intracellular extract (IE) and heat-killed cells (HK) of *L. acidophilus* La12, *L. bulgaricus* Lb23, *B. longum* Bl36 and *S. thermophilus* St28 on *t*-BHP-induced cellular damage in HepG2 cells. The cell viability was detected by MTT assay as described in Materials and Methods. Untreated cells served as the control. Each bar represents the mean ± SD (n = 3). Bars with different letters represent significant differences among different treatments ($p < 0.05$).

Table 1. Effects of lactic acid bacteria on liver enzyme LDH and ALT leakages

Strains	LDH leakage (% of control)	ALT leakage (U/L)
3 mM *t*-BHP	162 ± 7 ^{a}	83 ± 2 ^{a}
Control	100 ± 3 ^{fg}	56 ± 7 ^{d}
Intracellular extracts		
L. acidophilus La12 + 3 mM *t*-BHP	70 ± 3 ^{h}	75 ± 4 ^{b}
L. bulgaricus Lb23 + 3 mM *t*-BHP	100 ± 6 ^{fg}	73 ± 4 ^{b}
B. longum Bl36 + 3 mM *t*-BHP	147 ± 6 ^{b}	67 ± 8 ^{bc}
S. thermophilus St28 + 3 mM *t*-BHP	105 ± 10 ^{ef}	71 ± 2 ^{b}
Heat-killed cells		
L. acidophilus La12 + 3 mM *t*-BHP	123 ± 2 ^{c}	64 ± 2 ^{c}
L. bulgaricus Lb23 + 3 mM *t*-BHP	113 ± 6 ^{de}	73 ± 3 ^{b}
B. longum Bl36 + 3 mM *t*-BHP	118 ± 7 ^{gd}	73 ± 5 ^{b}
S. thermophilus St28 + 3 mM *t*-BHP	92 ± 11 ^{g}	64 ± 3 ^{c}

Untreated cells served as the control. Each number represents the mean ± SD (n = 3). Numbers with different letters represent significant differences among different treatments ($p < 0.05$).

those obtained from MTT assay. The levels of LDH and ALT that were released were significantly higher ($p < 0.05$) in the *t*-BHP-treated group than in the control group. Again, both heat-killed cells and intracellular extracts of lactic acid bacteria prevented the leakage of LDH and ALT from HepG2 cells treated with *t*-BHP.

IV. *Effect of Lactic Acid Bacteria on Intracellular ROS Generation*

To confirm the reduction of *t*-BHP- induced oxidative stress in HepG2 cells by lactic acid bacteria, intracellular ROS production was determined by monitoring the fluorescent dye, DCFH-DA. There was a 78% ($p < 0.05$) increase in ROS generation in the *t*-BHP stressed cells when compared with the control cells. HepG2 cell pretreated with either intracellular extracts or heat-killed cells significantly reduced the formation of ROS by 52% to 73% ($p < 0.01$) (Figure 3).

V. *Effect of Lactic Acid Bacteria on Lipid Peroxidation and GSH levels in HepG2 Cells*

Figure 4A shows that the amount of TBARS increased 18-fold in *t*-BHP treated HepG2 cells when compared with control cells. The extent of lipid peroxidation was reduced slightly in HepG2 cells pre-cultured with lactic acid bacteria, but there was no significant change when compared with *t*-BHP-treated HepG2 cells. Only intracellular extracts of *L. acidophilus* La12 and *B. longum* Bl36 showed a significant decrease in TBARS formation when compared with *t*-BHP-injured cells. To investigate the role of lactic acid bacteria in reducing *t*-BHP stress, intracellular reduced glutathione levels (GSH) were determined. As shown in Figure 4B, there was a significant decrease of GSH level in cells treated with *t*-BHP. The GSH depletion by *t*-BHP was significantly reduced by the pretreatment of cells with intracellular extracts of lactic acid bacteria prior to exposure to pro-oxidant *t*-BHP. However, heat-killed intact cells of *L. bulgaricus* Lb23 and *B. longum* Bl36 showed no significant protective effects on GSH levels when compared with *t*-BHP-stressed cells. These results suggested that most of the lactic acid bacteria strains in this study exhibit protective properties against *t*-BHP-induced oxidative damage.

