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Full Length Research Paper

Cytotoxicity assay and antioxidant activities of the lactic acid bacterial strains

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The present study was designed to evaluate the safety degree of lactic acid bacterial (LAB) usage on mammalian cells and anti-oxidative ability of their intact cells, and both extra-and intra-cellular extracts. The non-toxic doses of eleven different LAB strains were detected by cytotoxicity assay test on peripheral blood mononuclear cells (PBMC) using neutral red stain. The antioxidant properties of LAB strains were evaluated biochemically using OH^- scavenging assay, superoxide scavenging assay, hydrogen peroxide scavenging assay, DPPH radical scavenging assay, reducing ability assay, inhibition of lipid peroxidation assay, and inhibition of human blood erythrocyte haemolysis assay. In addition, *in-vitro* antioxidant evaluation was carried out by using Flow Cytometric assay, on which the ROS produced by the stress factor *Helicobacter pylori* LPS on PBMC. The cytotoxicity results clarified that the safe doses of LAB extracellular extracts reached up to 20% while, the intracellular extracts safe doses reached up to 14%. Out of eleven tested LAB strains, *Lactobacillus plantarum*, *Lactobacillus bulgaricus* DSMZ 20080 and *Lactobacillus acidophilus* showed maximum free radical scavenging abilities at two levels: at intact cell level and at extract levels. In addition, *in-vitro* assay showed that ROS scavenging ability reached up to 100% in case of using mixture of both extracts of these strains.

Key words: Lactic acid bacteria, antioxidant, DPPH, flow cytometer.

INTRODUCTION

As we age, our bodies produce less endogenous antioxidant defenses (superoxide dismutases, H_2O_2-removing enzymes, metal binding proteins) to combat the ongoing destruction caused by free radicals. This increases our risk of developing serious health problems. For instance in diabetes, increased oxidative stress which co-exist with reduction in the antioxidant status has been postulated: Oxygen free-radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure membranes, and play a role in the long term complication of diabetes (Sabu and Kuttan, 2002; Boynes, 1991; and function of collagen basement and other Collier et al., 1990). Similarly, in carcinogenesis, reactive oxygen species are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma (Tsao et al., 2004). Hence, therapy using free-radical scavengers (antioxidants) has potential to prevent, delay or ameliorate many of these disorders (Delanty and Dichter, 2000). At the moment, the most important frequently used antioxidant food sources are LABs (Kullisar et al., 2003; Sinyavskiy, 2005; Villani et al.,

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2005, Grajek et al., 2005) and medicinal plants (Halliwell, 2008, Zhu et al., 2004). It has been given high priority in the production of LABs and the extraction of bioactive components from plant materials by enzyme and fermentation technology to reduce loss of these compounds as well as by genetic engineering to intensify their biosynthesis. Most lactic acid bacteria have systems to cope with oxygen radicals. According to Stecchini et al. (2000) the most common systems are superoxide dismutase and high internal concentrations of Mn2+. Knauf et al. (1992) also reported that some species of lactobacilli produced a heme-dependent catalase, which can degrade H_2O_2 at a very high rate, blocking the formation of peroxyl radicals. The ability of lactic acid bacteria to create low oxidation-reduction potential needed for their optimum growth probably is related to some of these systems.

The aim of this work was to compare the LAB in view of their antioxidant activity. Eleven strains were selected with respect to: popularity, and the conventional use.

MATERIALS AND METHODS

LAB strains

Streptococcus thermophilus, *Streptococcus lactis* subspecies *Cremoris*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *L. delbrueckii* subspecies *bulgaricus* DSMZ 20080 and 20081 T, *Lactobacillus fermentum* DSMZ 20049, *Lactobacillus acidophilus* DSMZ 20079 T, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* subsp. *plantarum* DSMZ 20174 and *Bifidobacterium longum* subsp. *Longum* DSMZ 200707, were collected from culture collection of faculty of agriculture Ain Shams University, faculty of agriculture Kafr El-Sheikh University and faculty of science Tanta University.

Preparation of intracellular and extracellular extracts

LAB were cultured in De Man-Rogosa- Sharpe (MRS) (Biokar Diagnostics, Beauvais, France), *Streptococcus thermophilus*, Reinforced clostridial (RCM) and modified MRS agar media and incubated at 30^0C under aerobic and anaerobic conditions; cultures were collected at the early stationary phase of incubation and centrifuged at 10,000 rpm at 2^0C for cells re-suspended in PBS followed by ultrasonic disruption in five 1-min intervals in an ice bath. Cell debris were removed by centrifugation at 10,000 rpm at 2^0C for 10 min, and the resulting supernatant was used as the intracellular extract, both extracellular and intracellular extracts stocks were filtered through a 0.22 µm pore-size filter (Millipore, Bedford, Mass.) and stored frozen at -80^0C till used. The total cell numbers were adjusted to10^9 CFU/mL for the preparation of both extracellular and intracellular extracts.

Cell lines

Peripheral blood mononuclear cells (PBMC) are the primary source of lymphoid cells for investigation of the human immune system. Its use is facilitated by Ficoll-Hypaque density gradient centrifugation (Berthold, 1981). Cells were cultured in RPMI medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS) and 1% penicillin-streptomycin solution in flat bottom 6-12-24 or 96 well plates, incubated at 37 °C in a humidified atmosphere of 5% CO_2, 95% air for 24 h.

Cytotoxicity assay

For the determination of treatments concentration that does not exert a toxic effect on PBMC cells, the cytotoxic assay was performed. A cell suspension of 6 × 10^4 cell/ml was collected and seeded in 96-well plates (100 µl cell suspension per well). The plates were incubated at 37^0C in humidified 5% co_2 for 24 h, the exhausted old medium were discarded and 100 µl of different treatment concentrations (previously prepared in RPMI) or medium (as a negative control) were added. The cell plates were incubated at the same growth conditions for 3 days. After 3 days, culture medium was discarded, 100 µl of neutral red stain (100 µg /ml) was added to each well and incubated for 3 h at 37^0C in humidified 5% CO_2 (Borenfreund and Puerner, 1985). Only living cells are permeable to neutral red and incorporated it into liposomes providing a quantitative assay to the cytotoxic effects. Excessive dyes were discarded and the stained cells were fixed with 100 µl fixing solution (0.5% formalin with 1% $CaCl_2$) for 1 min, then cells were destained in 100 µl destaining solution (50% ethanol with 1% acetic acid glacial) for 5 min by shaking. The stain intensity was assayed using automated ELIZA microplate reader adjusted at 540 nm (reference filters 620 nm).

