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The High Anti-cytotoxic Effects of Novel *Lactobacillus Plantarum* HBM-IAUF-1 and *Lactococcus Lactis* HBM-IAUF-8 against SK-BR-3 Breast Cancer Cell Line

Ziba Harandi
Department of Microbiology, Faculty of Biological Sciences, Islamic Azad University, Falavarjan Branch, Falavarjan, Isfahan, Iran, z_harandi@yahoo.com
keivan Beheshti Maal*
Department of Microbiology, Faculty of Biological Sciences, Islamic Azad University, Falavarjan Branch, Falavarjan, Isfahan, Iran, kbeheshtimaal@yahoo.com
Ramesh Monajemi
Department of Biology, Faculty of Biological Sciences, Islamic Azad University, Falavarjan Branch, Falavarjan, Isfahan, Iran, r_monajemi@yahoo.com

Abstract

Introduction: Recently, breast cancer therapy is challenging worldwide. The probiotics could be used as an alternative for breast cancer prohibition and treatment among which Lactic Acid Bacteria, LAB, have beneficial effects on host health.

Materials and methods**:** Lactic acid bacteria were isolated from yogurt and cheese samples using bacterial culture on MRS broth and MRS agar. Identification of LAB was carried out by macroscopic, microscopic, and molecular analysis. Using MTT assay, the cytotoxicity of LAB on SK-BR3 cancer cells was investigated with bacterial concentrations of 100, 250, 500, and 1000 µg/ml.

Results: The molecular analysis showed that the two isolates obtained in this study were related to the *Lactococcus lactis* sp. They were named *Lactobacillus plantarum* HBM-IAUF-1 and *Lactococcus lactis* HBM-IAUF-8 and their 16s-rDNA genome sequences were deposited in GenBank, NCBI under the accession numbers of MG757680 and MG757734, respectively. The *Lactobacillus plantarum* HBM-IAUF-1 showed the highest cytotoxicity on SK-BR3 cancer cells at the maximum time of 72 hours at the concentration of 1000 µl/ml. Also, *Lactococcus lactis* HBM-IAUF-8 showed the highest cytotoxicity at the maximum time of 72 hours at the concentration of 500 µl/ml.

Discussion and conclusion: This is the first report of identifying the novel *lactococcus lactis* strains from traditional Iranian dairy products that had cytotoxic effects on breast cancer SK-BR-3 cells. The use of organic dairy products and the increasing of probiotics applications in the food industry could be an asset in cancer prohibition as well as advantageous in food and medical biotechnology.

Key words: Breast Cancer, Lactic Acid Bacteria, *Lactobacillus Plantarum, Lactococcus Lactis*, Probiotics, SK-BR-3 Cell Line