VI. *Effect of Lactic Acid Bacteria on Antioxidant and Detoxification Enzyme Activities*

SOD, CAT, GPx, GRd and GST are antioxidant and detoxification enzymes that protect cells against oxidative stress from highly reactive free radicals. Activities of these enzymes will be induced when free radicals are generated in cells. To study the enzymatic antioxidant activity changes in HepG2 cells in culture in the presence of *t*-BHP, the activities of SOD, CAT, GPx, GRd and GST were determined, as shown in Table 2. Compared with control cells, SOD, CAT, GPx, GRd and GST activities increased 1.2 to 2.9-fold in HepG2 cells alone exposed to *t*-BHP. The increase in antioxidant enzyme activities represented resistance against ROS production. SOD activities observed in the four heat-killed strains of lactic acid bacteria were significantly lower than in the *t*-BHP group, while no significant protective effect was induced by intracellular extracts of *L. acidophilus* La12 or *S. thermophilus* St28. SOD activities in the *L. bulgaricus* Lb23 and *B. longum* Bl36 intracellular extract groups also decreased when compared with the *t*-BHP group. As shown in Table 2, all groups except intracellular extracts of *L. acidophilus* La12 produced significant protective effects against *t*-BHP-induced liver injury. The *t*-BHP-induced increase in enzyme activity of GPx was found to be dramatically diminished by intracellular extracts of *L. bulgaricus* Lb23, *B. longum* Bl36, heat-killed *L. acidophilus* La12 and *L. bulgaricus* Lb23. In all 8 groups, intracellular extracts and heat-killed cells of lactic acid bacteria significantly prevented an increase in the activity of GR induced by *t*-BHP. In the case of GST, most lactic acid bacteria were able to reduce the GST activities, with only the intracellular extract of the *S. thermophilus* St28 strain found to be ineffective. These results implied that most of the intracellular extracts and heat-killed cells of lactic acid bacteria enhance the cell defense system and render cells more resistant to subsequent oxidative damage.

DISCUSSION

Human hepatoma G2 (HepG2) cells, a well-differentiated transformed cell line, are a reliable model, easy to culture, well-characterized and extensively used for the evaluation of dietary compounds on liver antioxidant defense mechanisms at the cellular level[37,38]. In addition,

