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ANTIOXIDANT PROPERTIES OF *LACTOBACILLUS* AND ITS PROTECTING EFFECTS TO OXIDATIVE STRESS CACO-2 CELLS

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ABSTRACT

This study was designed to analyse the antioxidant capacity of lactic acid bacteria (LAB) in order to provide characteristic parameters and theoretical basis for its further utilization as a natural antioxidant. Further experiment was carried out to study its protection mechanism through oxidative stress Caco-2 cell model in vitro. For this purpose, antioxidative activity of cells and different cellular fractions *of Lactobacillus delbrueckii* (ATCC7830) was evaluated by scavenging capacity of superoxide anion free radicals (SAFR)， reducing power and total antioxidant capacity assay. Low dose of H_2O_2 (100μmol/L) was used to establish oxidative damaged model on Caco-2 cells, the bacteria's protecting effect on the damaged cells was further researched. The results showed that extracellular secretions of *Lactobacillus delbrueckii* has the strongest antioxidant activity, following by cells, and cells can clear superoxide radicals. The ability to scavenge SAFR of this LAB was (42.4±0.32) % (cell lysates, the highest), the reducing power was (3.01±0.20, cell extracellular secretions, the highest), and the total antioxidant ability was (4.32±0.36, cell extracellular secretions, the highest). The above antioxidative character made this LAB possible to protect Caco-2 cells being damaged by low dose of H_2O_2. Further analysis showed that the total antioxidant capacity (T-AOC) of culture supernatant and lysate were significantly increased compared with the negative control group and the oxidative stress group (p<0.01). Also increased the anti-superoxide anion (O^{2-}) free radicals (p<0.01) level. All the results indicate that different fractions from lactic acid bacteria have various capabilities on reducing power, scavenging superoxide anion free radicals ability and total antioxidant capacity. Extracellular secretions have the most obvious antioxidant character. *Lactobacillus delbrueckii* could protect Caco-2 cells under oxidative stress by increasing its antioxidative function in vitro.

Key words: Antioxdiative, *Lactobacillus,* oxidative stress, Caco-2 cell, in vitro.

INTRODUCTION

Free radicals or reactive oxygen species (ROS), a class of highly reactive molecules, including SAFR (O^{2-}), HFR (OH^-), the hydrogen peroxide (H_2O_2) and singlet oxygen ($1O_2$) often generated as byproducts of biological reactions or from exogenous factors are believed to be involved in many health disorders. There is considerable evidence that reactive oxygen species (ROS) are important mediators of, or at least contribute to, the development of intestinal pathologies, inflammatory bowel disease and colon cancer, etc (Grisham, 1994; Valko *et al.*, 2001). ROS production and scavenging have been studied intensively in recent years. It has gradually get consensus that oxidative stress is a risk factor for chronic diseases and was previously shown to be independently associated with obesity (Wonisch *et al.*, 2012). Antioxidant-based drugs and formulations for the prevention and treatment of complex diseases have attracted a great deal of research interest in natural antioxidants. It is necessary to screen probiotics for their antioxidant potential. Various tests like reducing power, ferric reducing antioxidant power, linoleic acid assay, DPPH, nitric oxide, superoxide and hydrogen peroxide radical scavenging assays for evaluation of antioxidant activity were carried out in vitro (Ravi Narayan Venkatachalam, 2012).

Lactobacillus (Lactic Acid Bacteria /LAB) are commensal bacteria of oral, gastrointestinal tract and female genitourinary tract, and are well known for their popular use in industrial food fermentations based on their excellent probiotic properties. They can be acquired through the intake of probiotics or fermented foods (eg, cheese, yogurt, olives) (Borriello *et al.*, 2003). Probiotics are live organisms that confer a health benefit to the host when administered in adequate amounts (Yu *et al.*, 2007), the most abundantly used strains coming from the genus *Lactobacillus*. A study on *lactic acid bacteria* and *bifidobacteria* has shown that implementation of probiotic strains may increase health benefits (Parker, 1974). LAB provide several potential health and nutritional benefits, including improving the nutritional value of food, controlling gastrointestinal infections, improving digestion of lactose, controlling serum cholesterol levels, and controlling some types of cancer (Ouwehand *et al.*, 2002; Saxelin *et al.*, 2005) . These