*Corresponding Author

Introduction

Breast cancer is the most frequently diagnosed cancer in women and ranks second among causes for cancer-related death in women (1). Some of risk factors associated with breast cancer include higher body mass index, high glycemic diet, alcohol consumption, familial history of breast cancer, menopause age, and menopausal hormonal therapy (2-5). Advancement in the screening and treatment of breast cancer has steadily decreased its mortality, particularly for HER-2 and luminal cancers, but for triple negative cancers, it remains at higher rates (6). Most of the studies on breast cancer patients suggest that there is a reduction in immune responses, including increased response to delayed sensitivity, cytolytic function, reduced immune cell proliferation, and as a result, reduced cytokines due to breast cancer (7). Therefore, the consumption of immune-booster agents plays an important role in controlling breast cancer. Probiotics are one of the most important agents in boosting the immune system. Probiotics enhance the immune system and anti-inflammatory activities. Long-term consumption of probiotics was significantly associated with the suppression of breast cancer formation and proliferation. Probiotics, as functional foods, play a pivotal role against breast cancer development in vivo and in vitro (8). With the dramatic increase of the resistance of cancer cells to chemical anticancer agents, finding alternative strategies to prevent or treat a variety of cancers has become inevitable, so probiotics such as Lactic Acid Bacteria (LAB) are one of the appropriate alternative for this purpose. The most important LAB in fermentative foods are the genera of *Bifidobacterium, Enterobacter, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus,* and *Weissella* (9, 10). Several reports are indicating the anti-carcinogenic effects of LAB against various cancer cell lines such as *Bifidobacterium infantis* and *Bifidobacterium bifidum* on breast cancer cell line of MCF7 (11, 12), *Lactobacillus bulgaricus* and *Streptococcus thermophiles* against gastrointestinal cancers (13), *Lactobacillus acidophilus* and *Lactobacillus casei* on colorectal tumor LS315 cell line (14) and *Lactobacillus casei* and *Lactobacillus paracasei* against bone marrow cancer K562 cell line (15, 16). Among LAB, Lactococci are homo-fermentative non-motile gram positive catalase negative mono-, diplo- and streptococci with short chains that are able to grow in dairy products at 7 °C $\geq$ temperatures with the type sp. of *Lactococcus lactis* (17). So far there has been no report indicating the anti-carcinogenic effects of *Lactococcus* spp. against breast cancer cell lines. Also many health benefits associated with the consumption of probiotics have been proven by researchers; therefore, the present study was conducted to isolate and identify the lactococci from dairy products and to evaluate their cytotoxic effects on breast cancer SK-BR-3 cell line.

Materials and Methods

Sample Collection and Isolation of LAB: Traditional cheese from Broujen city (Chahamahal and Bakhtiari Province, Iran) and yogurt from Meymeh city (Isfahan Province, Iran) were collected in sterile ice-cooled tubes and transferred to the microbiology laboratory. To prepare bacterial suspension from yogurt and cheese samples, 10 g of each sample was added to 100 ml sterile distilled water and then shacked for 30 minutes. Then, 10 ml of bacterial suspension was separately transferred to 200 ml MRS broth and then incubated anaerobically at 37 °C for 24 hours. Then dilution was performed separately from each sample and 10 µl from each dilution streaked on MRS medium and incubated at 37 °C for 72 hours. For the

purification purposes, the individual colonies were isolated with sterile loop and streaked on MRS agar and incubated at 37 °C for 24 hours (18). All isolated *Lactobacillus* species were identified by macroscopic, microscopic and biochemical examinations. They were initially tested for colony morphology, gram reaction and catalase activity. Gram-positive and catalase-negative bacillus colonies were selected and stored at -80 °C in MRS broth containing 25% (v/v) glycerol (19).

DNA Extraction and Amplification of LAB 16S-rDNA Gene Sequences: The pure colonies of LAB were transferred to 50 ml sterile distilled water from the culture medium and 10 ml of this suspension was transferred to a sterile 15 ml Falcon and centrifuged at 5000 g for 15 minutes. The supernatant was discarded and the pellet was transferred to a sterile tube. DNA extraction kit (Bioneer, South Korea) was used for DNA extraction from 1 mg of bacterial mass. PCR amplification was carried out in a thermo cycler system (Bio Rad), using a pair of LAB-specific universal primers (Table 1). Each PCR reaction contained 1 µl of each primer (200 pM), 0.75 µl of $MgCl_2$ (1.5 mM), 0.5 µl of dNTP mix (200 mM), 0.2 µl of Taq polymerase (1 U), 2.5 µl of PCR Buffer, 2 µl of DNA template and 19.05 µl of distilled water. The PCR program contained initial denaturation at 94 °C for 5 minute, followed by 32 cycles amplification where each cycle involved three separate stages of denaturing at 94 °C for 45 seconds, annealing at 58 °C for 45 seconds and extension at 72 °C for 45 seconds. The final extension was performed at 72 °C for 5 minutes. The PCR products were resolved by electrophoresis in a 1% (w/v) agarose gel and visualized by ethidium bromide staining. Finally, the PCR products were purified and sequenced by Taligene Pars Co., Isfahan Science and Technology Town, Isfahan, Iran. The sequence similarity was determined by GenBank BLASTN analysis (20).