Antioxidant activities of the LAB bacterial strains

5-1-Hydroxyl radical scavenging capacity of the LAB strains

OH^- scavenging assay was performed according to a literature procedure of (Wang et al., 2008). The scavenging activity was calculated using the following Equation (1):

Scavenging or inhibition rate % rate = [1 - (A1 -A2)/A0] × 100

where A0 is the absorbance of the control (without sample), A1 is the absorbance of the sample addition and A2 is the absorbance without sodium salicylate.

5-2- Superoxide scavenging capacity of the LAB strains

0.2 ml of bacterial treatments was added to 5.7 ml of 50 mM Tris–HCl buffer (pH 8.2). The mixture was incubated at 25^0C for 10 min and 0.1 ml of 6 mM pyrogallol (25^0C) was added. The absorbance of the reaction mixture was measured at 320 nm every 30s. until the reaction proceeded to 5 min (the same concentration treatments were used as the blank to eliminate interference). O^-_2 scavenging activity was expressed by the oxidation degree of a test group in comparison to that of the control. The percentage of inhibition effect was calculated according to Equation (1), where A0 is the absorbance of the Tris–HCl buffer with pyrogallol, A1 is the absorbance of the treatment addition and A2 was the absorbance of blank, (Su et al., 2009).

5-3- Hydrogen peroxide scavenging capacity of the LAB strains

Hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Bacterial treatments were added to 0.6 ml of hydrogen peroxide solution, then the reaction absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide, (Soares et al., 2009). The percentage of inhibition effect was calculated according to Equation 1.

5-4- 2, 2-diphenyl-picrylhydrazyl radical scavenging activity of the LAB strains

The capacity of the LAB bacterial treatments to reduce the 2, 2-diphenyl-picrylhydrazyl (DPPH) stable free radical was assessed using the method in a literature (Vitalini et al., 2006). The percentage of DPPH scavenging activity was calculated according to absorbance difference, as has been depicted in Equaton 1, where A0 is the absorbance of DPPH alone, A1 is the absorbance of DPPH + sample and A2 is the absorbance of the sample only.

5-5-Reducing ability of the LAB bacterial strains

The reducing ability of LAB bacterial treatments was assayed as described in a reference (Tsai et al., 2006). The reducing ability was weighed by the absorbance value of the reaction solution and a higher absorbance indicated increased reducing power (Jayaprakasha and Bhimanagouda, 2007).

5-6- Inhibition of lipid peroxidation in rat liver homogenate by the LAB strains

The inhibition effect on lipid peroxidation was determined according to the thiobarbituric acid method. FeCl2– H_2O_2 was used to induce the liver homogenate peroxidation (Yen and Hsieh, 1998). The absorbance was recorded at 532 nm (Buege and Aust, 1978). The percentage of inhibition effect was calculated according to Equation1, where A0 is the absorbance of the control (without sample), A1 is the absorbance of the sample addition and A2 is the absorbance without liver homogenate.

5-7- Inhibition of erythrocyte haemolysis in human blood by the used LAB strains

The blood sample was obtained and made to 0.5% erythrocyte suspension for the assay. The reaction mixture was consisted of 1.0 ml of erythrocyte suspension (0.5%), 1.0 ml sample and 0.1 ml H_2O_2 (100 mM). The mixture was incubated at 37^0C for 60 min, and then four times volume distilled water was added to the mixture and centrifuged at 1000 rpm for 10 min (Su et al., 2009). The absorbance of the supernatant was read at 415 nm. The percentage of erythrocyte haemolysis inhibition effect was calculated according to Equation1, where A0 is the absorbance of the supernatant without sample, A1 is the absorbance of the sample addition and A2 was the absorbance of sample solution.

5-8-Detection of induced intracellular ROS in cell line by *Helicobacter pylori* lipopolysaccharide

Isolation of *Helicobacter pylori* lipopolysaccharide

In order to isolate *H. pylori* lipopolysaccharide (LPS) that has an essential role in free radicals and immune response induction, a modified method by Uchida and Mizushima (1987) was used. Previous isolated bacteria were microaerophilic cultured at 37^0c for 24 h in brucella broth supplemented with 10% fetal bovine serum after incubation; cells were collected by centrifugation at 10,000 rpm and 2^0C for 30 min and then washed with sterilized PBS, the cells were lyophilized. LPS was extracted from the lyophilized cells in three steps: recovery of LPS in insoluble form together with some denatured proteins, solubilization of LPS and Precipitation of LPS.

The total intracellular ROS generated during *H. pylori* infection was detected in PBMC using 2`, 7`-dichlorofluorescein diacetate (DFCH-DA) method. DFCH-DA enters cells and further oxidized by ROS forming fluorescent product (DFC). PBMC cells were grown in 96 well plates in complete RPMI medium at 37°C in humidified 5% CO_2 for 24 h, 100 µl of the recorded non-toxic doses of both intracellular and extracellular lactic acid bacterial extracts were added to cells and incubated at the previous incubation conditions for 24 h, after that, the treatments were removed and cells were washed again with prewarmed PBS. Cells were incubated with DFCH-DA at a final concentration 50 µM for 30 min, after incubation, ROS was induced by loaded cells with *H. pylori* LPS in RPMI. In the control groups, 100 µl RPMI was added. After incubation for 1 h, the stimulants were discarded and cells were washed three times with prewarmed PBS, all cell samples were analyzed using a BD FACSCalibur flow cytometer (Hafer et al., 2008) with CellQuest software. Cells were excited at 485 nm and DCF fluorescence was read on FL1 (530 nm) in log scale with FL1 gain set to 443. The inhibition percentage of ROS production was calculated by formula:

$(F_0\text{-}Ft/F_0)\times100$

Where, F_0: fluorescence of the control sample; Ft: fluorescence of the treated sample.

RESULTS

Cytotoxicity assay

The cytotoxicity results (Tables 1 and 2) indicated that, generally, the extracellular extracts were safer than the intracellular extracts and have no TC_{50} even in the maximum concentrations; only the extracellular extract of *Bifidobacterium longum* has $TC_{50} \leq 18\%$. In spite of the all used bacterial strains listed in the same bacterial groups (lactic the extracellular extracts of acid producing bacteria), there are a huge varity in the cytotoxicity percentage among them. The safe doses of extracellular extracts ranged from 10 to 20% while, the intracellular extracts safe doses ranged from 6 to 14%.

OH^- Scavenging activity

As shown in Table 3, in case of the whole culture and extracellular extract, *Lactobacillus plantarum* has been recorded as the highest scavenging activity on OH^-. With ratio 90 ± 0.88% and 95 ± 0.33% respectively. For intracellular extract, the highest scavenging activity was recorded for *Streptococcus lactis* (89 ± 1.3%). Generally, the intracellular extracts of seven tested strains out of eleven have been highly contributed to the hydroxyl radical capacity.