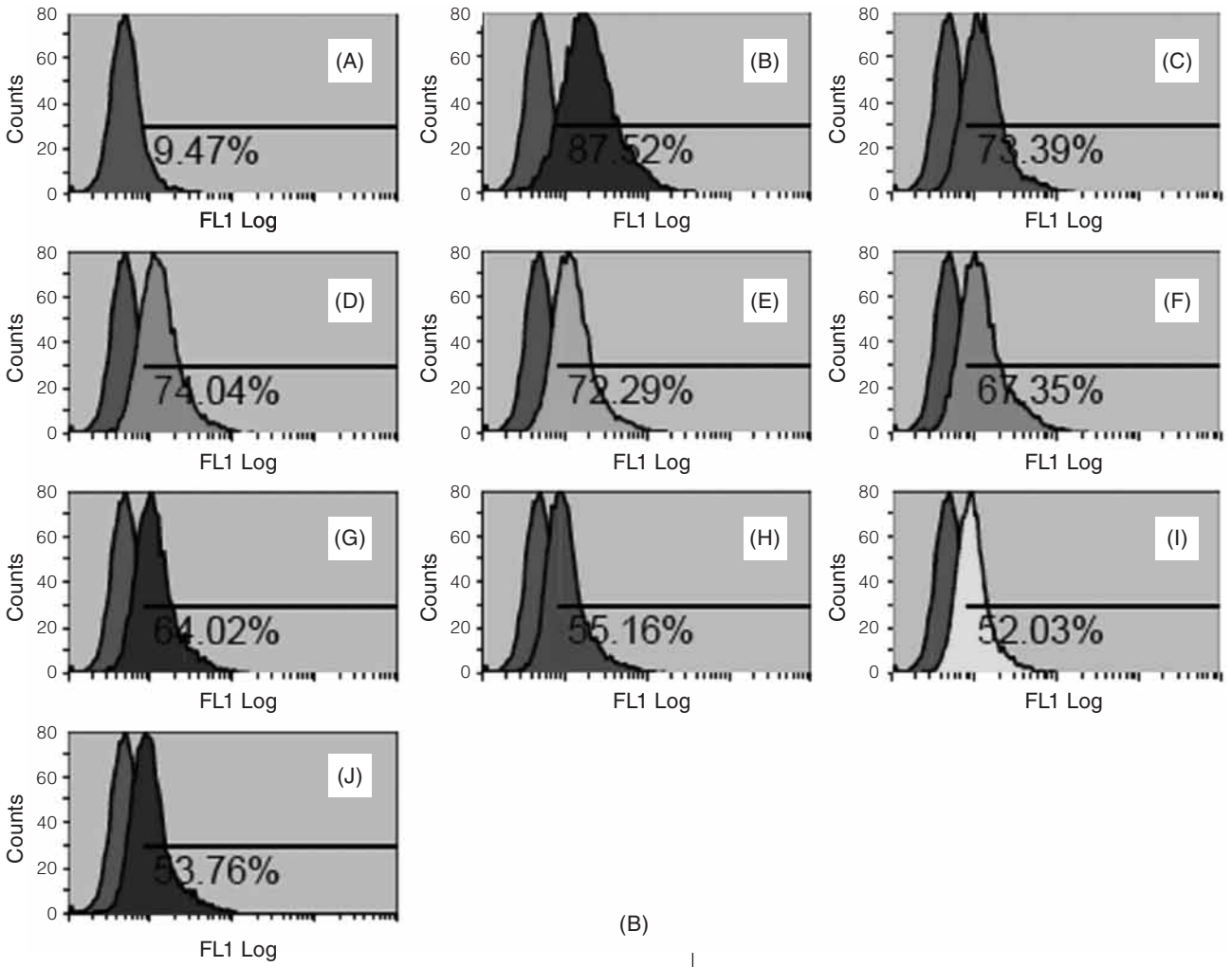

Figure 3. The effect of lactic acid bacteria on the scavenging of intracellular ROS. The generated intracellular ROS were detected with flow cytometry after DCF-DA treatment, reflected by the rightward shift of the histogram. The data shown is a representative of three independent experiments with similar shape in outcomes. In Figure 3A, A: control; B: 3 mM *t*-BHP; C: intracellular extracts of *Lactobacillus acidophilus* La12 + 3 mM *t*-BHP; D: intracellular extracts of *Lactobacillus bulgaricus* Lb23 + 3 mM *t*-BHP; E: intracellular extracts of *Bifidobacterium longum* Bl36 + 3 mM *t*-BHP; F: intracellular extracts of *Streptococcus thermophilus* St28 + 3 mM *t*-BHP; G: heat-killed cells of *Lactobacillus acidophilus* La12 + 3 mM *t*-BHP; H: heat-killed cells of *Lactobacillus bulgaricus* Lb23 + 3 mM *t*-BHP; I: heat-killed cells of *Bifidobacterium longum* Bl36 + 3 mM *t*-BHP; J: heat-killed cells of *Streptococcus thermophilus* St28 + 3 mM *t*-BHP. Figure 3B, the percentage of with significant ROS generation was analyzed by flow cytometry, reflected by the rightward shift of the histogram.

steady-state functioning of the antioxidant defenses in HepG2 is relatively higher than that in hepatocytes and other non-transformed cells. This makes variations of responses in HepG2 to different conditions more easily detected[39]. Therefore, we choose HepG2 cells as a cell model to examine the potential hepatoprotective effect of lactic acid bacteria against oxidative stress induced by a potent pro-oxidant, *tert*-butyl hydroperoxide (*t*-BHP).