Table 1- The Sequences and the Names of Universal Primers used in PCR

Primer name	Primer Sequences
BU1(Forward)	5'-AACTGGAGGAAGGTGGGGAT-3'
BU1(Reverse)	5'-AGGAGGTGATCCAACCGCA-3'

Evaluation of the Cytotoxic Effect of LAB Isolates on SK-BR-3 Breast Cancer Cell Line using MTT Assay: SK-BR-3 breast cancer cell line was provided from Pasteur Institute of Iran. The isolated bacteria were initially incubated at MRS broth for 24 hours until the bacteria reached the highest amount (cfu/ml). Then, the cytotoxic effects of isolate secretion metabolites on SK-BR-3 cell line were evaluated by microculture MTT assay. SK-BR-3 cells were seeded into each well of microplate with 180 µl of RPMI growth medium containing 5×10^3 cells in suspension. Then, the cells were treated with three replications, with 20 µl of each bacterial concentration (100, 250,500 and 1000 µl/ml). Then, the 20 µl of RPMI 1640 medium was added to the first well of each row and was selected as the negative control, for the second well of each row, 20 µl of Doxorubicin (200 µg/ml) was added and this row considered as the positive control. Then, 20 µl of various concentrations of bacterial suspensions were added to wells except for negative and positive controls, and subsequently all plates were incubated at 37 °C for 24, 48, and 72 hours. Finally, 20 µl of MTT was added to each plate and incubated at 37 °C for 3 hours. The supernatant was then removed gently, and 150 µl of DMSO was added to the wells and pipetted until the formazan crystals dissolved. Immediately after pipetting, the OD of each well was measured by employing a microplate ELISA reader at 540 nm (21).

Preparation of Various Concentrations of Bacterial Suspension: The purified bacteria were initially incubated in MRS

broth for 24 hours until the highest growth rate was reached. To prepare the desired concentrations of 100, 250, 500 and 1000 µl/ml of bacterial dilution, the RPMI 1640 medium was applied. Each bacterial concentration was added to microplate well with 3 replications.

Statistical Analysis: Statistical analysis was performed using SPSS software version 19.0. Statistical differences in multiple groups were determined by one-way Anova, Two-way Anova, Friedman Test, and Kruskal Wallis Test. Multiple mean comparisons were performed using Mann-Whitney U-test and Wilcoxon Signed Ranks Test. All numerical data were presented as mean ± standard deviation and $P \leq 0.05$ was considered to be statistically significant (22).

Results

Isolation and Identification of LAB using Macroscopic, Microscopic and Molecular Analysis: Results of gram staining showed that all isolated strains from yogurt and cheese samples were bacilli, gram positive, and without spore and their colonies were round-shaped, mucoid, and milky. Molecular identification of strain isolated from the yogurt sample using 16S-rDNA sequencing showed that the isolate was related to *the Lactococcus lactis* species. The sequence analysis of 16S-rDNA of *Lactococcus lactis* showed that the total nucleotide sequence of this isolate was 355 bp. (Fig. 1). The alignment of this isolate sequence with all available sequences for 16S-rDNA in NCBI, GenBank, showed the high similarity (%99) to *Lactobacillus plantarum* 16S-rDNA gene. This new isolate was identified as *Lactobacillus plantarum* HBM-IAUF-1 and its partial sequence was deposited in GenBank under the accession No. MG757680. Figure 2 shows the phylogenetic tree of *Lactobacillus plantarum* HBM-IAUF-1. The molecular identification of the isolated strain from cheese sample using 16S-rDNA sequencing showed that total nucleotide sequence of this isolate was 349 bp. (Fig. 1).