health benefits derive from a diverse range of biological activities and mechanisms (Parvaneh *et al.*, 2014). Some LAB strains have been used as probiotics due to their resistance to host gastrointestinal conditions, adhesion to host intestinal epithelium and prevent the growth or invasion of pathogenic bacteria into the animal intestine (Chiu *et al.*, 2007). Most important LAB belongs to the genera *Bifidobacterium* and *Lactobacillus*. moreover, species belonging to the genera *Lactococcus*, *Enterococcus, Saccharomyces* and *Propionibacterium* are also considered due to their health-promoting effects (Blandino *et al.*, 2003; Silva *et al.*, 2013). Present results indicate that LAB is a good candidate for development of new nutraceuticals. However, little is known about the antioxidant potential of *Lactobacillus delbrueckii* (ATCC7830), even the effect of these microorganisms with the gastro-intestinal epithelia cells under oxidative stress. In order to establish the potential health benefit of probiotic candidates/nutritional substitutes, appropriate tests in vitro are required particularly on their antioxidant capacity.

Caco-2 is derived from a well-developed colon ade-nocarcinoma that spontaneously differentiates on confluence to exhibit features indicative of normal fetal ileal-like epithelium. Such features include polarization of the monolayer, development of brush borders, and enhanced alkaline phosphatase, sucrose isomaltase, and aminopeptidase activities (Pinto, 1983). So Caco-2 monolayers are routinely used as a good in vitro model to study intestinal oxidative stress (Bestwick and Milne, 2001).

Above all, the aim of the current work was to analyse the antioxidant capacity of *Lactobacillus delbrueckii* (ATCC7830) so to provide characteristic parameters and theoretical basis for its further utilization as a natural antioxidant. Further experiment was carried out to study its protect mechanism to oxidative stress Caco-2 cell model in vitro.

MATERIALS AND METHODS

Chemicals and kits: Total antioxidant capacity (T-AOC) kit were obtained from Jiancheng Bioengineering institute (Nanjing, China). DMEM (Dulbecco's Modified Eagle's Medium) high glucose cell culture medium was product of Gibco, USA. All other regents were of the analytical purity, commercially available.

Bacterial strain and preparation: The probiotic bacteria *Lactobacillus delbrueckii*（ATCC7830） was preserved by biotechnology Lab. Dalian medical university, China. The strain was cultured in MRS medium (Man, Rogosa and Sharpe Medium) (Merck, France) and cryopreserved at -80℃ with 15% glycerol. The bacteria was sub cultured twice aerobically in MRS broth at 37℃ for 20h before experimental use. Bacterial cells were collected by centrifugation at 1,0000rpm (revolutions per minute) for 8 min, washed three times with sterile phosphate buffered saline(PBS，pH7.4) and resuspended in sterile 0.9% NaCl solution to final concentration of 1×10^9CFU/ml viable bacterial cells per milliliter. Cell lysates were obtained through break the previous cells by Ultrasonic Wave Cell Pulverizer (JY96-Ⅱ, China). Supernatant collected through the above centrifugation was extracellular secretions sample. They all prepared for the further measurement of reducing power、total antioxidant capacities (T-AOC) and the ability to clear superoxide anion free radical of extracellular secretions、cell lysates and intact cells.

Cell cultivation and intervention: Caco-2 cells was obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cells were routinely cultured in DMEM high glucose medium, which contained 2mM L-glutamine, 10% FBS(Fetal Bovine Serum), 100IU/ml penicillin, 100g/l streptomycin at 37℃， under 5% CO_2 incubator(Thermo, USA). Medium was changed every 2-3 days, and the cells were passaged once they reached approximately 80% confluence. Before intervention, Caco-2 cells identically cultured in 6 well tissue culture plate were randomly divided into three groups, each carried out in triplicate. Change the old culture medium (containning penicillin-streptomycin) with the fresh medium(no penicillin-streptomycin) and the total volume is equal: control group (0 μmol/L H_2O_2) and oxidative stress group(100μmol/L H_2O_2)，treatment group(added with *Lactobacillus* (at final concentration of 1×10^9CFU/mL) in the condition of oxidative stress group)， respectively. After incubation for 18 and 42 hours, each antioxidative activities were measured in culture supernatant and lysate. Supernatant was obtained by transfer culture supernatant to a new collecting tube directly; washed the adherent cells three times with Hank's Balanced Salt solution（Gibco，USA）quickly, blowed well after adding 1ml Triton X-100（1%） into each well，centrifuged 15 minutes at 2000rpm ， the supernatant was the sample of cell lysate.

