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Effects of *Lactobacillus plantarum A7* with probiotic potential on colon cancer and normal cells proliferation in comparison with a commercial strain

Hojjat Sadeghi-Aliabadi [1]*, Fatemeh Mohammadi [1], Hossain Fazeli [2], Maryam Mirlohi [3]

[1] Department of Biotechnology, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran
[2] Department of Microbiology, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran
[3] Food Security Research Center, School of Nutrition and Food Science, Isfahan University of Medical Sciences, Isfahan, Iran

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ABSTRACT

Objective(s): Several beneficial effects have been attributed to the probiotic lactic acid bacteria. It was determined that lactobacilli can exert antiproliferative effects on the various cancer cell lines including colon cancer. Effects of lactic acid bacteria on colon cancer may vary from strain to strain and there is a need to find the new probiotic strains with tumor suppressing properties through *in vitro* studies.

Materials and Methods: Anti-proliferative activities of heat-killed cells and cell-free supernatants of a native strain of *Lactobacillus plantarum A7* and a commercial strain of *lactobacillus rhamnosus GG* were assessed on human colon cancer cell lines (Caco-2 and HT-29) and normal cells (L-929), using MTT assay. Cells were seeded at 2×10^4 cells/mlin 96 well plates and incubated for 24 hr. Then heat-killed cells (OD_{620}: 0.025, 0.0.05, 0.1) and cell-free supernatants of bacteria were added at concentration of 2.5, 5 and 10 mg/ml. After 48 hr incubation MTT (5 mg/ml) was added and the absorbance was measured at 540 nm using ELISA plate reader.

Results: Results showed that heat-killed cells and cell-free supernatants of both probiotic strains reduced the growth rate of cancer and normal cells. These results suggested that anti-proliferative effect may not be an exclusive characteris ticwhich is dedicated to officially approved probiotics.

Conclusion: *L. plantarum A7* could be considered as colon cancer biological product, most likely due to its advantages in significant organic acid production.

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Introduction

Probiotics are beneficial microorganisms which provide health benefits to the host when administered in adequate number (1, 2). Strains of Lactobacilli and bifidobacteria are widely used in human consumption (3). Beneficial aspects of probiotic bacteria are regarded as: preventing and curing of gastrointestinal disorders, serum cholesterol reduction and immune responses enhancement (4-6). Newly emerged studies have concentrated on anticancer properties of probiotics. In vitro and animal studies as well as epidemiological studies have shown the positive role of probiotics against cancer (7). Colorectal cancer (CRC) has been widely considered in such studies. The most proposed mechanisms involved in anticancer characteristics of probiotics are changing the metabolic activities of gut microflora and colon physicochemical condition, removing the carcinogens, production of anti-tumorigenic or anti-mutagenic substances and increasing the host's immunity (8-10). Strains belonging to *L. rhamnosus*, *L. acidophilus, L. casei, B. longum, B. infantis, B. adolescentis* and *B. breve*species have been shown to be effective in suppression of colon tumor incidence (11). Various types of lactic acid bacteria (LAB) preparations such as whole cells, heat-killed (HK) cells, cell wall, peptidoglycans, polysaccharides and cytoplasmic fractions display the anti-proliferative effects on human cancer cell lines (12, 13). Health promoting effects of probiotic bacteria are stated to be very strain dependent and their positive role on colon cancer may also vary from one strain to another. Therefore there is a need to find new probiotic strains with anticancer properties. Recently *L. plantarum A7* (*L. A7*) has been isolated from fecal flora of healthy infants; and characterized with probiotic potential (14).

This study aimed to evaluate anti-proliferative effects of *L.* A7 as a native and *L. rhamnosus GG* (*L. GG*) as a commercial strain on human colon cancer cell lines (Caco-2 and HT-29) and also normal cells (L-

**Corresponding author:*Hojjat Sadeghi-Aliabadi.Department of Biotechnology, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran. Tel: +98-311-7922564; Fax: +98-311-6680011; email: sadeghi@pharm.mui.ac.ir

929). To examine whether cellular fractions or produced metabolites by the tested strains could inhibit cell growth; both HK cells and cell-free supernatants of *lactobacillus* strains were investigated in two independent series of experiments. To determine whether the non-probiotic bacteria have the anti-proliferative effect, *Escherichia coli (E. coli)* as a facultative anaerobic microflora of the human body (15) was considered as a negative control in this study.

Materials and Methods

Bacterial strain and culture medium

L.A7 strain was provided from the microbial collection of food microbiology laboratory of Industrial University of Isfahan. *L. GG* strain was purchased from pharmaceutical product (Culturelle, USA). *L. A7* and *L.GG* strains were grown in MRS broth (Merck, Germany) (16) and *E. coli* strain (ATCC: 25922) was grown in BHI broth (Merck, Germany) at 37˚C for 18 hr under aerobic conditions. All strains were sub-cultured at least three times prior to use.