The main findings of this study indicated that *t*-BHP-induced hepatotoxicity can be significantly alleviated by the pre-treatment of HepG2 cells with intracellular extracts or heat-killed cells of lactic acid bacteria. These positive effects are provided by modulating GSH content, ROS production and antioxidant enzyme activities.

Cell viability and LDH leakage are known to be general indices of hepatic cytotoxicity[40]. The decreased cell viability caused by hepatic damage is believed to be due to breakage of the structure of the cellular membrane, followed by leakage of LDH and ALT[41]. Therefore, LDH and ALT, hepatic enzyme markers, are commonly used as indicators of cellular damage[41,42]. As shown in Table 1, LDH and ALT leakage induced by *t*-BHP were significantly lowered when HepG2 cells were pre-incubated with intracellular extracts or heat-killed cells of lactic acid bacteria. This finding indicates that intracellular extracts and heat-killed cells of lactic acid bacteria protect cells against *t*-BHP- induced damage. Intracellular extracts and heat-killed cells may stabilize HepG2 cell membranes and maintain their integrity. Similar results by other researchers have shown that some *Lactobacillus* strains[43] and plant-derived lactic acid bacteria[44] have the ability to improve liver function *in vivo*. The increase of serum AST and ALT levels caused by ethanol intake are

Table 2. Effect of lactic acid bacteria on the activities of antioxidant and detoxification enzymes in t-BHP-treated HepG2 cells

Strains	SOD activity (μmol/min/ mg protein)	CAT activity (U/mg protein)	GPx activity (nmol NADPH/ min/mg protein)	GRd activity (nmol NADPH/ min/mg protein)	GST (mU/mg protein)
3 mM *t*-BHP	4 ± 0.6 ^{a}	17 ± 0.6 ^{a}	185 ± 21 ^{a}	20 ± 3 ^{a}	37 ± 0.9 ^{b}
Control	3 ± 0 ^{c}	14 ± 0.8 ^{bc}	84 ± 20 ^{cd}	7 ± 3 ^{bc}	30 ± 1.0 ^{e}
Intracellular extracts					
L. acidophilus La12 + 3 mM *t*-BHP	4 ± 0.4 ^{a}	15 ± 1.3 ^{bc}	150 ± 27 ^{ab}	8 ± 3 ^{bc}	33 ± 0.4 ^{c}
L. bulgaricus Lb23 + 3 mM *t*-BHP	3 ± 0.1 ^{c}	14 ± 0.4 ^{bc}	127 ± 21 ^{bc}	12 ± 4 ^{bc}	32 ± 0.3 ^{d}
B. longum Bl36 + 3 mM *t*-BHP	3 ± 0.2 ^{bc}	14 ± 1.6 ^{bc}	67 ± 41 ^{d}	8 ± 4 ^{bc}	33 ± 0.9 ^{c}
S. thermophilus St28 +3 mM *t*-BHP	4 ± 0.9 ^{ab}	13 ± 1.8 ^{c}	149 ± 21 ^{ab}	6 ± 2 ^{c}	44 ± 0.1 ^{a}
Heat-killed cells					
L. acidophilus La12 + 3 mM *t*-BHP	3 ± 0 ^{bc}	13 ± 1.7 ^{c}	114 ± 17 ^{bcd}	8 ± 3 ^{bc}	20 ± 0.4 ^{f}
L. bulgaricus Lb23 + 3 mM *t*-BHP	3 ± 0.2 ^{c}	13 ± 1.1 ^{bc}	123 ± 36 ^{bc}	9 ± 2 ^{bc}	19 ± 1.0 ^{g}
B. longum Bl36 + 3 mM *t*-BHP	3 ± 0.1 ^{c}	13 ± 0.4 ^{bc}	167 ± 32 ^{ab}	8 ± 3 ^{bc}	18 ± 0.5 ^{h}
S. thermophilus St28 + 3 mM *t*-BHP	3 ± 0.2 ^{c}	14 ± 0.5 ^{bc}	149 ± 17 ^{ab}	3 ± 3 ^{c}	19 ± 0.6 ^{gh}

Untreated cells served as the control. Each number represents the mean ± SD (n = 3). Bars with different letters represent significant differences among different treatments ($p < 0.05$).

