Evaluation of Antioxidant and Antitumor Activities of Lactobacillus acidophilus Bacteria Isolated from Egyptian Infants

Khalid Abd EL Ghany, Ekkal Abd Elhafeez, Ragaa A. Hamouda, Hoda Mahmoud, Fattah Allah H. Ahmed and Hanafy A. Hamza
National Organization of Drug Control and Research (NODCAR), Egypt
Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt

Abstract: Probiotic bacteria are considered beneficial microorganisms and have been widely used. They protect against harmful bacteria that can cause diseases. This study was carried out to evaluate the potential for L. acidophilus intact cells and cell lysates as antioxidant activities and also to evaluate the potential of this bacteria and their exopolysaccharides (EPS) as antitumor activity in vitro and in vivo against Ehrlich Ascites Carcinoma (EAC) cell line. Lactobacillus acidophilus demonstrated having antioxidant activities for both cell lysates and intact cells with increasing antioxidative properties for cell lysates against 2, 2-diphenyl-1-picrylhydrazyl (DPPH), also resistance to hydrogen peroxide and hydroxyl radical. The EPS produced by L. acidophilus showed a powerful antitumor effect in vivo and in vitro in comparison with L. acidophilus itself while, E. coli increasing solid tumor volume than control.

Key words: Antioxidant, antitumor, Lactobacillus acidophilus, Escherichia coli, exopolysaccharide

INTRODUCTION

Cancer is a group of diseases characterized as uncontrolled cellular proliferation and differentiation (Ponder, 2001). Cancer is one of the most prevalent groups of disorders in populations of many countries worldwide (Jamal et al., 2006). Most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells and cause immunotoxicity which affects not only tumor development but also aggravate patient's recovery (Zandi et al., 2010). The discovery and identification of new antitumor drugs with low side effects on immune system has become an essential side effect in many studies of immunopharmacology (Xu et al., 2009). With this aim, much attentions had been paid to natural compounds in plants, marine organism and microorganisms. Regarding the low side effects of plants and other natural compounds, scientists are interested in working on them to find new medications (Zandi et al., 2010). Lactic Acid Bacteria (LAB) is considered beneficial microorganisms and have been widely used. The potential benefits of lactic acid bacteria for human health include improvement on lactose intolerance, prevention of intestinal infection, reduction of serum cholesterol, stimulation of the immune system, anticarcinogenic action and antioxidative effects (Fernandes and Shahani, 1989; Gilliland, 1990; Lin, 1995; Lin and Yen, 1999; Hitchins and McDonough, 1989; Sanders, 1993). Studies have suggested that utilization of Lactobacilli in foodstuffs and medicines prevents infection by pathogenic bacteria (Reid and Burton, 2002; Chen et al., 2005) as well as cancer formation (Bolognani et al., 2001). The precise mechanisms by which these organisms exert the anti-tumorigenic effects are uncertain. Probiotics may retard colon carcinogenesis by influencing metabolic, immunological and protective functions within the colon and it is possible that they may stimulate tumor cell apoptosis. (Butler et al., 1999). The antioxidative effect of lactic acid bacteria has been reported only recently (Lin and Yen, 1999; Ahotupa et al., 1996; Sanders et al., 1995). Many reports suggested that LAB and their fermented products have anti-tumor effects (Schiffirin et al., 1995). The fermented products of some LABs, such as Bifidobacterium infantis and L. acidophilus are known to have anti proliferative effect against the growth of breast cancer cells (Schiffirin et al., 1995). Kefir (YK-1) has the ability to activate the immunosuppressive response from spleen cells of a mouse when treated with immuno- suppressed substances against Ehrlich carcinoma (El-Gawad et al., 2004). In addition, certain cancer preventing LABs strains, such as L. casei and L. acidophilus exopolysaccharides (EPSs) that had received a lot of attention on their contribution to improvement in texture and viscosity of fermented food products. Also, the lactic acid bacteria produce EPS probably as a protective function of their natural environment such as against deiscation,
phagocytosis, phage attack, osmotic stress, antibiotics or toxic compounds (Rusas-Madhoo et al., 2002). The EPS may have a role in cell recognition, adhesion to surfaces and formation of biofilms that facilitate colonization to various ecosystems, this is a beneficial attribute for probiotics in their endeavor to colonize the gastrointestinal tract. Health benefits have been attributed to some exopolysaccharides. They have been reported to possess antitumor, anti-atherosclerotic, modulating and cholesterol lowering effects and anti-oxidative stress of L. acidophilus (De Vuyst and Degeest, 1999).

So the aim of the present study is to investigate the antitumor and antioxidant activity of L. acidophilus bacteria and its exopolysaccharides as natural agents against EAC cell line in vitro and in vivo.