O^-_2 inhibition activity

According to the methodology used, Spontaneity oxidation of pyrogallol will proceed in slightly alkaline condition and the O^-_2 was produced with absorption at

Table 1. Cytotoxicity assay of LAB extracellular extract on peripheral blood mononuclear cells (PBMC).

Treatments	Cytotoxicity percentage								
	2%	6%	8%	10%	12%	14%	16%	18%	20%
Streptococcus thermophilus	0.20	0.23	0.57	2.07	3.13	6.00	6.17	6.33	7.90
Lactobacillus rhamnosus	0.70	0.90	3.54	6.44	8.57	9.00	10.10	10.34	11.2
Lactobacillus casei	1.43	3.03	3.43	3.50	4.50	5.8	7.53	8.83	9.23
Streptococcus lactis	1.40	1.96	2.56	4.10	4.66	5.96	6.16	7.73	8.33
Lactobacillus bulgaricus DSMZ 20081 T	2.30	2.37	2.40	3.60	5.48	6.44	7.77	9.74	10.00
L. bulgaricus DSMZ 20080	2	4.53	4.468	10.1	13.2	19.2	26.13	28.8	33.2
Lactobacillus bulgaricus NCTC 12197 T	1.27	3.04	4.79	5.91	10.7	11.82	16.93	26.52	33.71
Lactobacillus acidophilus	0.87	6.00	7.23	8.60	9.00	10.57	11.23	11.63	12.34
L. fermentum	1.45	2.9	3.48	9.1	23.37	27.43	30.48	32.8	35.7
Lactobacillus plantarum	1.5	4.1	6.66	10	18.46	19.54	20	27.69	30.26
Bifidobacterium longum	2.56	4.918	6.55	10	18.03	34.975	36.75	55.19	59.016

Table 2. Cytotoxicity assay of the LAB intracellular extract on peripheral blood mononuclear cells (PBMC).

T treatment	Cytotoxicity percentage											
	2%	4%	5%	6%	8%	9%	10%	12%	14%	16%	18%	20%
Streptococcus thermophilus	1.62	1.81	2.007	2.41	5.29	6.41	7.521	8.75	**9.61**	14.85	17.47	24.54
Lactobacillus rhamnosus	0.3	1.55	3.425	5.3	9.71	12.11	14.5	27.48	31.69	34.03	37.52	41.07
Lactobacillus casei	7.59	8.64	10.2	19.1	21.73	28.93	36.13	40.84	41.62	41.88	42.93	47.38
Streptococcus lactis	2	3.5	4.75	6	8	9.2	10	12	14	16	18	20
Lactobacillus bulgaricus DSMZ 20081 T	2	2.75	4.37	6	8	9.5	10	12	14	16	18	20
L. bulgaricus DSMZ 20080	2	2.375	4.19	6	8	9.63	10	12	14	16	18	20
Lactobacillus bulgaricus NCTC 12197 T	1.47	2.36	2.77	3.17	5.21	9.31	14.6	16.77	20.06	23.809	25.96	28.004
Lactobacillus acidophilus	1.07	2.37	3.67	4.14	5.99	7.53	9.06	9.98	11.67	13.67	17.82	18.74
L. fermentum	2.28	5.13	7.96	9.11	12.5	17.27	22.04	33.66	37.05	38.22	39.41	41.59
Lactobacillus plantarum	0.589	1.93	3.23	4.79	5.98	7.14	8.3	10.47	38.32	42.15	48.62	51.4
Bifidobacterium longum	0.9	1.69	2.48	2.27	4.06	4.55	5.04	7.804	8.78	13.3	17.07	24.88

320 nm. The antioxidant can inhibit the spontaneity oxidation of pyrogallol but it has been found that the reaction is instable in 1 to 5 min. Repetitious tests showed the reaction was stable in 4 to 5 min and consequently the data at 5 min was adopted to evaluate the inhibition activity. The inhibition activity was higher in six *Lactobacillus* spp out of eight as shown in Table 4. *Lactobacillus fermentum* and *S. lactis* have been recorded for the highest capacity of intracellular extract and the later has the maximum O^{-}_{2} Scavenging activity for intact cells and extracellular.

Hydrogen peroxide scavenging activity

Generally, the higher hydrogen peroxide activity was recorded when the intact cells of eleven strains were assayed as whole culture. At the top of all tested strains, *Lactobacillus plantarum* showed high activity in all cases, where its whole culture, extracellular and intracellular extract recorded the maximum activities (Table 5).

DPPH radical scavenging activity

An incubation time-response was found in DPPH scavenging activity of lactic acid bacterial extracts, where an increase in incubation time is synonymous of an increase in scavenging capacity, this relationship prolonged till constant values in DPPH scavenging activity after 6 hours incubation (Table 6a). At the end of incubation (Table 6b), the higher activity was for extracellular extracts (ten strains out of eleven), both *Bifidobacterium longum and L. plantarum* had strong DPPH scavenging effect with percentages of 89.8 ± 0.03 and 89.8 ± 0.08 respectively. In case of the whole cultutre, *Lactobacillus bulgaricus* DSMZ 20081 T has been recorded for the highest DPPH radical scavenging

Table 3. Hydroxyl radical scavenging capacity of the LAB strains.

Strain	OH scavenging rate (%)		
	Culture	Extracellular extract	Intracellular extract
Streptococcus thermophiles	22c ± 0.88	59c ± 1.2	61b ± 0.33
Streptococcus lactis	69a ± 1.52	77a ± 0.88	89 ± 1.3
Lactobacillus bulgaricus DSMZ 20080	27b ± 1.15	60c ± 1.15	77a ± 0.88
Lactobacillus bulgaricus DSMZ 20081 T	22c ± 1.15	36 ± 0.57	75a ± 0.33
Lactobacillus bulgaricus NCTC 12197 T	68a ± 0.89	75a ± 0.88	61b ± 0.57
Lactobacillus fermentum	33 ± 0.57	70b ± 0.88	31 ± 0.57
Lactobacillus acidophilus	29b ± 1.11	50 ± 1.4	59b ± 0.88
Lactobacillus rhamnosus	18c ± 0.577	45 ± 0,88	80 ± 0.33
Lactobacillus casei	45 ± 0,573	70b ± 0.88	75a ± 0.35
Lactobacillus plantarum	90 ± 0,88	95 ± 0.33	62b ± 0.3
Bifidobacterium longum	68b ± 0.84	81b ± 0.33	45 ± 0.51
F value	598.246***	328.774***	567.763***

***. The F values are highly significant at the p≤ 0.001 level; a, b, c Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments), Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.

Table 4. Superoxide scavenging (O-2) capacity of the LAB strains.

