اثر عصارهٔ سلولی باکتری (بومی) پروبیوتیک لاکتوباسیلوس کازئی روی مرگ سلول‌های آدنوکارسینومای پانکراس (AsPC-1) و بیان ژن‌های آپپتوزی Bcl2، bax و DPC4

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چکیده

مقدمه: امروزه باکتری‌های پروبیوتیک مانند لاکتوباسیل‌ها به‌عنوان یکی از عوامل پیشگیری‌کنندهٔ ابتلا به بسیاری از بیماری‌ها ازجمله سرطان شناخته شده‌اند. هدف مطالعهٔ حاضر، بررسی آثار سمیت سلولی محلول رویی باکتری پروبیوتیک *لاکتوباسیلوس پاراکازئی* (IBRC_10784) روی ردهٔ سلولی سرطان پانکراس (ASPc-1) و آنالیز بیان ژن‌های آپپتوزی bax، Bcl2 و DPC4 بود.

مواد و روش‌ها: مطالعهٔ حاضر روی ردهٔ سلولی سرطانی پانکراس انجام شد و اثر محیط‌کشت و فراوردهٔ *لاکتوباسیلوس پاراکازئی* روی بیان ژن‌های دخیل در آپوپتوز و همچنین ژن DPC4 مطالعه شد. باکتری در محیط‌کشت اختصاصی MSR broth کشت داده و در انکوباتور CO_2 دار به‌مدت ۴۸ ساعت گرماگذاری شد. پس از مشاهدهٔ رشد باکتری، محیط‌کشت در شرایط ۹۰۰۰ rpm به‌مدت ۵ دقیقه سانتریفیوژ و محلول رویی جدا شد. سپس با استفاده از فیلتر سرنگی، این محلول استریل و در مراحل بعدی استفاده شد. در ادامه، سلول‌های AsPC-1 با محلول رویی کشت باکتری تیمار ۲۴ ساعته شدند و پس از آن، استخراج RNA و ساخت cDNA انجام شد. سپس واکنش Q-PCR انجام شد.

نتایج: نتایج نشان دادند محلول رویی کشت باکتری *لاکتوباسیلوس پاراکازئی* می‌تواند میزان بیان ژن‌های مطالعه‌شده را تغییر دهد؛ به‌طوری‌که بیان ژن bax را ۱/۸۶ برابر نسبت به ژن رفرنس افزایش داد. همچنین، بیان ژن bcl-2 را نسبت به ژن رفرنس ۴/۵۶ برابر کاهش داد. نکتهٔ درخور توجه در تغییر بیان ژن DPC4 بود؛ به‌طوری‌که بیان این ژن نسبت به ژن رفرنس به مقدار ۲/۶۷ برابر افزایش یافت و این مقدار تغییر بیان، معنادار ($P< 0.005$) بود.

بحث و نتیجه‌گیری: مطابق با نتایج ما به نظر میرسد که افزایش بیان ژن سرکوبگر تومور DPC4 که از عوامل ضروری در مسیر TGF-β میباشد می تواند در سرکوب سرطان پانکراس مهم باشد.

واژه‌های کلیدی: *لاکتوباسیل پاراکازئی*، پروبیوتیک، ردهٔ سلولی AsPC-1، سمیت سلولی، آپپتوز

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The Effects of Native *Lactobacillus Paracasei* Supernatant Solution on Death of Pancreatic Adenocarcinoma Cells (AsPC-1) and Expression of bax, bcl2, and DPC4 Apoptotic Genes

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Abstract

Introduction: Nowadays, probiotic bacteria such as Lactobacilli have been recognized as promising agents to prevent a large number of diseases, including cancer. This study aimed to evaluate the cytotoxic effects of *Lactobacillus paracasei* (IBRC_10784) extract on pancreatic cancer cell line ASPc-1 and to analyze the expression of bax, Bcl2, and DPC4 apoptotic genes**.**

Materials and methods**:** This study was conducted on ASPC-1 pancreatic adenocarcinoma cell line to assess the effects of culture medium and *Lactobacillus paracasei* products on the expression of genes involved in apoptosis as well as DPC4 gene expression. *Lactobacillus paracasei* was cultured on specialized MSR broth medium and incubated for 48 hours in a CO_2 incubator. After observing the growth of bacteria, the culture medium was centrifuged for 5 minutes at 9000 rpm and the supernatant was removed. Then, the supernatant was sterilized by a 0.22-micron syringe filter and was used in the subsequent steps. In the next step, AsPC-1 cells were treated with supernatant solution for 24 hours, followed by RNA extraction and cDNA synthesis. The Q-PCR reaction was performed using specific primers.