**MATERIALS AND METHODS**

**Microbial strains:** Probiotic strain candidate (one strain of L. acidophilus P185) was identified by Mahrous (2006) which was isolated from healthy breast-feeding infants (15-day-old) and used after selection and identification according to Bergey's Manual of Determinative Bacteriology, 9th edition (Holt et al., 1994) with confirm identification by SDS-PAGE technique and API System. The strain was tested for its probiotic characteristics i.e., gastric acid resistance, bile salt tolerance, antibacterial activity, adhesion to human mucus. Lactobacillus strain was cultivated in MRS (de Man Rogosa Sharpe) broth (Lab M, IDG, UK) and incubated at 37°C in BBL anaerobic jar (Becton Dickinson Microbiology Systems, Sparks, MD) provided with disposable BBL gas generating pack (CO2 system envelopes, Oxoid, Ltd., West Heidelberg, Victoria, Canada).

The culture was preserved in reconstituted skim milk in eppendorf tubes, stored at -80°C with glycerol (20%, v/v). Prior to use, strain was a subcultured (1%, v/v) twice in MRS broth and adjusted at 1×10^6 CFU mL^-1.

The E. coli (10536) strain obtained from ATCC was cultivated in nutrient broth (Lab M, IDG, UK) and incubated at 37°C for 24 h and preserved in reconstituted skim milk in eppendorf tubes.

**Extraction of exopolysaccharides from L. acidophilus:** The EPS was isolated and purified according to Cerning et al. (1994) with some modification. The growth culture was heated at 100°C for 5 min to inactivate enzymes potentially capable of polymer degradation and the cells were removed by centrifugation at 8000 rpm for 5 min at 4°C. The EPS was precipitated using 2 vols of absolute ethanol. After standing overnight at 4°C, the resultant precipitate were collected by centrifugation at 8000 rpm for 20 min. The EPS was dissolved in deionized water, dialyzed against deionized water at 4°C for 24 h and freeze-dried. The freeze-dried powder was dissolved in 10% (w/v) trichloroacetic acid to remove proteins. The supernatant was dialyzed at 4°C against deionized water for 5 days and freeze-dried. These preparations were referred to as purified EPS and were stored at 4°C.

**HPLC analysis:** The EPS sample was analysed using a High Performance Liquid Chromatograph (HPLC) under the following conditions; mobile phase: 0.65 m m sulfuric acid; columns: Aminex HPX-87H (30×7.8 mm); temperature 75°C, flow rate, 0.7 mL min^-1, injection volume: 20 μL, detection at UV 205 nm by using reference standard from sigma-addrich company, D(+)- glucose (G8270), D(+)-glucose (5G388) and D-glucaric acid (G5269).

**Cell lysates and intact cells:** Cells were harvested by centrifugation at 4°C for 30 min (5,000 g) after overnight incubation at 37°C and the pellet was washed twice with 20 mM sodium phosphate buffer (SPB, pH 7.4), then re-suspended in SPB. Washed cell suspension was disrupted with a ultrasonic cell disruptor (Brandson 4°C) and filtration (0.45 μm, Millipore). Cell debris was removed from centrifugation (10,000 g for 10 min and concentration was measured by the Bradford method (Bio-Rad Laboratories) and adjusted to 1 mg mL^-1. For the preparation of intact cells, cells were washed twice with SPB and re-suspended in SPB. The total cell number was adjusted to 10^9 CFU mL^-1 (Kim et al., 2006).

**Resistance to hydrogen peroxides and hydroxyl radical:** Hydrogen peroxides and hydroxyl radical were observed by the method of Kullisaar et al. (2002). For the measurement of Lactobacillus resistance in the presence of hydrogen peroxide, cells were suspend at the level of 10^7 CFU mL^-1 in Sodium Phosphate Buffer (SPB) and incubated with 1.0 mM hydrogen peroxide (30%) at 37°C. At 1 h time intervals, the number of viable cells was estimated at MRS agar plates. For resistance to hydroxyl radicals, cells (10^7 CFU mL^-1) were incubated with the solution containing 10 mM THAs (terephthalic acid, Sigma) in SPB and 0.01 mM CuSO4·5H2O. The reaction was started by the addition of 1 mM hydrogen peroxide and the number of viable cells was estimated (Kim et al., 2006).