Strain	O-2 scavenging rate (%)		
	Culture	Extracellular extract	Intracellular extract
Streptococcus thermophiles	58.65 ± 0.052	63.94 ± 0.026	60.67 ± 0.012
Streptococcus lactis	90.87 ± 0.006	95.19 ± 0.003	87.02a ± 0.027
Lactobacillus bulgaricus DSMZ 20080	53.36 ± 0.012	70.19 ± 0.018	75.96 ± 0.012
Lactobacillus bulgaricus DSMZ 20081 T	47.12 ± 0.017	78.58 ± 0.014	70.01 ± 0.031
Lactobacillus bulgaricus NCTC 12197 T	46.15a ± 0.015	30.16 ± 0.013	71.63b ± 0.008
Lactobacillus fermentum	63.5 ± 0.02	86.05 ± 0.016	87.98a ± 0.005
Lactobacillus acidophilus	28.85 ± 0.016	81.25 ± 0.028	85.09 ± 0.01
Lactobacillus rhamnosus	77.4 ± 0.066	71.15b ± 0.014	86.54 ± 0.018
Lactobacillus casei	46.15a ± 0.0152	71.63b ± 0.02	85.58 ± 0.005
Lactobacillus plantarum	74.04 ± 0.018	45.67 ± 0.01	71.63b ± 0.012
Bifidobacterium longum	59.13 ± 0.021	85.57 ± 0.026	83.65 ± 0.018
F value	483882.312***	1090868.02***	249345.06***

***. The F values are highly significant at the p ≤0.001 level; a, b Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments), Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.

activity.

Reducing ability assay

Table 7 showed a significant difference between the whole bacterial culture and both extracellular and intracellular extract, where the whole culture has been recorded for the highest reducing power. The only exception was for *Lactobacillus acidophilus* intracellular extract that recorded slightly higher reducing power than the whole culture. The highest record was for *B. longum*, with percentage 0.669 ± 0.00.

Inhibition of lipid peroxidation in rat liver homogenate

Lipid peroxidation is a well-known mechanism of liver injury induced by free radicals and malondialdehyde (MDA) is one of its end products. Thus, MDA is a good indicator of the degree of lipid peroxidation (Abado-Becognee K, 1998). $FeCl_2$–H_2O_2 system was used to induce lipid peroxidation in rat liver homogenate. In this assay, the potentiality of lactic acid bacterial extract as a strong antioxidant was confirmed where, they have strong inhibition for lipid peroxidation (low MDA concentrations) as seen on Table 8. The whole culture of

Table 5. Hydrogen peroxide scavenging capacity of the LAB bacterial strains.

Strain	H_2O_2 scavenging rate (%)		
	Culture	Extracellular extract	Intracellular extract
Streptococcus thermophilus	64.9 ± 0.03	46.3^{c} ± 0.012	60.4 ± 0.033
Streptococcus lactis	70.1^{a} ± 0.05	50.7^{b} ± 0.088	48.8 ± 0.057
Lactobacillus bulgaricus DSMZ 20080	71.6^{a} ± 0.17	47.8^{c} ± 0.57	46.1 ± 0.066
Lactobacillus bulgaricus DSMZ 20081 T	53.7 ± 0.066	46.9^{c} ± o.o33	53.2 ± 0.088
Lactobacillus bulgaricus NCTC 12197 T	71.3^{a} ± 0.088	67.9 ± .057	62.7^{a} ± 0.066
Lactoba cillus fermentum	51.4 ± 0.066	50.9^{b} ± 0.088	57.1 ± 0.088
Lactobacillus acidophilus	59.8 ± 0.12	59.7^{a} ± 0..057	58.2 ± 0.057
Lactobcillus rhamnosus	65.7 ± 0.088	59.7^{a} ± 0.033	64.9 ± 0.088
Lactobacillus casei	73.4 ± 0.12	67.2 ± 0.033	70.9 ± 0.033
Lactobacillus planta rum	76.9 ± 0.088	71.1 ± 0.057	75.4 ± 0.14
Bifidobacterium longum	66.4 ± 0.055	60.1^{a} ± 0.011	62.7^{a} ± 0.066
F value	7949.34***	71.089***	6659.49***

***. The F values are highly significant a t the p≤ 0.001 level; a ,b Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments), Values with different letters are significantly different (p < 0.05). Data are means ± SD of triplicate experiments.

seven tested strains gave higher percentage inhibition in comparison with both extracts (Table 8) and the top of this inhibition was for *L. fermentum* and *L. acidophilus* (83.33^{a} ± 0.034). The remaining four strains gave higher inhibition in case of intracellular extract where**,** the *Lactobacillus casei* was the highest one (91.6 ± 0.208).

Inhibition of erythrocyte haemolysis

Oxidant damage of cell film which induced by H_2O_2 can result in erythrocyte haemolysis. The current results showed that erythrocyte haemolysis was effectively inhibited by the whole lactic acid bacterial strains as shown on Table 9, where, nine strains out of eleven gave higher activity in case of intact cells. The higher inhibition of the whole culture was recorded for *Lactobacillus bulgaricus* NCTC 12197 T (83.6 ± 0.100). The extracellular extract of *Lactobacillus acidophilus* showed high erythrocyte inhibition percentage (85.6 ± 0.15) while, and the intracellular extract was obvious for *L. casei* (65.4 ± 0.20).

The effects of selected lactic acid bacterial treatments on ROS production using flow cytometric analysis

The mixture of extracellular extracts and intracellular extracts of the most potent free radical scavenging capacity treatments were analyzed by flow-cytometric analysis to detect the total induced ROS scavenging capacity (Figure 1). By combination, the ROS scavenging capacity of both *L. plantarum* and *L. bulgaricus* DSMZ 20080 reached 100% followed by *L. acidophilus*, *S. lactis* and *B. longum* with scavenging percentage ranged from 88.05 to 95.52% (Table 10).

DISCUSSION

The cytotoxicity assays

Acute oral toxicity testing is used to characterize the risk of hazard associated with human exposure to a substance. In October, 2000, the International Workshop on *in vitro* Methods for Assessing Acute Systemic Toxicity reviewed the validation status of *in vitro* methods directed toward reducing and refining the use of laboratory animals for acute toxicity testing (ICCVAM, 2001a). One approach considered was the use of *in vitro* cytotoxicity assays to predict acute *in vivo* lethality (Spielmann et al., 1999). One of the workshop recommendations for reducing and refining the use of animals for lethality assays in the near-term was the publication of guidance for using *in vitro* cytotoxicity assays to estimate the starting dose for acute oral lethality assays (ICCVAM, 2001b). The recommended publication provides details and examples on how to execute such an approach. Cytotoxicity assay was done to determine the non-toxic doses TC_0 (recommended doses) and the concentration killing 50% of the cells (TC_{50}) using serial dilutions of the extracts not exceed 20%; the safe doses should have maximum inhibition percentage not exceed 10% that is, the percentage of survival cells not less than 90%, neutral red (NR), a weak cationic supravital dye that readily penetrates cell membranes by non-ionic diffusion and predominately accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such

Table 6a. DPPH radical scavenging activity of the LAB strains.

