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Full Length Research Paper

Antioxidative activity of lactic acid bacteria in yogurt

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The antioxidative effect of intact cells and cell-free extract of *Lactobacillus casei* subsp. *casei* SY13 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LJJ, isolated from the traditional yogurt, was evaluated by various antioxidant assays. The results showed that two *Lactobacillus* strains had good antioxidant capacity, inhibiting the peroxidation of linoleic acid by 62.95% and 66.16%. The cell-free extract showed excellent scavenging superoxide anion and hydroxyl radicals activity. The intact cells on 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging ability and chelating ferrous ion capacity were superior to cell-free extract. The highest reducing activity was equivalent to 305 and 294 µM L-cysteine. This study suggests that two strains are high antioxidative bacterial strains. Antioxidative property of lactobacilli would be useful in the dairy manufacturing industry. They could beneficially affect the consumer by providing dietary source of antioxidants.

Keywords: *Lactobacillus*, reactive oxygen species, antioxidative.

INTRODUCTION

Aging is characterized by a progressive decline in biochemical and physiological functions of various tissues and organs in an individual. The causes of this decline have been proposed that increased oxidative stress and disorders in energy metabolisms might play an important role (Lin and Beal, 2003). According to the free radical theory of aging (Muller et al., 2007), the disruption of the delicate balance between generation of reactive oxygen species (ROS) and antioxidant scavenging systems with increasing age could lead to a shift to an oxidative cellular milieu, and eventually lead to serious health problems such as diabetes and Alzheimer's disease. Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases (Bellino, 2006; Getoff, 2007). In recent years, search for novel type of antioxidants from traditional Chinese traditional food has achieved considerable attention (Cai et al., 2004).

Lactic acid bacteria (LAB) have some probiotic functions, such as adjusting the balance of intestinal flora, reducing serum cholesterol, inhibiting and reducing the risk of tumors, and revitalizing the immune system etc (Gilliland, 1999; Leroy and Vuyst, 2004). The anti-oxidative effect of LAB has been reported only recently (Lin and Yen, 1999a, b; Lin and Chang, 2000c; Kullisaar et al., 2002). Among lactic acid bacteria, *Lactobacillus* have attracted a lot of attention for their potential probiotic effects in human health. *Lactobacillus* spp. are important members of the healthy human microbiota (Naaber et al., 1998). Some lactobacilli has been shown that possessing antioxidative activity, and are able to decrease the risk of accumulation of ROS during the ingestion of food.

In previous studies, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus fermentum* and *Lactobacillus sake* (Lin and Chang, 2000c; Kullisaar et al., 2002; Amanatidou et al., 2001a) had been shown possessing antioxidative activity, and were able to decrease the risk of accumulation of ROS. In this study, we mainly evaluate the antioxidative activity of *Lactobacillus casei* subsp. *casei* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which isolated from Chinese traditional yogurt. The aim of this study was to

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evaluate antioxiative activities including inhibition of linoleic acid peroxidation, the scavenging effect of their intact cells and cell-free extract on DPPH radicals, superoxide anion radicals and hydroxyl radical, the resistance to hydrogen peroxide. In addition, the chelating capacity of ferrous ion and reducing activity was also examined.

MATERIALS AND METHODS

Strains

L. casei subsp. *casei* SY13 and *L. delbrueckii* subsp. *bulgaricus* LJJ were isolated from the yogurt collected from Beijing, China and obtained from our frozen stock collection. *L. rhamnosus* GG (LGG) was isolated from a commercially available fermented yogurt of Inner Mongolia Yili Industrial Group Co., Ltd and as a positive strain.

They were propagated in MRS (Luqiao, Beijing) at 37°C for 18 h. Agar plates were made by adding 1.5% agar (Guangda, Beijing) to broth for viable cell counts and were incubated at 37°C for 48 h. All strains were serially transferred at least three times prior to use in studies.