For blanks, the cells were suspended at the level of 10^7 CFU mL^-1 in SPB and incubated at 37°C for 7 h. At 3 h time intervals, the number of viable cells was estimated on MRS agar plates and we examined that the number of viable cell did not affect for 7 h.
DPPH freed radicals scavenging assay: Evaluation of Antioxidant activity by in vitro technique with 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH (0.1 Mm) was prepared by dissolving 1.9 mg from DPPH to absolute methanol in 100 mL, then keeping in the dark place for 1 h and 1.0 mL of this solution was added to 3.0 mL of extract solution in methanol at different concentrations. Thirty minute later, the absorbance was measured at 517 nm. A blank was prepared for adding extract. Concentrations (1-18 µg mL⁻¹) were used as standard; lower absorbance of the reaction mixture indicates higher free radicals scavenging activity. The scavenging ability was defined as follows:

\[
\frac{A_{517} \text{ (sample)}}{A_{517} \text{ (blank)}} \times 100
\]

**In vitro assessment of anti tumor activity of L. acidophilus bacteria and its EPS**

**Tumor cells:** The initial inoculums of Ehrlich Ascites Carcinoma (EAC) were purchased from the National Cancer Institute, Cairo University, Egypt. EAC cells were propagated in NODCAR laboratories by weekly intraperitoneal injection of 0.2 of 1:5 mL⁻¹ saline solution of freshly drawn ascetic fluid (0.2×10⁶ EAC cells) from a donor mouse bearing 6-8 day old ascetic tumor, into three mice to ensure that the ascetic fluid will still propagated and can then be drawn from at least on life mouse. Transplantation was carried out using sterile disposable syringes under aseptic conditions. The tumor growth was as rapid as to killing the mice within 18-20 day due to the accumulation of ascetic fluid and rarely the tumor showed distal metastasis or spontaneous regression. After making appropriate dilution, the non-viability was tested for trypan blue exclusion method, (Freshney, 2005), as shown follows:

\[
\frac{\text{No. of non-viable cells}}{\text{Total cells}} \times 100
\]

By this method we can determine the effective dose of bacterial extracts induced more regression of tumors.

**Effect of different doses from bacteria on the growth of solid tumors in male albino mice:** Adult Male Swiss albino mice with an average body weight 20-25 g were used as experimental animals throughout the study. The mice were housed in specially designed cages and maintained in a thermostatically controlled room during the experimental period. They were fed an ordinary pellet diet and given up tap water ad-libitum in (NODCAR) these mice were divided into 4 groups having 5 animals each:

**Group 1:** Healthy mice injected subcutaneous in thigh with 1×10⁶ tumors cell/mouse (control)

**Group 2:** Healthy mice oral inoculated with daily dose of 500 µg mL⁻¹ from L. acidophilus (1×10⁶) CFU for 14 day, then the animals injected with 1×10⁶ tumor cell/mouse subcutaneous, then oral inoculated by L. acidophilus (1×10⁶) CFU three dose weekly for two week

**Group 3:** Healthy mice injected intra peritoneal with daily dose of 500 µg mL⁻¹ of conc. 1 mg mL⁻¹ from EPS extracted from L. acidophilus then the animals injected with 1×10⁶ tumor cell/mouse subcutaneous and injected with EPS three times weekly for two week

**Group 4:** Healthy mice oral inoculated with daily dose 250 µg mL⁻¹ Escherichia coli (1×10⁶) CFU mL⁻¹ for 14 day, then some animals injected with 1×10⁶ tumor cell subcutaneous, then oral inoculated by three dose E. coli (1×10⁶) CFU mL⁻¹ weekly for two week

**Statistical analysis:** Data obtained was subjected to analysis of variance and the means were compared using the Least Significant Differences (LSD) tested for the 0.05 level, as recommended by Snedecor and Cochran (1982). *In vitro* experiments were conducted in triplicate.

**RESULTS AND DISCUSSION**

**Resistance of L. acidophilus bacteria to hydroxyl radicals:** Figure 1 and 2 show the resistance of L. acidophilus to hydrogen peroxide and hydroxyl radicals. *Lactobacillus acidophilus* was viable for 7 h in concentrations of 1.0 mM hydrogen peroxides and 0.01 mM hydroxyl in the presence of highly damaging effect, both these intact cell and cell lysate, had strong anti-oxidative activities with increasing inhibition percentage in cell lysate than intact cell. Anti-oxidative activity of microorganisms is one of the reasons for their increased resistance to Reactive Oxygen Species (ROS). Kullisaar et al. (2002) reported that the antioxidant strain L. acidophilus had significantly high resistance to ROS. Kim et al. (2006) proved that L. acidophilus was the most active strain which hydroxyl radicals scavenging activity in cell lysates was 70 and 53% in intact cells. The scavenging of different types of ROS was thought to be one of the main antioxidant mechanisms of the antioxidant action exhibited by lactic acid bacteria (Namiki, 1990).