Strain		Incubation time (h) DPPH scavenging rate (%)					
		1	2	3	4	5	6
Streptococcus thermophilus	Culture	13.8	28.5	35.7	39.3	60	60
	Extracellular extract	20	35	42	43	60	61
	Intracellular extract	1	25	30	30	30	30
Streptococcus lactis	Culture	32.5	33.9	35	62	70	70
	Extracellular extract	35	37	39	69	75	75
	Intra cellula r extra ct	25	30	35	70	70	70
Lactobacillus bulgaricus DSMZ 20080	Culture	3.5	14.2	44.6	62.5	76.6	76
	Extracellular extract	14	25	45	65	80	85
	Intra cellular extract	62.5	65	75	75	75	75
Lactobacillus bulgaricus DSMZ 20081 T	Culture	33.3	40	45	73.2	87.5	87.5
	Extracellular extract	20	45.4	46	75	89.6	88.5
	Intracellular extract	1	40	41	41	41	41
Lactobacillus bulgaricus NCTC 12197 T	Culture	46	50	51	70	70.3	70.5
	Extra cellular extract	46.3	50	55	73	75	78
	Intra cellular extract	1	37.5	50	50	50	50
Lactobacillus fermentum	Culture	10	16	50	83	85	85
	Extra cellular extract	30	35	55	83.3	85	85
	Intracellular extract	1	20	37.5	37.5	37.5	37.5
Lactobacillus acidophilus	Culture	37	60	75	82	86	86
	Extracellular extract	42	60.3	76	85	86	86.1
	Intracellular extract	30	60	65	65	65	65
Lactobacillus rhamnosus	Culture	10.7	20	53	64	84	86
	Extracellular extract	12	23.3	53.9	66	84.9	85
	Intracellular extract	10	12	50	50	50	50
Lactoba cillus casei	Culture	40	55	60	70	75	75
	Extracellular extract	44.3	56	62.3	70.9	76	76
	Intracellular extract	37.5	50	62	62	62	62
Lactobacillus plantarum	Culture	51.7	60.7	62.5	78.5	86.3	86.5
	Extracellular extract	58.9	64.2	64.9	78.9	89.9	89.8
	Intra cellular extract	50.3	59.6	60.3	78	78	78
Bifidobacterium longum	Culture	10.7	28.5	64.2	78.2	86	86.5
	Extracellular extract	29.2	30.5	64.4	80	89	89.8
	Intracellular extract	20	25	60	88	88	78

changes produced by toxic substances decreased the uptake and binding of NR, making it possible to distinguish between viable, damaged, or dead cells via spectrophotometric measurements.

The antioxidant activities

LAB considered as a bio active safe microorganisms that normally transit the gastrointestinal tract and colonized intestinal microbial ecosystem promoting host health (Chu-Chyn et al., 2009). Lactic acid bacteria 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. Although survival is higher for intestinal strains, certain numbers of these bacteria are lysed and

Table 6b. The final DPPH scavenging rate of LAB extracellular extract after 6 h incubation.

Strain	DPPH scavenging rate (%)		
	Culture	Extracellular extract	Intracellular extract
Streptococcus thermophilus	60 ± 0.70	61 ± 0.13	30 ± 0.0088
Streptococcus lactis	70^{c} ± 0.13	75 ± 0.12	70 ± 0.066
Lactobcillus bulgaricus DSMZ 20080	76^{b} ± 0.12	85^{b} ± 0.26	75 ± 0.13
Lactobacillus bulgaricus DSMZ 20081 T	87.5 ± 0,28	88.5 ± 0.10	41 ± 0.14
Lactobacillus bulgaricus NCTC 12197 T	70.5^{c} ± 0.28	78 ± 0.1.	50^{b} ± 0.11
Lactobacillus fermentum	85 ± 0.13	85^{b} ± 0.12	37.5 ± 0.28
Lactobacillus acidophilus	86^{a} ± 0.12	86.1 ± 0.16	65 ± 0.06
Lactobacillus rhamnosus	86^{a} ± 0.03	85^{b} ± .03	50^{b} ± 0.00
Lactobacillus casei	75^{b} ± 0.1	76 ± 0.43	62 ± 0.084
Lactobacillus plantarum	86.5^{a} ± 0.28	89.8^{a} ± 0.03	78^{a} ± 0.06
Bifidobacterium longum	86.5^{a} ± 0.28	89.8^{a} ± 0.08	78^{a} ± 0.08
F value	603.708***	1406.085***	15387.305***

***. The F values are highly significant at the p≤ 0.001 level; a, b, c Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments), Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.

Table 7. Reducing ability of the LAB strains.

Strain	Absorbance A 700		
	Culture	Extracellular extract	Intracellular extract
Streptococcus thermophilus	0.443^{a} ± 0.008	0.287^{a} ± 0.011	0.171^{a} ± 0.003
Streptococcus lactis	0.381 ± 0.009	0.207^{b} ± 0.008	0.276 ± 0.0088
Lactobacillus bulgaricus DSMZ 20080	0.576 ± 0.008	0.167^{d} ± 0.005	0.163^{a} ± 0.005
Lactobacillus bulgaricus DSMZ 20081 T	0.617 ± 0.006	0.168^{d} ± 0.005	0.1530.012
Lactobacillus bulgaricus NCTC 12197 T	0.647 ± 0.003	0.182^{c} ± 0.002	0.223 ± 0.008
Lactobacillus fermentum	0.456^{a} ± 0.005	0.281^{a} ± 0.008	0.205 ± 0.003
Lactobacillus acidophilus	0.346 ± 0.006	0.334 ± 0.006	0.376 ± 0.006
La ctoba cillus rhamnosus	0.304 ± 0,018	0.164^{d} ± 0.001	0.236 ± 0.014
La ctoba cillus casei	0.40 ± 0.005	0.248 ± 0.005	0.187 ± 0.008
Lactobacillus plantarum	0.546 ± 0.008	0.173^{cd} ± 0.008	0.244 ± 0.0011
Bifidobacterium longum	0.669 ± 0.006	0.180^{b} ± 0.011	0.290 ± 0.088
Control		0.160 ± 0.0066	
F value	541.059***	287.367***	537.017***

Higher absorbances indicated increased reducing power; ***. The F values are highly significant at the p≤ 0.001 level; a , b, c, d Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments), Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.

their intracellular extracts released into the gut (Isolauri et al., 2004). Therefore, it is of interest to compare the antioxidative ability of intact cells and both extra- and intracellular extracts of LAB strains. In this study, the free radical scavenging ability of lactic acid bacterial strains was first compared using biochemical methods and then *In-vitro* fluorescent method in mammalian cell line by fluorescent prob. As shown in results section, there were significant differences in scavenging ability among bacterial strains either as intact cells or both extracts (intra- and extra-cellular). This finding suggests that levels of the antioxidative factor contributed by each strain were not comparable. All of intact cells and intracellular cell-free extracts of all tested strains were effective in inhibiting the hydroxyl, superoxide and H_2O_2-induced oxidative damage and erythrocytes haemolysis. However, in most strains, the intact cells and intracellular extracts, in comparison with the extracellular extracts, 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. A similar finding has been reported by Saide and Gilliland (2005) that intracellular extracts of lactobacilli possessed markedly increased antioxidative activity than intact cells.