Preparation of HK cells

Overnight bacterial cultures were harvested by centrifugation (Hettich, Germany) at 5,000 g for 15 min. Pellets were washed twice with phosphate-buffered saline (PBS, pH: 7.2), heated at 95˚C for 1 hr and then lyophilized (Christ, alpha 2-4 LD plus, Germany) (15). The lyophilized cells were resuspended in appropriate medium and final OD_{620} were adjusted to 0.025, 0.05 and 0.1 for cell viability assays.

Preparation of cell-free supernatant

To prepare cell-free supernatant, cultured bacterial cells were centrifuged at 5,000 g for 15 min and the supernatants were filtered using a 0.22 µm bacterial filter. This cell-free supernatant was subjected to freeze-drying and then various concentrations (2.5, 5 and 10 mg/ml) were prepared for cell viability assays (17).

Cell culture

Human colon cancer cell lines (Caco-2 and HT-29) and a non-cancerous cell line (L-929) were obtained from Pasture Institute of Iran in Tehran. HT-29 and L-929 were cultured in RPMI-1640 medium (Sigma, Germany) and Caco-2 cells were cultured in DMEM (Sigma, Germany) consisting 10-15% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (50 IU/ml and 50 µg/ml respectively) and incubated at 37˚C in a humidified atmosphere with 5% CO_2. Trypsin-EDTA (PAA, Austria) was used for cells detachment after confluence.

Assessment of cell proliferation using MTT assay

The MTT assay is based on the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide (MTT, Merck, Germany), by actively growing cells. Briefly, 180 µl of cells were seeded on 96-well microplates at a concentration of2×10^4 cells/ml and incubated for 24 hr. Then 20 µl of various concentrations of HK cells and cell-free supernatants were added and incubated at 37˚C. After 48 hr incubation, 20 µl of MTT solution (5 mg/ml) was added to each wellandincubated for another 3 hr. The medium was then removed and the blue formazan crystals were solubilized with 150 µl of dimethyl-sulfoxide (DMSO, Merck, Germany). MTT converted to formazan by metabolically viable cells and its absorbance was measured using anELISA reader (Awareness, USA) at 540 nm.

Statistical analysis

One-way analysis of variance (ANOVA) was applied using SPSS (version 16.0) to evaluate the experimental data. The significant differences were accepted at $P<0.05$.

Results

Anti-proliferative effects of bacterial HK cells

The effects of different concentrations of bacterial HK cells on the conversion of the MTT tetrazolium

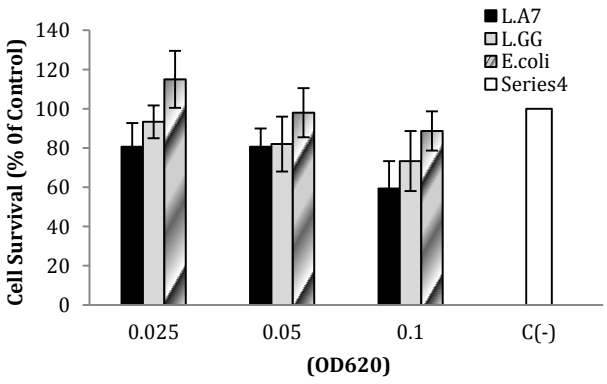

Figure1. The inhibitory effects of bacterial HK cells on the growth of Caco-2 colon cancer cell line using MTT assay. Data are expressed as mean±SD of three separate experiments. C(-) represent untreated cells (negative control) and assumed 100% survival

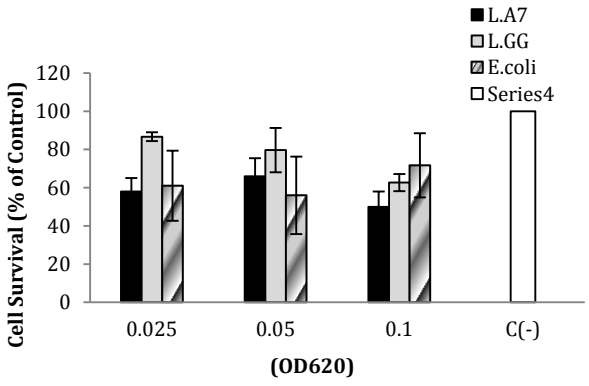

Figure2. The inhibitory effects of bacterial HK cells on the growth of HT-29 colon cancer cell line using MTT assay. Data are expressed as mean±SD of three separate experiments. C(-) represent untreated cells (negative control) and assumed 100% survival