Reducing power activity: The reducing power was determined according to the modified method of Oyaizu (Oyaizu, 1986). A concentration of sample under test (0.5ml intact cell, cell lysate or extracellular secretions) was mixed with 0.5ml of 0.2M sodium phosphate buffer (pH 6.6) and 0.5ml of 1% potassium ferricyanide (w/v), and the resultant mixture was cooled rapidly after incubated at 50℃ for 20 min. The mixture was centrifuged at 3000 rpm for 5 min after adding 0.5 ml of 10% trichloroacetic acid (w/v). The upper layer (1ml) was mixed uniformly with 1ml of 0.1% ferric chloride (w/v), still 10 minutes, and the absorbance at 700 nm was measured with the blank of double-distilled water: higher

absorbance indicates higher reducing power. The assays were carried out in triplicate and the results are expressed as means ± standard deviation.

Determination of T-AOC (Total Antioxidant Capacity): Many antioxidants in organism, can make Fe^{3+} reduct into Fe^{2+}, which can combine with ferrozine to form solid chelation showing various color. Base on this theory, antioxidative ability can be determined by chemical colorimetry. In this work, total antioxidant capacity was determined according to the modified method of Dinis (Dinis *et al.*, 1994). All the operation were proceeded according to the specifications requirements strictly. Briefly, the reaction was initiated by the addition of 0.2 ml of ferrozine, vortexed thoroughly and the absorbance at 520 nm against blank was read after 10 minutes. The results were expressed as unit per milligram sample protein: Absorbency value of the reaction system increases 0.01, in every minute, every miligram sample protein, 37℃, called one T-AOC unit. T-AOC was calculated according to the following equation:

$$\text{T-AOC (U/mg protein)} = \frac{A_{sample} - A_{control}}{0.01} \div 30 \times \frac{\text{Total reaction volume}}{\text{Sample volume}} \times \text{dilution multiple} \div \text{protein content (mg)}$$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents, except the sample solution), and A_{sample} is the absorbance of the test sample. Coomassie(Bradford) protein assay was adopted to determine protein concentration. Tests were carried out in triplicate.

Scavenging superoxide anion free radical (SAFR) activity: Superoxide anion free radical (SAFR) scavenging ability was determined by a spectrophotometric method. SAFR was produced by 1, 2, 3-benzentriol autooxidation systems under alkaline condition. Each sample solution (0.1 ml) was mixed with 4.5 ml of Tris-HCl buffer (0.05M, pH8. 2) preheated at 25℃ constant temperature for 20 minutes, adding 0.4ml 1, 2, 3-benzentriol (0.25M, 25 ℃ preheated) into the mixture. The mixture was shaken vigorously and left to react for 4 minutes in 25℃ thermostatic bath. Reaction was terminated by adding 2 drops of hydrochloric acid (8M) and the absorbance was then measured at 320 nm against a blank by a spectrophotometer. Inhibition of free radical, SAFR, in percent (I%) was calculated according to formula:

$$I\% = (A_{control} - A_{sample}) / A_{control} \times 100\%;$$

where $A_{control}$ is the absorbance of the control reaction (0.05M Tris-HCl buffer, pH8. 2), and A_{sample} is the absorbance of the test sample. Tests were carried out in triplicate.

Data analysis: All data for biochemical analyses were statistically analyzed using the Statistical Product and Service Solutions 10.0 software (SPSS10.0) . To determine whether there were any differences between activity of samples, one-way analysis of variance (ANOVA) was applied and $p < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Antioxidative activities of cells and different cellular fractions of LAB: Figure1. shows the reducing power of cells and different cellular fractions of lactic acid bacteria. In this assay, the yellow color of the test solution changes to green or blue depending on the reducing power of each component. The presence of reducers in the test solution results in reduction of the Fe^{3+}/ferricyanide complex to ferrous form. As a general rule, sample reductive ability and oxidation resistance present linear correlation. In this study, the reducing power of three components was excellent, mean value were 0.899, 1.108, and 3.01, respectively. The reducing power of extracellular secretion was the highest, cell lysate take the second place.