**DPPH method:** DPPH is a relatively stable organic radical, it has been widely used in the determination of antioxidant...
activities. Figure 3 shows the results of DPPH scavenging potential of different concentrations of *L. acidophilus*, cell lysates and intact cells. The maximum antioxidant activity was 69.41 and 65.215% with 80 μg mL⁻¹ of both cell lysates and intact cells. It is noticed that the antioxidant activity of different concentrations of cell lysates was more effective than different concentrations of intact cells. Lin and Chang (2000) indicated that the radical scavenging ability of the intact cells and intracellular extracts of *B. longum* and *L. acidophilus* contribute good antioxidative effect on inhibiting linoleic acid peroxidation and scavenging the DPPH radical.

**Chemical composition of exopolysaccharides (EPS):** The results of exopolysaccharides content secreted by *L. acidophilus* are shown in Fig. 4, which shows different sugar content and concentrations. The sugar contents is glucose, galactose and D. glucoronic acid (71.9, 22.46 and 0.16 mg, respectively). The chemical composition of exopolysaccharides from *L. acidophilus* has been investigated and there is agreement that EPSs from LABs is polysaccharides with D. glucose and

**Fig. 1:** Percentage of resistance of *L. acidophilus* bacteria to hydrogen peroxide. Bacteria was suspended at the level of 10⁷ CFU mL⁻¹ with conc. 1 mg mL⁻¹ for both cell lysates and intact cells and incubated with 1.0 mM hydrogen peroxides

**Fig. 2:** Percentage of resistance of *L. acidophilus* to hydroxyl radical. Bacteria was suspended at the level of 10⁷ CFU mL⁻¹ with conc. 1 mg mL⁻¹ for both cell lysates and intact cells and incubated with 0.01 mM hydroxyl radicals

**Fig. 3:** Antioxidant activities of different concentrations of *L. acidophilus* cell lysates and intact cells against DPPH radical

**Fig. 4:** EPS by HPLC analyses containing D. glucoronic acid, D. lactose and D. glucose
D. galactose as main sugar constituents and the ratio of the two components varies (Laws et al., 2001).

In vitro assessment of the effective dose of *L. acidophilus* and its EPS against EAC cells: Different concentrations of *L. acidophilus* and its EPS (100, 200, 300, 400, 500, 600 and 700 µg mL⁻¹) was investigated on the non-viability of Ehrlich Ascites Carcinoma (EAC) cell line as shown in Table 1. Results showed that different concentrations of *L. acidophilus* and its EPS inhibited the proliferation of EAC cells. The EPS showed more antiproliferative effects against EAC cell line than *L. acidophilus*. The maximal percentage of inhibition of EAC cells line was (93 and 95%) with 700 µg mL⁻¹ of *L. acidophilus* and its EPS, respectively. This result is demonstrated by Doleyres and Lacroix (2005) which reported LAB EPS is provided beneficial physiological effects on human health, such as antitumourous activity, immunomodulating bioactivity and antimutagenicity.

Effect of *L. acidophilus* and its exopolysaccharides on the volume of solid tumors: Further in vivo study for the highly toxic *L. acidophilus* and its exopolysaccharides has been done on solid tumor bearing mice. As show in Fig. 5 and Table 2, *L. acidophilus* and its exopolysaccharides caused significant reduction in the tumor volume as compared to that of the positive control group and effects of *E. coli*. The reduction of tumor volume (1.02±0.022 and 1.21±0.019 mm³) were observed when mice-bearing solid tumor was treated with exopolysaccharides extracted from *L. acidophilus*. The EPS from *L. acidophilus* showed powerful reduction of solid tumor volume than *L. acidophilus* itself while *E. coli* causing propagation of solid tumor volume than control mice. Baldwin et al. (2010) suggested that probiotics may be used as adjuvants in anticancer chemotherapy. Daniluk (2012) reported that the anticancer activity through induction into a apoptosis of cancer cells seems to be promising to approach for use of some probiotic strains on support therapy or disease prevention. Probiotics also have a role in prevention of colon cancer (Dugas et al., 1999; Rafter, 2003). Different strains of probiotics had different effects on the gastrointestinal luminal environment, epithelial and mucosal barrier function and the mucosal immune system (Hirayama and Rafter, 2000).

Fig. 5: Effect of tested *Lactobacilli* and its exopolysaccharides on the volume of solid tumor (mm³)

**CONCLUSION**

This study demonstrated that *L. acidophilus* having antioxidant activities for both intact cells and cell lysates. The *L. acidophilus* and its exopolysaccharide (EPS) had antitumor activity in vivo and in vitro. The exopolysaccharide of *L. acidophilus* is more effective against Ehrlich Ascites Carcinoma (EAC) cells than *L. acidophilus*.

REFERENCES