Preparation of intact cells and intracellular cell-free extracts

Cells of *L. casei* subsp. *casei* SY13 and *L. delbrueckii* subsp. *bulgaricus* LJJ were harvested by centrifugation at 4000× *g* for 10 min after 18 h incubation at 37°C. For the preparation of intact cells, cells pellets were washed three times with phosphate buffer solution (PBS: 0.85% NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4) and resuspended in PBS. For the preparation of intracellular cell-free extracts, cell pellets were then quickly washed twice with deionized water and resuspended in deionized water followed by ultrasonic disruption (JY-92 sonicator, Scientz, Ningbo). Sonication was performed for five 1-min intervals in an ice bath. Cell debris was removed by centrifugation at 10000 × *g* for 10 min, and the resulting supernatant was the intracellular cell-free extract. Total cell numbers were adjusted to 10^9 cfu ml^{-1} for the preparation of intact cells and intracellular cell-free extracts.

Assays of antioxidative activity of Lactobacilli

Measurement of linoleic acid peroxidation

The measurement of antioxidative activity of *L. casei* subsp. *casei* SY13 and *L. delbrueckii* subsp. *bulgaricus* LJJ was performed by the thiobarbituric acid (TBA) method, based on the monitoring of inhibition of linoleic acid peroxidation by intact cells and by intracellular cell-free extracts according to the method (Lin and Chang, 2000c).

DPPH free radical scavenging ability

The DPPH radical scavenging activity was measured by the method (Shimada et al., 1992) with a minor modification. 0.8 ml of intact cells or intracellular cell-free extract and 1 ml of freshly prepared DPPH solution (0.2 mM in methanol) were mixed and allowed to react for 30 min. Blank samples contained either PBS or deionized water. The scavenged DPPH was then monitored by measuring the decrease in absorbance at 517 nm. The scavenging ability was defined as follows:

$$\text{Scavenging effect}(\%)=1-\frac{A517(\text{sample})}{A517(\text{blank})}\times 100\%$$

Resistance to hydrogen peroxide

Lactobacilli were grown at 37°C in MRS broth for 18 h and harvested by centrifugation (3000 × g) for 10 min, then suspended at the level of 10^9 CFU ml^{-1} in PBS and incubated with 1 mM hydrogen peroxide at 37°C. At 2 h time intervals, the removed aliquots were plated onto MRS Agar plates. The incubation of MRS agar for the cultivation of lactobacilli was performed at 37°C for 48 h.

Superoxide anion scavenging ability

A sample of Tris-HCl (150 mM, pH 8.2), 3 mM EDTA, 1.2 mM 1,2,3-Benzenetriol, 0.5 ml of intact cells or cell-free extract was mixed and the volume of total reaction was 3.5 ml. This mixture was incubated at 25°C for 10 min, and the absorbance was measured at 325 nm. The percentage of resistance to superoxide anions was defined as follows:

$$\text{Scavenging effect }(\%)=1-\frac{A11-A10}{A00-A01}\times 100\%$$

Where A00- no lactobacilli or 1,2,3-Benzenetriol, A01-no lactobacilli but have 1,2,3-Benzenetriol, A10-contain lactobacilli but no 1,2,3-Benzenetriol and A11-contain lactobacilli and 1,2,3-Benzenetriol.

Hydroxyl radical scavenging ability

The generation of hydroxyl radical was performed in solution containing 1 ml 1,10-Phenanthroline (Sigma) ,1 ml phosphate buffer (0.02 mM pH 7.4), 1 ml distilled water, and 1 ml $FeSO_4$ (2.5 mM). The reaction was started by adding 1ml H_2O_2 (20 mM). This mixture was incubated at 37°C for 1.5 h, and the absorbance was measured at 536 nm. The data was expressed as Ap. 1 ml H_2O_2 was replaced with 1 ml distilled water, and the data was expressed as Ab. 1 ml distilled water was replaced with 1 ml sample, and the data was expressed as As. The percentage of resistance to hydroxyl radicals was defined as follows:

$$\text{Scavenging effect }(\%)=\frac{As-Ap}{Ab-Ap}\times 100\%$$

Measurement of the chelating capacity of ferrous ion and reducing activity

Iron ion chelating assay was performed as described by Amanatidou et al. (2001a). Briefly, 0.5 ml of intracellular cell-free extract was mixed with 0.1 ml of ascorbate (1%, w/v), 0.1 ml of $FeSO_4$ (0.4 g l^{-1}), and 1 ml of NaOH (0.2 M). The mixture was incubated at 37°C in a water bath, and 0.2 ml of trichloroacetic acid (TCA:10%) was added into the mixture and incubated for 20 min. Supernatant was obtained by centrifugation at 1800 × g for 10 min, and 0.5 ml of o-phenanthroline (1 g l^{-1}) was added. After the 10 min reaction, the absorbance was measured at 510 nm.