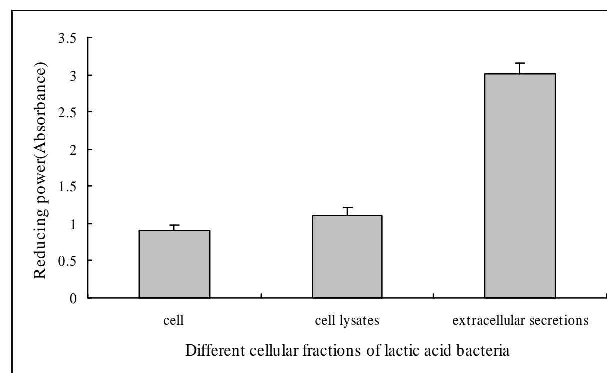

Figure 1. Reducing power of cells and different cellular fractions of lactic acid bacteria

Antioxidative ability, the most important organism member of defense sysetem, is closely related with health. Many factors, such as hunger, trace elements absorptive power, age and hormone level etc, influence the function of body's defense system. This hypofunction often lead to the happenning of various disease. It is easily to cause inflammation, cancer, diabetes, immune system and other diseases when the body's total antioxidant capacity reduced. Therefore determine the level of the total antioxidant capacity has important significance. In the total antioxidant capacity assay, standard curve of protein concentration was designed for Coomassie (Bradford) protein assay. Further conversion was done under the guidance of the equation to obtain final unit data of T-AOC. Result showed (Figure 2.) that extracellular secretions (4.32U) had the highest total antioxidant ability, whereas the intact cell (0.65) had the

lowest total antioxidant ability, cell lysate (1.52U) in the middle level.

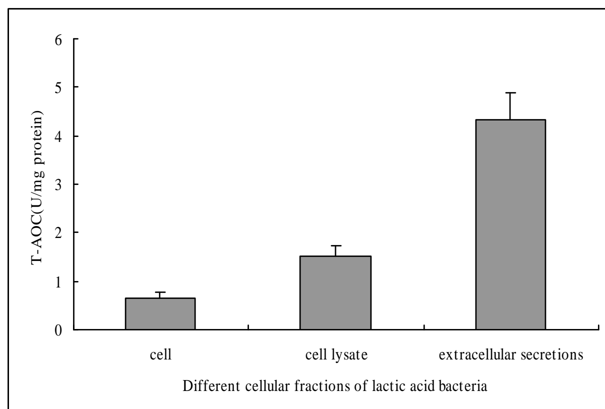

Figure 2. T-AOC of cells and different cellular fractions of lactic acid bacteria

SAFR and HFR are the most important ROS that play a key role in our body. They can proceed oxidation reaction with a variety of substance, cause a series of chain reaction and create new free radicals, further strengthens the oxidation reaction. Therefore, study about the medicine which can scavenge free radical has vital significance. Superoxide anion free radical also has been widely used to determine free radical-scavenging ability (Craft *et al.*, 2010). Figure 3. shows the SAFR scavenging ability of cells and different cellular fractions of lactic acid bacteria. In this assay, the scavenge percent (I%) was obtained by the ration between absorbance difference ($A_{control}$-A_{sample}) and control absorbance. Result showed that scavenge SAFR activity of three components was close to each other, mean value were 40.4%, 42.4%, and 41.2%, respectively.

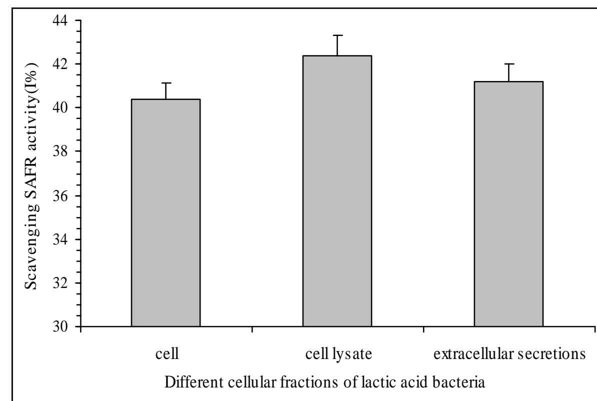

Figure 3. Scavenging SAFR activity of cells and different cellular fractions of lactic acid bacteria

Effect of *Lactobacillus* on oxidative stress Caco-2 cells: Effect of *Lactobacillus* on oxidative stress Caco-2 cell showed in Table 1. and Figure 4. as follows: compared with the control and the oxidative stress group the group added with *Lactobacillus* increased the total antioxidative capacity(T-AOC) significantly in cultured supernatant of Caco-2 cells at 18 and 42 hours(p<0.01)； also it increased the superoxide anion(O^{2-}) clear ability (p<0.01). The same results emerged in cell lysates of Caco-2 cells at 18 and 42 hours. These results indicate that the strains of *Lactobacillus* can increase the antioxidative function of Caco-2 cells under oxidative stress.