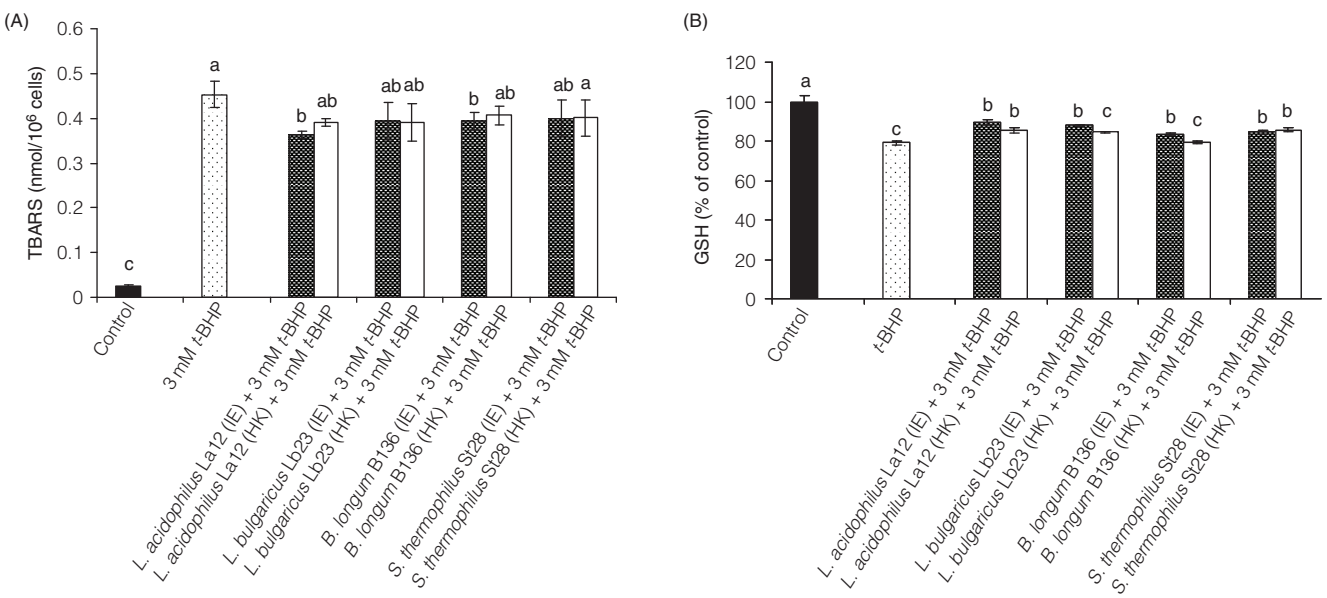

Figure 4. Lipid peroxidation status and GSH levels of HepG2 cells during treatment with intracellular extracts of (IE) and heat-killed cells (HK) of *L. acidophilus* La12, *L. bulgaricus* Lb23, *B. longum* Bl36 and *S. thermophilus* St28 for 1 h before exposure to 3 mM *t*-BHP. Untreated cells served as the control. Each bar represents the mean ± SD (n = 3). Bars with different letters represent significant differences among different treatments ($p < 0.05$).

significantly inhibited by oral administration of heat-killed *L. brevis* SBC8803 in C57BL/6N mice[45]. Results from animal model and human clinical trial show that lactic acid bacteria administered orally interact with digestive enzymes in the alimentary tract and follow their components after intestinal absorption to the liver where they exhibit probiotic effects.