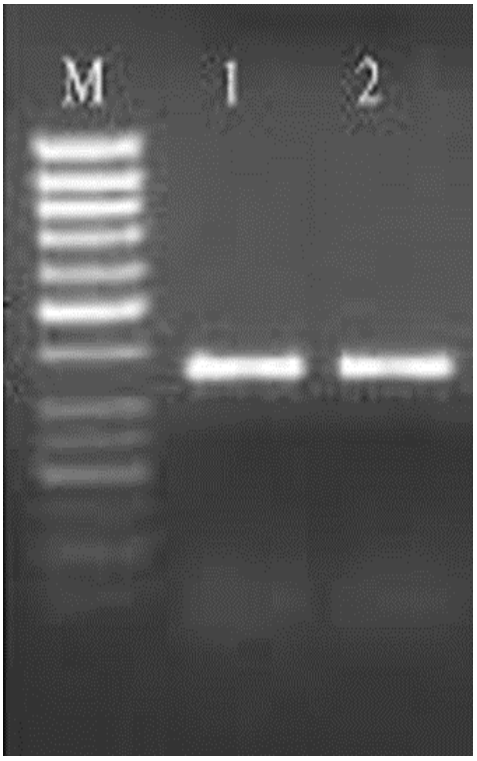

Fig. 1- The Gel Electrophoresis of 16s-rDNA Gene Amplification of *Lactobacillus Plantarum* HBM-IAUF-1 with MW of 355 bp (1) and *Lactococcus lactis* HBM-IAUF-8 with MW of 349 bp (2). M: 100 bp DNA Marker

The alignment of this isolate sequence with all available sequences for 16S-rDNA in NCBI, GenBank, showed the high similarity (%99) to *Lactococcus lactis* 16S-rDNA gene. This new isolate was identified as *Lactococcus lactis* HBM-IAUF-8 (accession No: MG757734) strain based on the results of phylogenetic analysis and nucleotide sequence of the 16S-rDNA gene of this strain (Fig. 3).

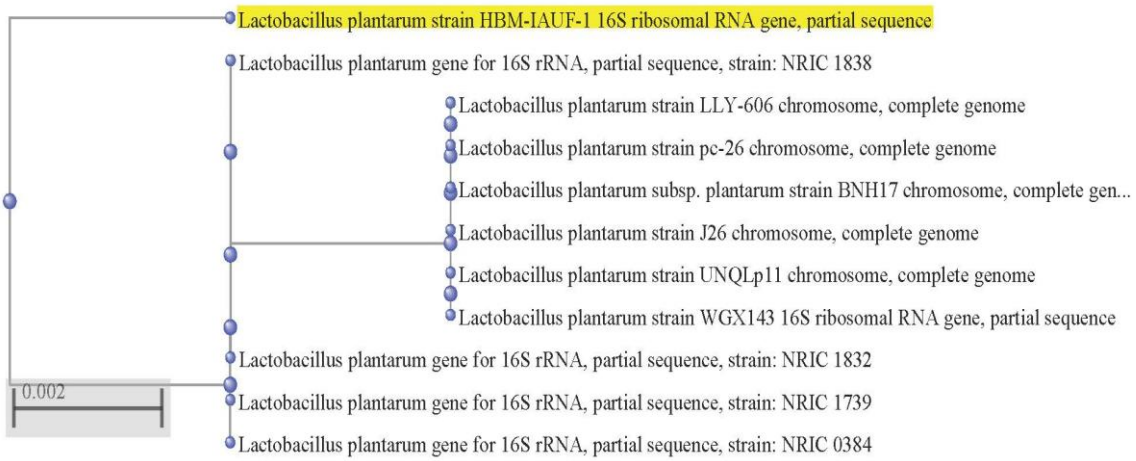

Fig. 2- Phylogenetic Tree of the 16s-rDNA Gene of *Lactobacillus Plantarum* HBM-IAUF-1