Table 8. Inhibition of lipid peroxidation in rat liver homogenate by LAB strains.

Strain	Inhibition of lipid peroxidation %			MDA concentration (µM/ml)		
	Culture	Extra. extract	Intra. extract	Culture	Extra. extract	Intra. extract
S. thermophilus	66.67[b] ± 0.015	46.66 ± 0.017	66.65[a] ± 0.25	0.41[a] ± 0.033	0.64 ± 0.058	0.4[a] ± 0.025
S. lactis	33.34 ± 0.03	33.34[c] ± 0.032	90 ± 0.025	0.80 ± 0.067	0.81[a] ± 0.033	0.095 ± 0.011
L. bulgaricus DSMZ 20080	56.67 ± 0.015	45[b] ± 0.25	60[b] ± 0.35	0.52 ± 0.077	0.66[b] ± 0.022	0.48[a] ± 0.089
L. bulgaricus DSMZ 20081 T	60 ± 0.3	50[a] ± 0.15	45 ± 0.208	0.36 ± 0.11	0.6[c] ± 0.32	0.66 ± 0.056
L. bulgaricus NCTC 12197 T	66.67[b] ± 0.017	45[b] ± 0.20	60[b] ± 0.36	0.4[a] ± 0.45	0.66[b] ± 0.18	0.62 ± 0.3
L. fermentum	83.33[a] ± 0.034	31.67 ± 0.15	36.67 ± 0.04	0.2[b] ± 0.54	0.8[2] ± 0.022	0.76 ± 0.41
L. a cidophilus	83.33[a] ± 0.032	45[b] ± 0.30	66.67[a] ± 0.017	0.2[b] ± 0.036	0.66[b] ± 0.04	0.4[a] ± 0.054
L. rhamnosus	66.60[b] ± 0.017	50[a] ± 0.17	80 ± 0.25	0.40[a] ± 0.87	0.60[c] ± 0.07	0.29 ± 0.05
L. casei	50 ± 0.10	33.30[c] ± 0.28	91.6 ± 0.208	0.6 ± 0.04	0.81[a] ± 0.07	0.125 ± 0.098
L. plantarum	66.67[b] ± 0.02	53.34 ± 0.10	25 ± 0.25	0.4[a] ± 0.07	0.65 ± 0.076	0.9 ± 0.055
B. longum	75 ± 0.15	33.34[c] ± 0.288	33.33 ± 0.03	0.3 ± 0.04	0.81[a] ± 0.08	0.81 ± 0.076
Control					1.2	
F value	40704.31 ***	6932.026 ***	30991.29 ***	636.4 ***	432.88 ***	315.6 ***

***. The F values are highly significant a t the p≤ 0.001 level. a, b, c Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments), Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.

Table 9. Inhibition of erythrocyte haemolysis in human blood by LAB strains.

Strain	Inhibition %		
	Culture	Extracellular extract	Intracellular extract
Streptococcus thermophilus	40 ± 0.577	29.6 ± 0.264	50.4 ± 0.10
Streptococcus lactis	77 ± 0.346	75.3b ± 0.152	54.1 ± 0.11
Lactobacillus bulgaricus DSMZ 20080	80.1[a] ± 0.20	75.2b ± 0.057	6 ± 0.288
Lactobacillus bulgaricus DSMZ 20081 T	72.2b ± 0.152	71.9 ± 0.058	5 ± 0.173
Lactobacillus bulgaricus NCTC 12197 T	83.6 ± 0.100	64.9 ± 0.50	6.7 ± 0.12
Lactobacillus fermentum	59.5 ± 0.208	80.8[a] ± 0.1	24.8 ± 0.15
Lactobacillus acidophilus	80.5a ± 0.321	85.6 ± 0.15	27.8 ± 0.14
Lactobacillus rhamnosus	72.5b ± 0.288	68.4 ± 0.28	21.8 ± 0.10
Lactobacillus casei	80a ± 0.173	70.6 ± 0.15	65.4 ± 0.20
Lactobacillus plantarum	74.6 ± 0.208	40.6 ± 0.17	15 ± 0.11
Bifidobacterium longum	81.4 ± 0.28	80.9[a] ± 0.15	6 ± 0.25
F value	5486.705***	22638.171***	54201.965***

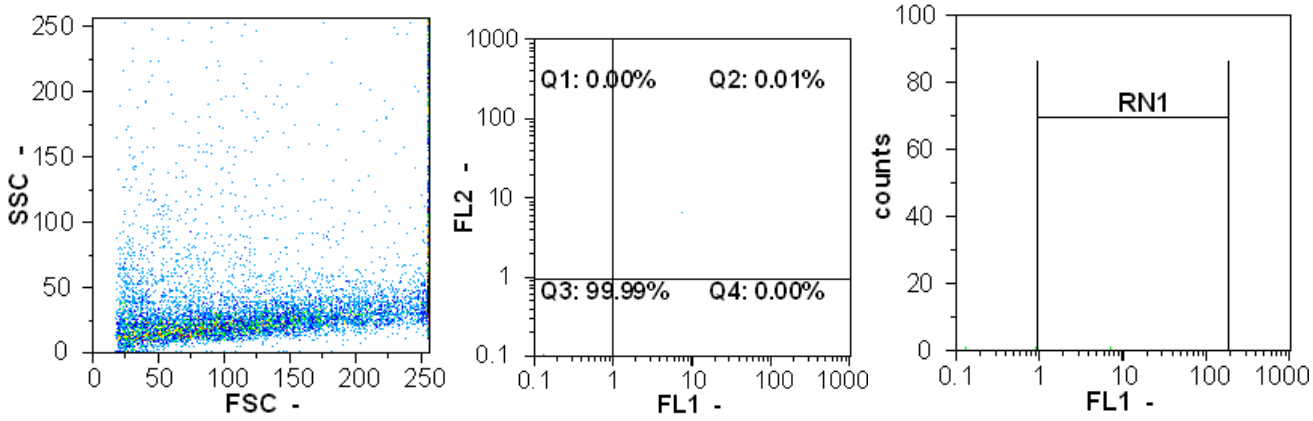
***. The F values are highly significant at the p≤ 0.001 level. a ,b Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments), Values with different letters are significantly different ($p < 0.05$). Data are means ± SD of triplicate experiments.