The reducing activity of samples was determined essentially according to the method of Wang et al. (2006). 0.5 ml sample or distilled water (control) was mixed with 1.0% potassium ferricyanide (0.5 ml, Sinopharm, China) and sodium phosphate buffer (0.5 ml, 0.02 M, pH 7). The mixture was incubated at 50°C for 20 min and then 10% trichloroacetic acid (0.5 ml) was added. The mixture was

Table 1. Inhibited capacity of two strains on linoleic acid peroxidation.

Strain	Viable cells(cfu/ml)	Cell morph	Inhibition (%)
SY13	2.54×10^9	Intact cells	62.95±1.57 [a]
		Cell-free extract	44.4±1.38 [b]
LJJ	2.10×10^8	Intact cells	59.63±1.42 [a]
		Cell-free extract	66.16±1.32 [a]

[a,b]Presented values are means of triplicate determinations; ±indicates standard deviation from the mean.
[a,b]Mean values (±standard deviation) within the same column followed by different superscript letters differ significantly ($P < 0.05$).

centrifuged at 780×g for 5 min. The upper layer (1.5 ml) was mixed with 0.1% ferrichloride (0.2 ml, Sinopharm, China) and the absorbance was measured at 700 nm. A higher absorbance of this mixture indicates a higher reducing activity, and the reducing activity of cysteine was used as a standard.

Statistical analysis

Statistical analysis of data was carried out using SAS V8 (SAS Institute Inc, Cary, USA). The mean values and the standard deviation were calculated from the data obtained from three parallel experiments. These data were then compared by the Duncan's multiple range method at the significance level of $P < 0.05$.

RESULTS

Inhibition of linoleic acid peroxidation

2 *Lactobacillus* strains were tested for their antioxidative capacity in both intact cells and intracellular cell-free extracts in this study. *L. rhamnosus* GG, which was shown to have an antioxidative activity in the previous reports (Lin and Chang, 2000c; Sun et al., 2010), was used as a positive control. For the determination of their antioxidative capacity, inhibition of lipid peroxidation is commonly used for analysis. Unsaturated fatty acids, such as linoleic acid, are typically used. As shown in Table 1, both intact cells and cell-free extract of SY13 and LJJ possess the inhibition on linoleic acid peroxidation. The intact cells of 10^9 cells of SY13 and 10^8 cells of LJJ demonstrated inhibition on linoleic acid peroxidation by 62.95 and 59.63%, and the cell-free extract by 44.4 and 66.16%. Therefore, the two strains presented excellent inhibition on linoleic acid peroxidation.

DPPH free radical scavenging ability

The DPPH radical scavenging method is widely used to evaluate antioxidant activities, because of its simplicity, rapidity, sensitivity and reproducibility compared with other methods (Milardovic et al., 2006). The principle of the assay is based on the reduction of ethanolic DPPH solution in the presence of a hydrogen-donating antioxidant, leading to the formation of non-radical form DPPH-H. The antioxidant is able to reduce the stable radical DPPH from purple to yellow-colored diphenylpicrylhydrazine. As shown in the Figure 1, the scavenging DPPH rate of intact cells was higher than that of cell-free extracts. The intact cells of SY13 and LJJ exhibited the scavenging DPPH radical by 27.50 and 23.99%, respectively. The results also showed that intact cells and cell-free extract of two strains exhibited obviously higher antioxidative activity in scavenging DPPH radical than LGG.

Resistance to hydrogen peroxide

Hydrogen peroxide is a relatively weak oxidant, but it is highly diffusive and has a long life time. Because of these two basic characteristics, hydrogen peroxide contributes to oxidative damage either directly or as a precursor of hydroxyl radicals.

To evaluate the resistance to ROS, we tested the survival of intact cells in the presence of 1 mM hydrogen peroxide. As shown in Figure 2, SY13 and LJJ were viable even after 8 h in the presence of 1 mM hydrogen peroxide. The number of viable cells of SY13 at the level of 3.4×10^8 drops to 6.0×10^4 after 8 h, and the number of viable cells of LJJ at the level of 1.4×10^7 drops to 4.5×10^5 after 8 h. The number of viable cells of SY13 after 8 hour was higher than that of LJJ. Therefore, the results of examination demonstrated that both strains are resistant to 1 mM hydrogen peroxide.