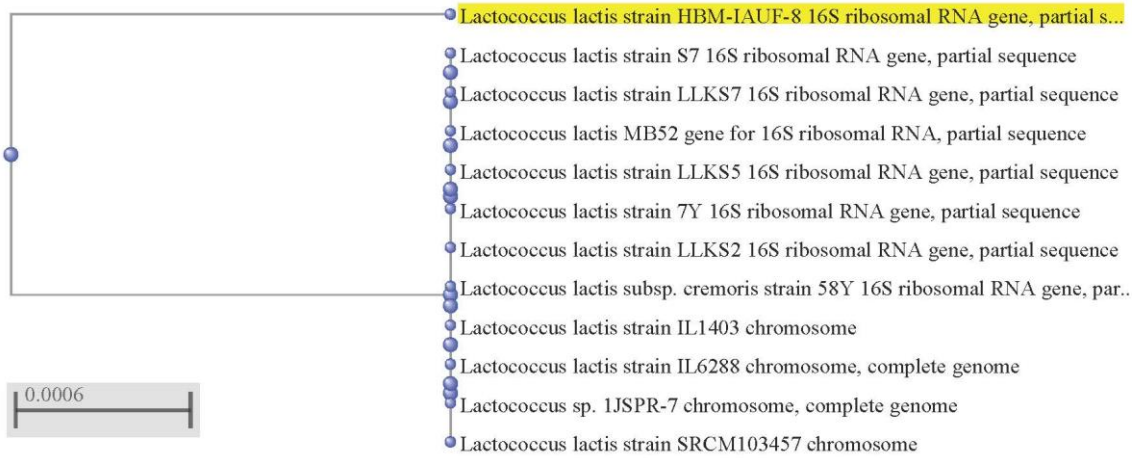

Fig. 3- Phylogenetic Tree of the 16s-rDNA Gene of *Lactococcus Lactis* HBM-IAUF-8

Evaluation of the **Cytotoxic Effect of LAB Isolates on SK-BR-3 Breast Cancer Cell Line using MTT Assay:** This method is usually used to evaluate cytotoxicity, proliferation and viability of living cells in a well plate format. In this study, cytotoxic effects, time of exposure, and the concentration of lactobacilli isolated from yogurt and cheese samples were examined on SK-BR-3 breast cancer cell line at different times of 24, 48 and 72 hours. The results of cytotoxic effects of *Lactobacillus plantarum* HBM-IAUF-1 on SK-BR-3 cell line after 24 hours showed that there was a significant difference ($P<0.05$) between mean values of two concentrations of 250 and 1000 µl/ml with the control. The results also showed that the least cell survival percentage of SK-BR-3 cell lines after 24 hours treatment with various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 suspensions belonged to 250 µl/ml (32.2 ± 44.95) and the most cell survival rate belonged to 500 µl/ml (166.18 ± 95.87). The IC50 for this isolate was measured as 507.20 ± 0.32 µl/ml. The results of cytotoxic effects of *Lactobacillus plantarum* HBM-IAUF-1 on SK-BR-3 cell lines after 48 hours showed that there was a significant difference between mean values of all concentrations with the control. Results also showed that the least cell survival percentage of SK-BR-3 lines after 48 hours of treatment with various