Since it has been indicated that *Lactococcus* expresses activity of antioxidative enzyme superoxide dismutase (SOD) (Sanders et al., 1995), it is possible that the significant increase in inhibitory activity of the intracellular extracts could be due to the greater accessibility of anti-oxidative enzymes to the oxidant substrates. According to Lin and Yen (1999), 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. (1993) demon-strated that the intracellular extract is also antioxidative *in vivo*. In addition, Kaizu et al. (1993) have demonstrated that haemolysis 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 improved the α-tocopherol deficiency status.

Table 10. The effects of the selected LAB treatments on ROS production using flow cytometric analysis.

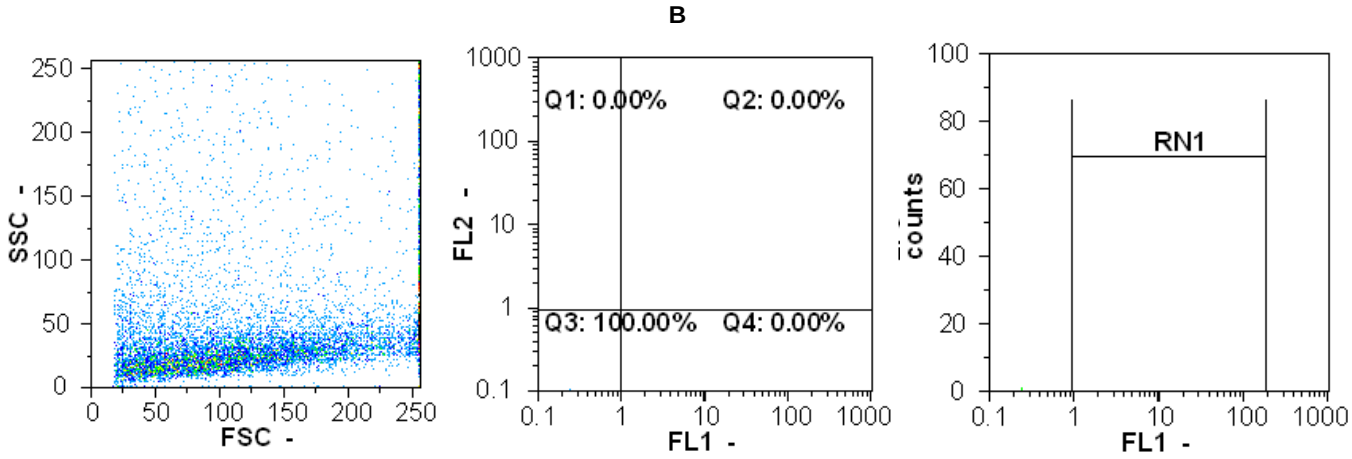
Lactic acid bacterial strains	Inhibition percentage of ROS production
Lactobacillus plantarum extracellular extracts and intra cellular extract	100
Lactobacillus bulgaricus DSMZ 20080 extracellular extracts and intra cellular extract	100
Lactobacillus acidophilus extracellular extracts and intracellular extra ct extracts	95.52
Streptococcus lactis extracellular and intracellular extract	89.55
Bifidobacterium longum extra cellular extracts and intracellular extracts	88.05

A



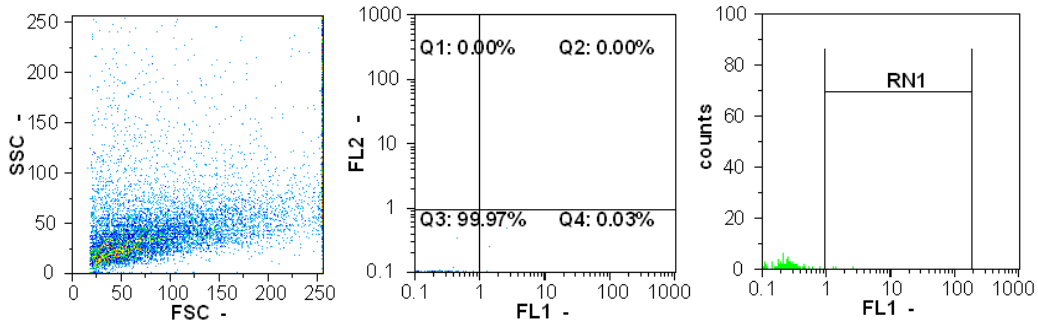
Region	Gate	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
RN1	<None>	1	-	0.01	7.45	0.00	-	-
Q1	<None>	0	-	0.00	-	-	-	-
Q2	<None>	1	-	0.01	7.45	0.00	6.36	0.00
Q3	<None>	9999	-	99.99	0.10	8.50	0.10	7.02
Q4	<None>	0	-	0.00	-	-	-	-

B



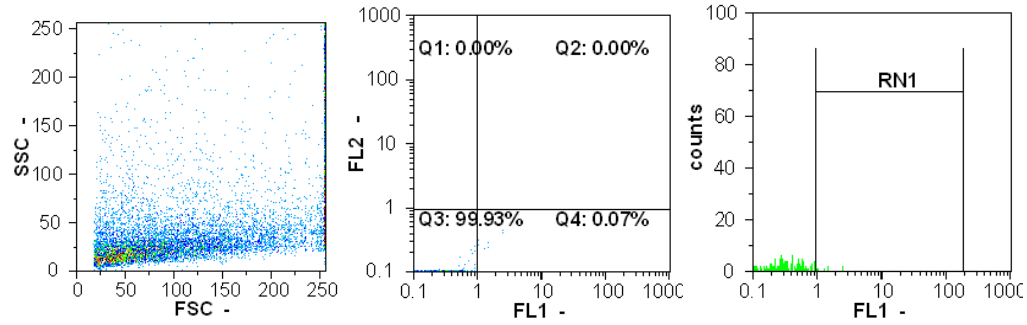
Region	Gate	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
RN1	<None>	0	-	0.00	-	-	-	-
Q1	<None>	0	-	0.00	-	-	-	-
Q2	<None>	0	-	0.00	-	-	-	-
Q3	<None>	10000	-	100.00	0.10	1.52	0.10	0.01
Q4	<None>	0	-	0.00	-	-	-	-