It has been reported that an overproduction of ROS derived from *t*-BHP metabolism inside the hepatocytes is responsible for the observed modulation of the cellular response to oxidative challenge[40]. Lipid peroxidation is the result of ROS attacking unsaturated fatty acids of cell membranes and lipids. TBARs, which indicate the level of lipid peroxidation, have been extensively used as markers of oxidative stress in biological systems[40]. Intracellular extracts and heat-killed cells of lactic acid bacteria markedly reduced *t*-BHP-induced ROS levels in HepG2 cells, indicating that both intracellular extracts and heat-killed cells of lactic acid bacteria are effectively ROS scavengers. GSH is an important intracellular antioxidant and redox potential regulator that provides an efficient detoxification effect and protects cells from damage by free radicals, peroxides and toxins[46]. The depletion of intracellular GSH may result from excess ROS production, and thus it is assumed that GSH depletion reflects intracellular oxidation[47]. Figure 4B shows that intracellular extracts and heat-killed cells of lactic acid bacteria significantly prevent the depletion of GSH levels induced by *t*-BHP. These results suggest that intracellular extracts and heat-killed cells of lactic acid bacteria attenuate *t*-BHP-induced redox disequilibria by raising the levels of GSH and lowering the levels of ROS.

Cells are normally able to defend themselves against ROS damage by antioxidant enzymes such as SOD, CAT, GRd and GPx. These antioxidant enzymes play critical roles in the system of defense against oxidative stress caused by free radicals within liver cells. Catalase, an essential cytosolic antioxidant enzyme[48], converts H_2O_2 to H_2O. GPx catalyzes GSH oxidation to oxidized glutathione (GSSG) by consuming H_2O_2 or other organic peroxides, while GRd recycles GSSG back to reduced glutathione using NADPH[39]. When the challenge of *t*-BHP to HepG2 cells leads to an increase in activities of antioxidant enzymes including SOD, GRd, GPx, and CAT[39], ROS are produced and antioxidant enzymes are involved in the system of defense against oxidative stress[49]. Moreover, recent studies have revealed that some lactic acid bacteria strains express manganese superoxide dismutase (Mn-SOD)[50]. In the present study, some intracellular extracts and heat-killed cells of lactic acid bacteria maintained the intracellular redox balance in *t*-BHP-injured hepatocytes by restoring the activities of antioxidative enzymes. These results suggest that intracellular extracts and heat-killed cells of lactic acid bacteria exhibit adaptive response to oxidative stress.

Recently, intracellular extracts of lactic acid bacteria have been widely studied and evaluated for their antioxidative capacity[15]. Intracellular extracts exhibit higher antioxidant activity than intact cells[15,21]. The greatest degree of antioxidant capacity is associated with the cell-free extracts of the cultures, which suggests that they may be important in delivering antioxidants to the intestines where they can be released when cells of the cultures encounter bile[21]. However, to date, research on the authentic components of lactic acid bacteria that exert hepatoprotective action is limited[45]. The cell wall of lactic acid bacteria consists mainly of polysaccharide-peptidoglycan complex and lipoteichoic acid[51,52]. The radical scavenging activity on DPPH assay showed that heat-killed *L. acidophilus* 606 exerted slightly higher antioxidative activity than the soluble polysaccharides isolated from this strain[22]. Moreover, what is known is that the intracellular cell-free extract is composed of DNA fragments, proteins and soluble carbohydrate compounds[53]. Mikelsaar and Zilmer[19] indicated that probiotics show different potent antioxidative effects due to the expression of antioxidant enzymes such as Mn-SOD and the components of the complete glutathione system (GSH, glutathione peroxidase and glutathione reductase). Recently, a systematic review described antioxidative peptides from food proteins[54]. Therefore, we speculated that intracellular extracts of lactic acid bacteria contain some antioxidative peptides which are involved in antioxidative activity. Further investigation of the cell components associated with antioxidative activity is required to identify the specific molecules that exert hepatoprotective effects by attenuating oxidative stress.

Lactic acid bacteria is a promising agent for protecting the liver[45] and very useful as a functional food for maintaining human health[8]. In the present study, lactic acid bacteria showed protective effects against oxidative damage by *t*-BHP in HepG2 cells, at least in part by decreasing the risk of accumulated ROS, slightly inhibiting lipid peroxidation reaction, reducing GSH depletion, and improving antioxidant enzyme activities. These results offer primary experimental data for further *in vivo* studies and for in-depth research regarding the protective mechanisms of lactic acid bacteria against *t*-BHP-induced liver damage.

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