concentrations of *Lactobacillus plantarum* HBM-IAUF-1 suspensions belonged to 250 µl/ml (18.10 ± 44.35) and the most cell survival rate belonged to 1000 µl/ml (140.90 ± 60.99). The measured IC50 for this isolate was 440.40 ± 0.29 µl/ml. The results of cytotoxic effects of *Lactobacillus plantarum* HBM-IAUF-1 on SK-BR-3 cell line after 72 hours showed that the mean value of both 500 and 1000 µl/ml concentrations were significantly lower than the control treatment. Results also showed that the least cell survival percentage of SK-BR-3 cell line after 72 hours of treatment with various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 suspensions belonged to 250 µl/ml (11.00 ± 24.65) and the most cell survival rate belonged to 1000 µl/ml (95.70 ± 89.61). The measured IC50 for this isolate was 365.60 ± 0.34 µl/ml (Fig. 4). The results of the cytotoxic effects of *Lactobacillus plantarum* HBM-IAUF-1 on SK-BR-3 cell line after 24, 48, and 72 hours showed that the mean value of SK-BR-3 cell survival percentage was significantly different in all concentrations. The least cell survival percentage of SK-BR-3 line after 72 hours of treatment with various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 suspension belonged to 1000 µl/ml (11.24 ± 0.65) and the most cell survival rate belonged to 1000 µl/ml (166.95 ± 18.87). The IC50 for this isolate was 365.6 ± 0.34 µl/ml (Fig. 5). The results of the cytotoxic effects of *Lactococcus lactis* HBM-IAUF-8 on SK-BR-3 cell line after 24 hours showed that the mean value of SK-BR-3 cell survival percentage in the concentrations of 100, 250 and 500 µl/ml was significantly higher than the control treatment. The least cell survival percentage of SK-BR-3 line after 24 hours treatment with various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 suspension belonged to 1000 µl/ml (81.17 ± 36.30) and the most cell survival rate belonged to 100 µl/ml (178.22 ± 58.46). The measured IC50 for this isolate was 669.02 ± 0.32 µl/ml. The results of cytotoxic effects of *Lactococcus lactis* HBM-IAUF-8 on SK-BR-3 cell line after 48 hours showed that the mean value of SK-BR-3 cell survival percentage in concentrations of 100 and 1000 µl/ml was significantly different with the control treatment. The least cell survival percentage of SK-BR-3 cell line after 48 hours of treatment with various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 suspension belonged to 1000 µl/ml (31.4 ± 33.00) and the most cell survival rate belonged to 100 µl/ml (128.10 ± 66.95). The measured IC50 for this isolate was 536.9 ± 0.25 µl/ml. The results of cytotoxic effects of *Lactococcus lactis* HBM-IAUF-8 on SK-BR-3 cell line after 48 hours showed that the mean value of SK-BR-3 cell survival percentage in all concentrations was significantly lower than the control treatment. The least cell survival percentage of SK-BR-3 line after 48 hours of treatment with various concentrations of *Lactococcus lactis HBM-IAUF-8* suspension belonged to 500 µl/ml (12.00 ± 56.66) and the most cell survival rate belonged to 100 µl/ml (66.7 ± 53.36). The IC50 for this isolate was 175.9 ± 0.29 µl/ml (Fig. 6). The results of the cytotoxic effects of *Lactococcus lactis* HBM-IAUF-8 on the SK-BR-3 cell line after 24, 48, and 72 hours showed that the mean value of SK-BR-3 cell survival percentage in the concentrations of 100 and 500 µl/ml was significantly different. The least cell survival percentage of SK-BR-3 cell line after 72 hours of treatment with various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 suspension belonged to 500 µl/ml (12.56 ± 0.66) and the most cell survival after 24 hours of treatment belonged to 100 µl/ml (178.58 ± 22.46) (Fig. 7).

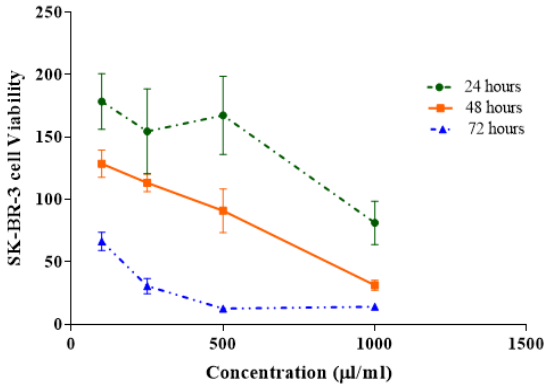

Fig. 4- The Effect of *Lactobacillus Plantarum* HBM-IAUF-1 Supernatant on the Viability of SK-BR-3 Cells for Various Concentrations and Incubation Times after 24, 48 and 72 Hours of Treatment. Data are expressed as the mean viability ratio ± SD.