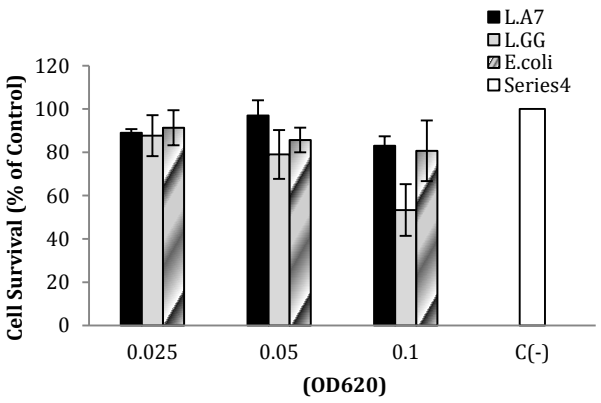

Figure 3. The inhibitory effects of bacterial HK cells on the growth of L-929 cell line using MTT assay. Data are expressed as mean ± SD of three separate experiments. C (-) represent untreated cells (negative control) and assumed 100% survival

salt in cells after 48 hr in comparison with the untreated control cells are illustrated in Figures 1-3. Several concentrations (OD_{620}:0.025, 0.05, 0.1) of HK cells obtained from microbial strains were tested against cell lines. Survival rate was assumed at 100 % in control group.

The HK cells of *L. A7* strain at the (OD_{620}: 0.1) have the most relative inhibitory effects and reduced the cell survival to 59.3%. There were no significant differences (P>0.05) between various concentrations of bacterial HK cells in the reduction of Caco-2 cell viabilities.

The HK cells of *L. A7* strain at the (OD_{620}: 0.1) have the most relative inhibitory effects on HT-29 cells and reduced the cell survival to 50%, although reduction of cell survival by this strain was not in a dose dependent manner. There were no significant differences (P>0.05) between various concentrations of bacterial HK cells in the reduction of HT-29 cell viabilities.

The HK cell of *L. GG* strain at the (OD_{620}: 0.1) have the most relative inhibitory effects on L-929 cells and reduced the cell survival to 53.3%. There were no significant differences (P>0.05) between various concentrations of bacterial HK cells in the reduction of L-929 cell viabilities.

Anti-proliferative effects of cell-free supernatants

The effects of various concentrations of bacterial cell-free supernatants on the conversion of the MTT tetrazolium salt in cells after 48 hr comparing with control groups are presented in Table 1. In each tested concentration, equal amount of MRS and BHI broth, as used in the test sample, were served as controls.

All cell lines were treated with increasing concentrations of bacterial cell-free supernatants. MTT assay was used to evaluate cell survival after 48 h incubation.

All tested concentrations of *L. A7* (2.5, 5, 10 mg/ml) and concentration levels of 5 and 10 mg/ml *L. GG* cell-free supernatants display significant (P<0.05) inhibitory effects on Caco-2 cells compared with the control groups; While *E. coli* cell-free supernatant displayed no significant inhibitory effects in tested concentrations. Statistical analysis showed significant differences (P<0.05) between the concentrations of 2.5 and 10 mg/ml *L. A7* and *L. GG* cell-free supernatants, respectively. At concentration of 5 mg/ml, there was no significant difference (P=0.24) between cell-free supernatants of *L. A7* and *L. GG* strains in the reduction of Caco-2 cell survival.

Concentration level of 10 mg/ml *L. A7* and *L. GG* cell-free supernatants display significant (P<0.05)

Table 1. The inhibitory effects of bacterial cell-free supernatants on the growth of three cell lines using MTT assay

Bacterial Strains	% Cell survival at three Concentrations(mg/ml)			pH at 10 mg/ml
	2.5	5	10	
Caco-2				
L.A7	51.0±18.0*	47.3 ±17.5*	8.6±3.1*	3.9±0.08
L.GG	79± 11.7	61.0 ± 3.5*	34.0 ± 5.7*	4.2± 0.09
Control (MRS broth)	107 ±14.1	108.6±7.8	90.6 ±10.7	
E. coli	115±6.2	87 ±14.8	73± 15.9	6.1 ±0.12
Control (BHI broth)	85.7 ± 22	80.7 ±20	70.3 ±6	
HT-29				
L.A7	46±2.6	15 ± 7	0*	3.9± 0.08
L.GG	54±14	19±2.9	0.4 ±0.75*	4.2±0.09
Control (MRS broth)	57 ± 3	38.5 ± 11.5	32± 1	
E. coli	58±17.5	47.7±9.6	41.3 ±6.6	6.1±0.12
Control (BHI broth)	48 ± 10	49.5±13.5	46.5±2.5	
L-929				
L.A7	42 ±4*	29.33 ±8*	0*	3.9 ±0.08
L.GG	43 ±8.7*	20 ± 2.6*	0*	4.2 ± 0.09
Control (MRS broth)	81 ±8	59.7± 2.3	42.3 ±2.1	
E. coli	83.3 ±5	46± 7.8*	70.7 ±7.5	6.1± 0.12
Control (BHI broth)	90.7 ± 5.8	69.3 ± 3.2	55.7± 8.7	