Superoxide anion scavenging ability

Superoxide anions are a precursor to active free radicals. It is normally formed first in cellular oxidation reactions. Superoxide anions would play important roles in the formation of some ROS such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA. It is the source of free radicals formed *in vivo* (Rout and Banerjee, 2007). In this study, superoxide anions were

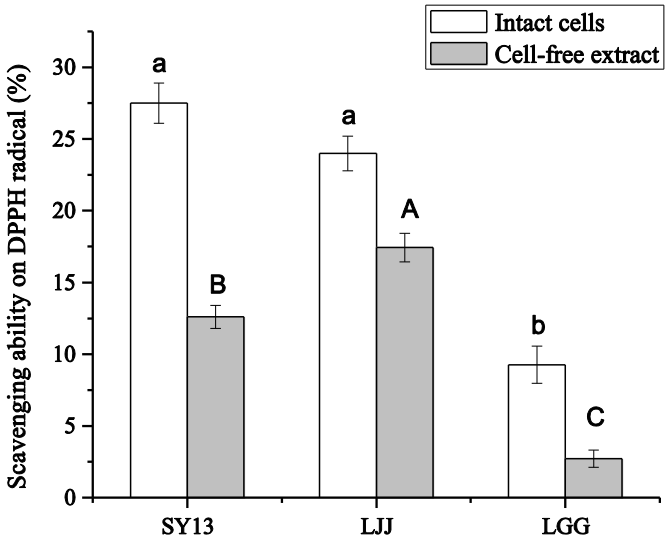

Figure 1. Scavenging ability of SY13 and LJJ on DPPH radical.

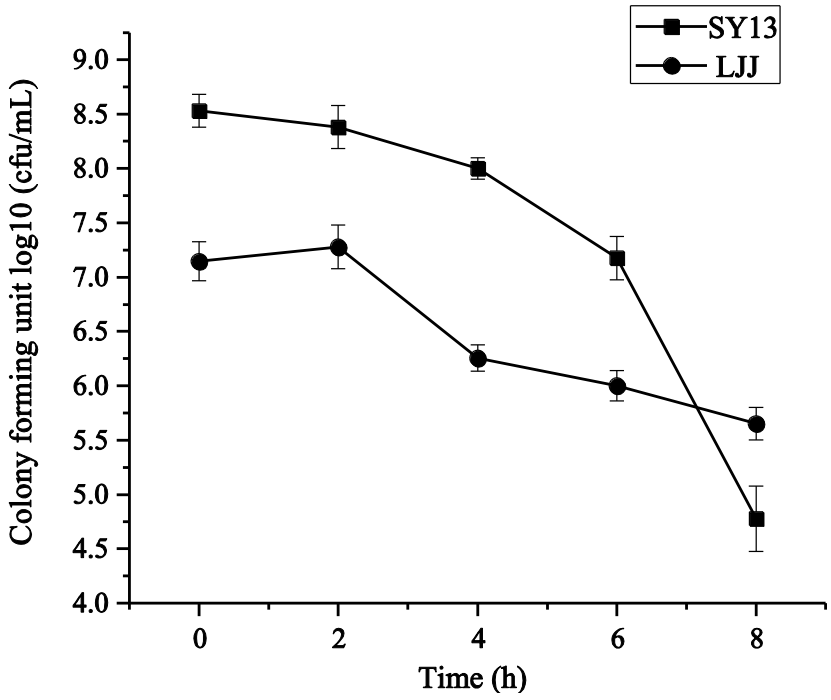

Figure 2. Survival of intact cells of two strains in the presence of 1 mM hydrogen peroxide. At 2 h time intervals, the number of viable cells was estimated by the plating the removed aliquots onto MRS agar plates.

generated by using 1,2,3-Benzenetriol hydrate that was readily reduced to stable, but oxygen-sensitive, cation radicals.

As shown in Figure 3, the cell-free extract of SY13, LJJ and LGG possessed the scavenging ability on superoxide anion. Besides, the intact cells of SY13and LGG showed scavenging ability on superoxide anion. It means that the two strains were resistant to superoxide anions, and the values of intact cells and cell-free extract of SY13 and LJJ scavenging superoxide anion were siginificantly different ($P<0.05$).