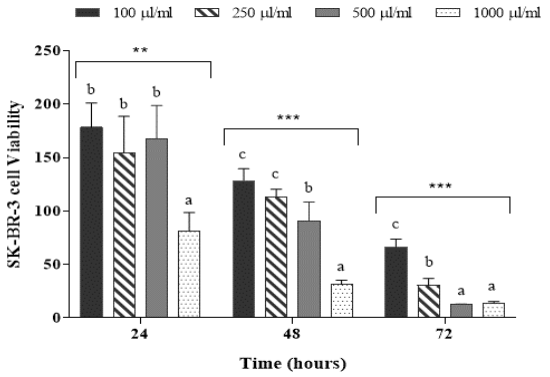

Fig. 5- The effects of various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 supernatant on the viability of SK-BR-3 cells in comparison with the time of exposure. Data are expressed as the mean viability ratio ± SD.

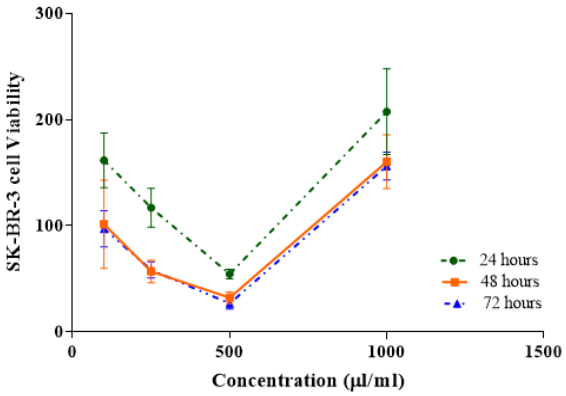

Fig. 6- The Effects of *Lactococcus Lactis* HBM-IAUF-8 Supernatant on the Viability of SK-BR-3 Cells for Various Concentrations after 24, 48 and 72 Hours of Treatment. The data are expressed as the mean viability ratio ± SD.

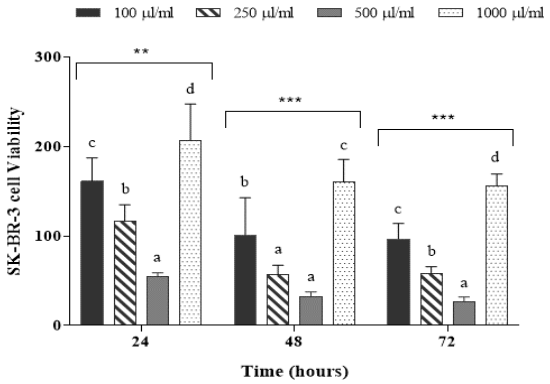

Fig. 7- The Effects of Various Concentrations of *Lactococcus Lactis* HBM-IAUF-8 Supernatant on the Viability of SK-BR-3 Cells in Comparison with the Time of Exposure. The data are expressed as the mean viability ratio ± SD.

Discussions and Conclusions

The precise mechanisms by which LAB may inhibit any cancer are currently unknown. However, such mechanisms might include alteration of the metabolic activities of intestinal microflora, alteration of physicochemical conditions in the colon and gut, binding and degrading potential carcinogens, quantitative and qualitative alterations in the intestinal microflora incriminated in producing putative carcinogens and promoters, production of anti-tumorigenic or anti-mutagenic compounds, enhancing the host's immune response and effects on the physiology of the host (23). The efficacy of anticancer drugs is measured by their ability to detect cancer cells and selectively promote their apoptosis. However, in the screening of beneficial compounds for cancer treatment, finding compounds that induce apoptosis is very important (24). Taverniti and Guglielmetti (2011) reported that *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, and *Lactobacillus casei* cause the survival rate reduction in HT-29 and Caco-2 cell lines (25). Liu and Pan (2010) reported that the human breast adenocarcinoma cells of MDA-MB-231 treated with local lactobacilli strains of *L. paracasei* ssp. *paracasei* NTU101, and *L.*