Dataareexpressed as mean±SDof three separate experiments; Controlversus treatments groups, *P<0.05

inhibitory effects on HT-29 cells compared with the control groups; While *E. coli* cell-free supernatant again displayed no significant inhibitory effects in tested concentrations. Statistical analysis showed that there were no significant differences between various concentrations of *L. A7* and *L. GG* cell-free supernatants in the reduction of HT-29 cellviabilities (P>0.05).

All tested concentrations of *L. A7* and *L. GG* and concentration level of 5 mg/ml *E. coli* cell-free supernatants showed significant (P<0.05) inhibitory effects on L-929 cells compared with the control groups. Statistical analysis revealed that there were no significant differences between various concentrations of *L. A7* and *L. GG* cell-free supernatants in reduction of L-929 cell viabilities (P>0.05).

Discussion

Probiotic bacteria convey a wide range of beneficial effects to their hosts (16). Recently many studies have concentrated on the effects of probiotics in reduction of cancer cell viability and tumor size. *L. rhamnosus GG* as a legally approved human probiotic strain has been examined in many studies (18-20) and was considered as one of the most efficient probiotic strains in this regard. Orlando *et al* (21) reported anti-proliferative effects of *L. GG* on gastric and colon cancer cells. They found that the highest concentrations of *L. GG* homogenate and cytoplasm extracts reduced the percentage of cell viability to nearly 55% and 65% in DLD-1 (colon) and HGC-27 (gastric) cancer cell lines, respectively. Choi *et al* (16) in another study reported that the HK cells of *L. rhamnosus GG* potently inhibited the viability of some cancer cell lines. In the present study HK cells of *L. GG* triggered anti-proliferation of Caco-2 and HT-29 colon cancer cells and reduced the cell viabilities to 73% and 62.7% at the highest prepared concentration respectively. The results presented in this article were in agreement with those two mentioned studies conducted on the same strains. Moreover, the results showed that there were no significant differences between the bacterial strains regarding their HK cells. But cell-free supernatant of probiotic strains were more effective in the growth inhibition of cancer and normal cells in comparison with *E. coli* strain. Since pH of cell-free supernatant of probiotic strains was identified to be lower than that of *E. coli* (Table 1), the given differences between examined strains in this study could be simply attributed to the higher concentration of organic acids in the supernatant of probiotics. Results published by Kim *et al* (17) showed that cell-free supernatant of a native strain *Bifidobacterium adolescentis* SPM0212 has more growth inhibitory effects on the colon cancer cells in comparison with *B. adolescentis* SPM0212 whole cells and H cells. As neutralization of the supernatant was not carried out in that study, it can be inferred that inhibitory effects of supernatant was resulted by the effect of organic acids. Antiproliferative effects of probiotic bacteria may partly be caused by the produced exopolysaccharides. Kim *et al* (22) reported that *Lactobacillus rhamnosus* ATCC 9595 reduced the growth of colon (HT-29) and pancreas (PANC-1) cancer cell lines. This reduction was attributed to two exopolysaccharides of bacteria; rEPS (released exopolysaccharides) and cbEPS (cell bound exopolysaccharides). According to their results rEPS was identified to be more effective than cbEPS in preventing cancer. In animal models also *L. plantarum* has been found to inhibit colon cancer. Asha and Gayathri (23) reported administration of *L. fermentum* and *L. plantarum* alone or in combination with vincristine have a synergistic impact on increase the body weight, decrease the ammonia concentration, decrease B glucosidase and B glucuronidase enzyme activity and a reduction in the number of crypts in the mice with 1,2-dimethylhydrazine (DMH) hydrochloride-induced colon cancer.

Conclusion

The anti-proliferative effect of the tested native strain by MTT assay was not different from officially approved probiotic strain and even more growth inhibitory effects on the tested cancer cell lines were observed using its cell-free supernatant. Considering these results and the results of studies in line, a conclusion can be drawn that anti-proliferative effect is not an exclusive probiotic characteristic and is belonged to the genera of lactobacilli. Since, all the strains belonging to this genus are organic acid producer; they are capable to suppress the cancer cells through *in vitro* experiments. Further studies are needed to clarify the mechanisms of this inhibition.

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