C



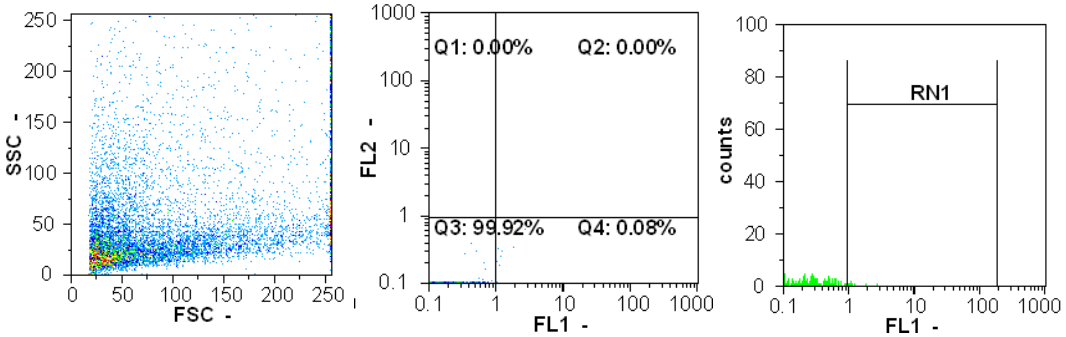
Region	Gate	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
RN1	<None>	3	-	0.03	1.81	40.32	-	-
Q1	<None>	0	-	0.00	-	-	-	-
Q2	<None>	0	-	0.00	-	-	-	-
Q3	<None>	9997	-	99.97	0.10	17.46	0.10	2.41
Q4	<None>	3	-	0.03	1.81	40.32	0.27	68.88

D

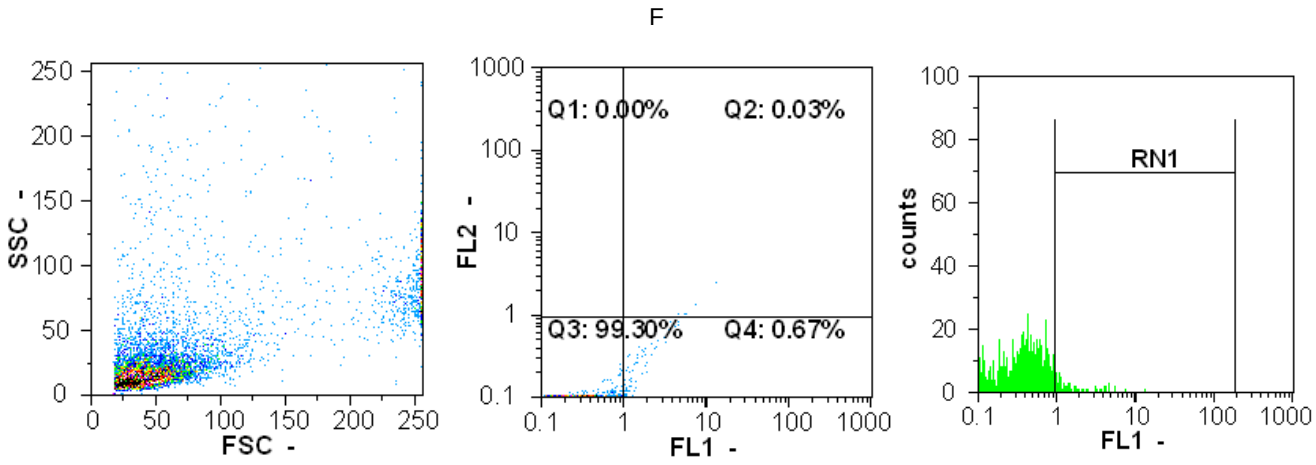


Region	Gate	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
RN1	<None>	7	-	0.07	1.62	37.69	-	-
Q1	<None>	0	-	0.00	-	-	-	-
Q2	<None>	0	-	0.00	-	-	-	-
Q3	<None>	9993	-	99.93	0.10	41.35	0.10	2.45
Q4	<None>	7	-	0.07	1.62	37.69	0.30	26.37

E



Region	Gate	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
RN1	<None>	8	-	0.08	1.43	42.32	-	-
Q1	<None>	0	-	0.00	-	-	-	-
Q2	<None>	0	-	0.00	-	-	-	-
Q3	<None>	9992	-	99.92	0.10	30.68	0.10	6.41
Q4	<None>	8	-	0.08	1.43	42.32	0.29	54.22



Region	Gate	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
RN1	<None>	70	-	0.70	2.25	88.51	-	-
Q1	<None>	0	-	0.00	-	-	-	-
Q2	<None>	3	-	0.03	9.02	45.67	1.55	45.27
Q3	<None>	9930	-	99.30	0.12	82.81	0.10	3.00
Q4	<None>	67	-	0.67	1.94	61.70	0.31	76.62

Figure 1. The Flow Cytometry results: Effect of extra- and intra-cellular extracts of selected LAB on ROS production in comparison with control, A represents *Lactobacillus plantarum,* B represents *Lactobacillus bulgaricus* DSMZ 20080, C represents *Lactobacillus acidophilus,* D represents *Streptococcus lactis*, E represents *Bifidobacterium longum*, F represents the control.

The production of free radicals and many diseases are closely related (Devasagayam et al., 2004). Therefore, the ability of LAB to scavenge DPPH, a free radical was studied. The results as shown on Table 6b, indicate that the radical scavenging ability of the extracellular extracts of the tested strains highly contribute to the antioxidative effect. The reason for that result might be due to the peptides which were generated (extracellular) from the digestion of milk proteins. Such peptides are generally small in size, with molecular weights of less than 3000Da (Pihlanto, 2006). This could be attributed to accessibility to the oxidant-antioxidant test systems being greater for small peptides and amino acids than for large peptides and proteins (intracellular), because large peptides are poorly soluble in assay solvents like methanol used in DPPH radical scavenging test.

The *in-vitro* total ROS scavenging activity (highly sensitive method) results came to confirm the previous results and revealed that both extracts are more potent when they work together. In this study, the DCFH method is adapted and refined for flow cytometry measurement of *H. pylori* LPS-induced ROS. The optimized flow cytometric method offers benefits in the measurement process, when DCF fluorescence is scored by flow cytometry, extracellular fluorescence is not measured and only induced intracellular ROS measured, in addition, flow cytometric system automatically accounted cell type-specific correction factor for cellular density (Wan et al., 2005), in which uniform cell numbers are used in all measurements.

Conclusion

Owing to the complexity of the antioxidant materials and their mechanism of actions, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of studied strains and a combination of different method is necessary. The results of the present study suggest that tested strains have moderate to potent antioxidant activity and free radical scavenging activity. It should be kept in mind that antioxidant activity measured by *in-vitro* methods may not reflect *in vivo* effects of antioxidants. Many other factors such as absorption/metabolism are also important. The finding of this study support this view that some LAB stains are promising sources of potential antioxidant and may be efficient as preventive agents in some diseases. The providing data can just enrich the existing comprehensive data of antioxidant activity of LAB.

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