plantarum NTU102 showed the highest inhibitory effect at G0/G1 phase in the cell cycle. Their results showed that the inhibitory effects on cancer cells were different upon various LAB strains. They reported that their Thailand local lactobacilli strains had strong anticancer activities (26). McGuir et al. (2015) have reported that the use of fermented milk provided by *Lactobacillus bulgaricus* and *Streptococcus thermophiles* resulted in the decrease of cancer occurrence frequency (13). It has been reported that the *Lactobacillus acidophilus* and *Lactobacillus casei* could increase the apoptosis in colorectal cancer LS315 cell line. Also, it has been proposed that these probiotic bacteria could be used with chemotherapeutic measures simultaneously (14). Some reports are indicating the cytopathic effects of *Lactobacillus casei* and *Lactobacillus paracasei* cytoplasmic extracts on bone marrow cancer K562 cell line. These LAB could induce the apoptosis commence in the mentioned cancer cells (15, 16). Biffi et al. (1997) reported the cytotoxic effects of fermented milk LAB of *Bifidobacterium infantis, Bifidobacterium bifidum, Bifidobacterium animalis, Lactobacillus acidophilus* and *Lactobacillus paracasei* on breast cancer cell line of MCF7 (11). There is a study clarifying the cytotoxic effects of *Lactococcus lactis* on the gastrointestinal cancer cell line of AGS (27). So far there has been no report of anti-carcinogenic effects of *Lactococcus lactis* strains against breast cancer cell lines. In this study, the authors isolated two lactococci spp. from local dairy products. According to the molecular analysis, the isolated LAB from cheese sample was *Lactococcus lactis* HBM-IAUF-8 and from yogurt sample was *Lactobacillus plantarum* HBM-IAUF-1. The cytotoxic effects of secretion metabolites of *Lactobacillus plantarum* HBM-IAUF-1 and *Lactococcus lactis* HBM-IAUF-8 were well observed. Microscopic observation of SK-BR-3 cells treated with various concentrations of *Lactobacillus plantarum* HBM-IAUF-1 and *Lactococcus lactis* HBM-IAUF-8 secretion metabolites showed that the metabolites had cytotoxic effect on SK-BR-3 cells. In order to evaluate the cytotoxic effect of this isolate on SK-BR-3 cells, the MTT method was employed as the validation test for isolates with maximum inhibition at lowest time. According to the results, both *Lactobacillus plantarum* HBM-IAUF-1 and *Lactococcus lactis* HBM-IAUF-8 showed significant anti-cancer effects on SK-BR-3 cells. The cytotoxic effects of *Lactococcus lactis* HBM-IAUF-8 on SK-BR-3 was dependent on the concentration of LAB and the incubation time. The cell survival percentage of SK-BR-3 was reduced upon the elevation of LAB concentration and treatment time. The cytotoxic effects of *Lactobacillus plantarum* HBM-IAUF-1 were dose-dependent after 24 and 48 hours of treatment, but it was not time-dependent. This could be due to the increase of metabolite secretion of bacteria with the increase of incubation time or due to the reaction of cancer cells after the exposure to those metabolites. Though the low genetic distance of two isolates, the difference in cytotoxic effects of both isolates may be related to the genetic divergence of two isolates. In conclusion, this is the first report of isolation and identification of novel lactococci from traditional Iranian dairy products (yogurt and cheese) that had anti-carcinogenic effects on breast cancer cell line of SK-BR-3. These isolates were named *Lactobacillus plantarum* HBM-IAUF-1 and *Lactococcus lactis* HBM-IAUF-8 and their 16s-rDNA genome sequences were deposited in GenBank, NCBI under the accession numbers of MG757680 and MG757734, respectively. The *Lactobacillus plantarum* HBM-IAUF-1 showed the highest

cytotoxicity on SK-BR3 cancer cells at the maximum time of 72 hours at the concentration of 1000 µl/ml. Also, *Lactococcus lactis* HBM-IAUF-8 showed the highest cytotoxicity at the maximum time of 72 hours at the concentration of 500 µl/ml. Accordingly, persuading people to use traditional organic dairy products and the increasing of probiotics applications in the food industry could be useful in health promotion against cancer development as well as a potential advantageous agent in food and medical biotechnology.

Conflicts of Interests

The authors declare no competing interests.

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