Hydroxyl radical scavenging ability

Hydroxyl radicals are considered to be the most reactive

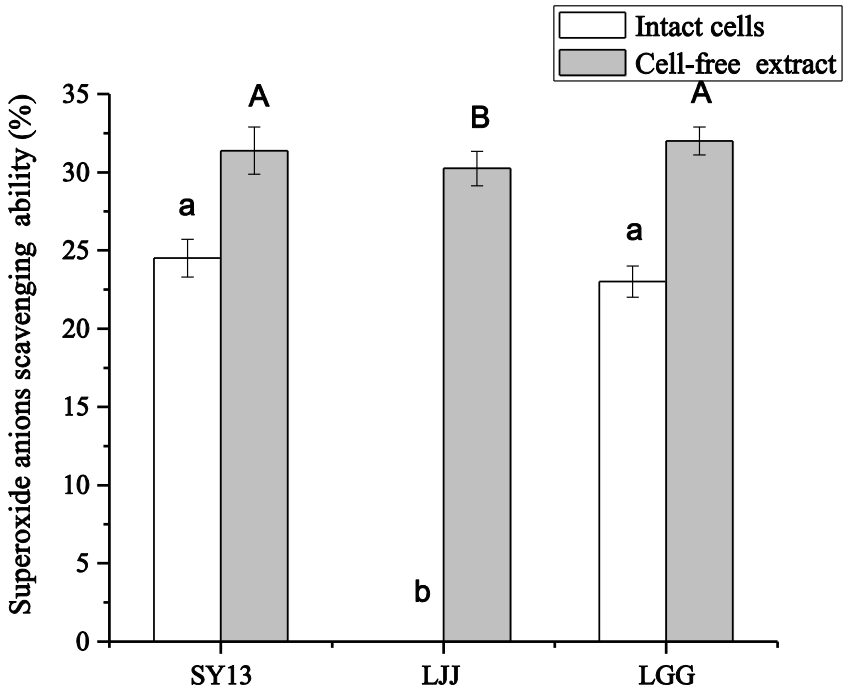

Figure 3. Scavenging ability of SY13 and LJJ on superoxide anion radical.

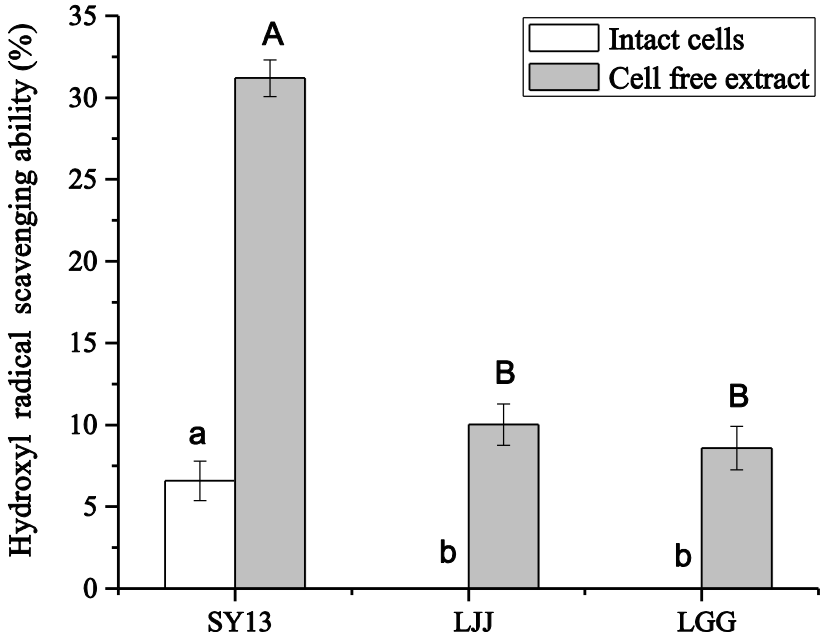

Figure 4. Scavenging ability of SY13 and LJJ on hydroxyl radical.

oxygen radicals, which can react with all biomacromolecules functioning in living cells and can induce severe damage to cells. Therefore, scavenging of hydroxyl radical plays a critical role in reducing oxidative damage. In this study, Fenton reaction system was used to determine the hydroxyl radicals scavenging capacity of the intact cells and cell-free extract of the strains.