Table 1. Antioxidative activities of cell supernatant and lysates after 18 and 42 hours

Sample/ Object	Time	Index	control group	oxidative stress group	treatment group
cell supernatant	18 hours	T-AOC (U/mg protein)	1.12±0.11	24.33±3.01	42.37±3.56*
		Superoxide anion clear ability (I%)	1.82±0.21	9.92±1.04	19.11±2.21*
	42 hours	T-AOC (U/mg protein)	5.91±0.47	13.63±1.37	36.58±3.14*
		Superoxide anion clear ability (I%)	5.31±0.68	10.58±1.34	16.89±1.96*
cell lysates	18 hours	T-AOC (U/mg protein)	0.14±0.07	2.96±0.34	18.41±2.02*
		Superoxide anion clear ability (I%)	1.06±0.14	2.85±0.46	7.30±0.98*
	42 hours	T-AOC (U/mg protein)	2.83±0.32	6.37±0.88	13.91±1.76*
		Superoxide anion clear ability (I%)	^	0.27±0.03	0.52±0.09*

* p<0.01, Compare with correspondence control and oxidative stress group.
^Data lower the minimium threshold, not detectable.

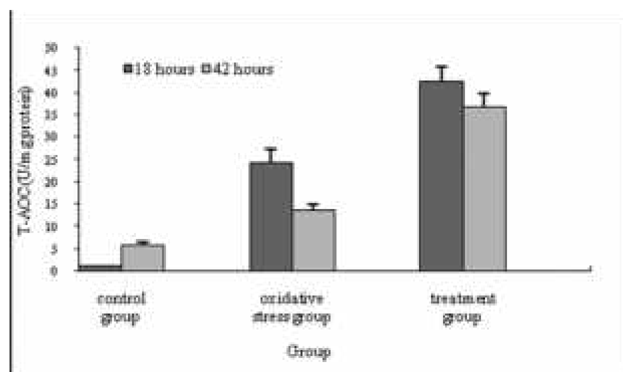

Figure 4. T-AOC of cell supernatant after 18 and 42 hours

Since the conceiving of free radical theory by Denham Harman in the 1950s, people gradually realized that oxygen free radicals produced during normal respiration would cause cumulative damage which would eventually lead to organismal loss of functionality, and ultimately death. Free radical damage within cells has been related to a range of disorders including cancer, atherosclerosis, Alzheimer's disease, and diabetes (Halliwell, 1994). More recently, the relationship between disease and free radicals has led to the formulation of a more generalized theory about the relationship between aging and free radicals. Almost all organisms are well protected against free radical damage by enzymes, such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherols and glutathione (Mau *et al.*, 2002; Hazra *et al.*, 2010). So in the physical status, the generation and clearance of ROS is in low levels of equilibrium state. Although organism itself have defense system that have evolved to protect them against antioxidant, but these systems are insufficient to resist or repair the damage brought by oxidation entirely (Simic, 1988). Oxidative stress is rightly caused by an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage and can induce diseases. However, antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage (Kullisaar *et al.*, 2002).

It was reported previously that probiotics play a key role in promoting intestinal health and preventing diseases, Their metabolic products, such as vitamin C, vitamin E, beta-carotene, polysaccharide, glutathione, superoxide dismutase (SOD), prototype coenzyme I (NADH) and some unknown substance, etc, are of great antioxidant activity (Dinis *et al.*, 1994; Craft *et al.*, 2010). In the acid environment of stomach probiotics can still keep the higher activity, so to improve the production performance of livestock and poultry, maintain intestinal balance, enhance the digestion rate. But little was studied about the antioxidant function of lactobacillus in vitro both at home and abroad. Our research showed that the LAB strain we used exhibit certain reducing power, scavenging superoxide anion free radicals ability and total antioxidant capacity. And the cell component of extracellular secretions has the most obvious antioxidant character. Also it can protect Caco-2 cells under oxidative stress by increasing its antioxidative function in vitro through eliminated excessive intracellular ROS by superoxide dismutase (SOD). *Lactobacillus* ATCC7830 may become the valuable candidate of probiotics. This could provide some basis for the further research and utilization of this LAB. Study on Lactic acid bacteria oxidation mechanism has important significance in screening high oxidizing *Lactobacillus* strain, developing new natural antioxidant and health foods, etc.

Conclusion and Recommendations: The current study revealed that tested *Lactobacillus* ATCC7830 showed a significant antioxidant property typical to those reported in the literature. Also it indicated the safety use of this LAB. It succeeded to prevent the hazards effects of H_2O_2 to Caco-2 cells. The protective role of this strain may be due to its antioxidant properties. Our study provide characteristic parameters and theoretical basis for its further utilization as a natural antioxidant.

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