As shown in Figure 4, the cell-free extract of SY13, LJJ and LGG possessed the eliminating ability on hydroxyl radicals. The cell-free extract of SY13and LJJ demonstrated excellent eliminating hydroxyl radical by 31.19 and 10.01%, and higher than that of LGG by

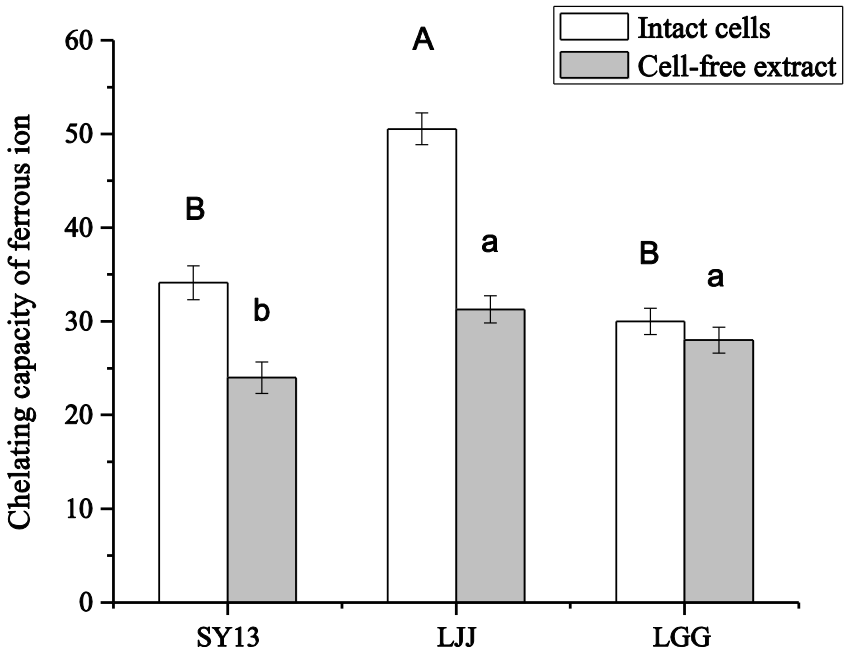

Figure 5. Chelating capacity of ferrous ion.

8.58%. The intact cells of SY13 also showed scavenging ability on hydroxyl radical. However, the intact cells of LJJ and LGG scavenging hydroxyl radical ability were not detected.

Measurement of the chelating capacity of ferrous ion (Fe^{2+})

Among the many metal ions that involved in catalyze oxidative reactions, iron ions are highly reactive, the most abundant, and important catalysts for ROS formation, resulting in the cell membrance damage. Therefore, the ability of the tested strains to chelate iron ions of the tested strains was investigated, and estimated the antioxidant activity.

From the Figure 5 to chelating ability, both the intact cells and cell-free extract of SY13, LJJ and LGG showed a wide range of Fe^{2+} chelating ability. And the chelating ability of the intact cells was higher than that of cell-free extract siginificantly. The intact cells of LJJ demonstrated the highest chelating ability by 50.55%, obviously superior to SY13.

Measurement of the reduction activity

Some enzymes (catalase, NADH oxidase, NADH peroxidase) and non-enzymatic compounds (glutathione, ascorbate, α-tocopherol) can minimize generation of ROS and control the transition metal ions to prevent oxidation reaction, which is called reduction activation. As shown in Table 2, two strains exhibited strong reducing capacity. The cell-free extract of SY13 showed higher reducing capacity than the intact cells, equivalent to 305 μM L-cysteine. However, the intact cells of LJJ showed higher reducing capacity than the cell-free extract, equivalent to 294 μM L-cysteine. The total reducing capacity of SY13 and LJJ are significantly different ($P< 0.05$), but values are not significantly different ($P>0.05$) between the intact cells and cell-free extract.

DISCUSSION

Two types of antioxidative pathways of lactic acid bacteria have been described: enzymatic and non-enzymatic defense systems. Antioxidant enzymes play a critical role in defense against ROS. Such as superoxide dismutase (SOD) eliminates direct toxicity of superoxide anions and glutathione peroxidase (GPx) scavenges hydrogen peroxide and hydroxyl radicals (Amanatidou et al., 2001b). However, to prevent excessive oxidative stress, cells and organisms had to develop non-enzymatic defense mechanisms, including reduction activity and the chelating capacity of metal ion, which can eliminate active oxygen (Saide and Gilliland, 2005 ; Lee et al., 2005).

The ability of *Lactobacillus* to scavenge free radicals has been shown previously. Lin and Chang (2000c) found that *B. longum* and *L. acidophilus* demonstrated antioxidative activity, inhibiting linoleic acid peroxidation by 28.3–33.2%, and also showed the ability to scavenge DPPH free radical, scavenging 43.2–52.1%. And the

Table 2. Reducing L-cysteine activity of strains SY13 and LJJ.

Strain	Viable cells(cfu/ml)	Cell morph	Equivalent L-cysteine (μM/l)
SY13	2.54×10^{9}	Intact cells	$215\pm0.8^{a,b}$
		Cell-free extract	$305\pm0.95^{a,b}$
LJJ	2.10×10^{8}	Intact cells	294 ± 1.21^{a}
		Cell-free extract	254 ± 0.68^{a}

[a,b]Presented values are means of triplicate determinations; ±indicates standard deviation from the mean.
[a,b]Mean values (±standard deviation) within the same column followed by different superscript letters differ significantly ($P < 0.05$)

inhibition rates on plasma lipid peroxidation rats of ranged from 11.3 to 16.2%. Amanatidou et al. (2001b) found that *Lactobacillus sake* possessed the inhibition on hydrogen peroxide, and its cell-free extract showed ability of scavenging hydroxyl radicals, chelating to ferrous ion and reducing activity. The tested *Lactobacillus fermentum* showed a high scavenging ability against DPPH, superoxide and hydroxyl radicals which was dose dependent (Wang et al., 2009). Our results confirm these findings as our data showed that SY13 and LJJ had significant ability to scavenge the DPPH, hydroxyl and superoxide radicals.

Over 50% of SY13 and LJJ intact cells were viable after 8 h of incubation in an environment of 1 mM hydrogen peroxide. The finding that intact cells are not particularly susceptible to hydrogen peroxide is not surprising given the fact that one of the mechanisms that allows lactobacilli to successfully compete in the gastrointestinal tract is their production of antimicrobial compounds such as organic acids, bacteriocins and hydrogen peroxide (Ouwehand and Vesterlund, 1998).

The finding of a high viability of SY13 and LJJ in hydrogen peroxide supported the findings of Talwalkar and Kailasapathy (2003) who found that *L.acidophilus* demonstrated no significant decrease in cell viability even after being exposed to 30 000 mg l^{-1} of hydrogen peroxide for 1 min. However, Kullisaar et al. (2002) found that *L. fermentum* E-3 survived only 180 min in the presence of 1 mM hydrogen peroxide. The reason for this apparent discrepancy may be due to differences in the initial number of lactobacilli or due to the different *Lactobacillus* strains used.

Lactobacillus strains also showed strain specific ability to chelate ferrous ion. Lee et al. (2005) found that *L. casei* KCTC 3260 seems to remove metal ions instead of increasing SOD level, resulting in blocking of the oxidative chain reaction. In our study, the ability of the tested strains to chelate iron was investigated. Our results showed a wide range of Fe^{2+} chelating ability, the highest chelating ability by 50.55%. Besides, the tested strains showed the excellent reducing ability. The highest reducing activity was equivalent to 305 μM and 294 μM L-cysteine, which contribute to the antioxidative activity of the strains.

It has been shown that some lactobacilli possess antioxidative activity and are able to decrease the adverse effect of ROS. *L. casei* subsp. *casei* SY_{13}, *L. delbrueckii* subsp. *bulgaricus* LJJ in this study may be candidate as an antioxidative strain that showed high resistance to ROS. Such antioxidative strains, with desirable properties, may be a promising material for both applied microbiology and scientific food industry, considering the fact that human microbiota have to be tolerant to endogenous and exogenous oxidative stress.

Conclusion

In conclusion, the *L. casei* subsp. *casei* SY13 and *L. delbrueckii* subsp. *bulgaricus* LJJ used in the present study exhibited significant free radical-scavenging capacity. This study suggests that two strains are high antioxidative bacterial strains. Antioxidative property of lactobacilli would be useful in the dairy manufacturing industry. They could beneficially affect the consumer by providing effective and security dietary source of antioxidants or by providing probiotic bacteria with the potential of producing antioxidants during their growth in the intestinal tract.

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