Results: The findings of this research showed that the supernatant solution of *Lactobacillus paracasei* culture was able to change the expression of the studied genes, so that the expression of bax gene increased 1.86 times relative to the reference gene. Moreover, the expression of bcl-2 gene decreased by 4.56 folds compared to the reference gene. A notable point was the changing expression of DPC4 gene, which significantly increased to 2.67 folds in comparison to the reference gene ($P<0.005$)**.**

Discussion and conclusion: According to the results of the present study, it seems that the increased expression of DPC4 tumor suppressor gene, which is one of the essential factors in TGF-β pathway, may be important in suppressing pancreatic cancer.

Key words: *Lactobacillus Paracasei*, Probiotic, AsPC-1 Cell Line, Cytotoxicity, Apoptosis

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Introduction

Probiotics are live and useful microorganisms having beneficial effects on the host health when used in humans, animals or as microbial flora of the body (1, 2). Most probiotics belong to a large group of major bacteria in human colon microflora. Some of these microorganisms are strains of Lactobacilli that are rarely pathogenic to humans and animals and their application has been demonstrated in the production of food products without any adverse effects (3, 4). According to studies conducted on probiotic bacteria, several Lactobacilli species including Bifidobacteria and yeasts have had higher efficacy than a variety of probiotics (5). Lactobacilli are polymorphic, immobile, and facultative anaerobic bacteria that produce energy through the fermentation of sugars and lactic acid accounts for at least half of their products (6, 7). In general, probiotics generate various agents like short-chain fatty acids, acetate, lactate, succinate, butyrate, H_2O_2, and bacterioc in compounds that affect both gram-positive and gram-negative bacteria (8, 9). Investigations indicated that probiotics play an effective role in coping with cancer by affecting digestive enzymes of animals and humans, inhibiting carcinogenic agents in vivo and in vitro, suppressing mutations, cancer-inducing compounds and tumors in laboratory animals (10-13).

Generally, cancer is a heterogeneous genetic disorder and the second leading cause of mortality in the world after cardiovascular diseases. Pancreatic cancer is highly aggressive and lethal, so that its mortality rate equals its incidence rate (14). Most pancreatic cancers are in advanced stages upon diagnosis since primary pancreatic tumors have unclear symptoms or are asymptomatic (15). In vitro studies have shown that probiotics play a role in suppressing primary neoplastic lesions and tumors in mouse models (16). Anticancer effects of probiotics are exerted by preventing the conversion of procarcinogens to carcinogens, binding and inactivating mitogenic compounds, decreasing the growth of procarcinogenesis bacteria, reducing the absorption of mitogens, and enhancing the immune function. Moreover, anti-proliferation effect on cancer cells is a functional mechanism of probiotics like Lactobacilli (17, 18).

Apoptosis is a programmed cell death that plays a key role in regulating the number of cells (19). In many cancers, the reduced ability to promote apoptosis impairs the process of cell proliferation. Regulation of viability and death of cells by molecules acting on the apoptotic process can have a significant role in the prevention and treatment of cancers (20). Several pieces of evidence indicated that probiotics can have a role in regulating cell proliferation and apoptosis (21). Bax and Bcl2, important genes involved in apoptosis, are implicated in the mitochondrial pathway of apoptosis. Since the balance of proliferation and apoptosis is disrupted in cancer cells and because cells need to inhibit apoptosis for their uncontrolled growth and proliferation, there is a direct link between the expression of these genes and the process of malignancy. Therefore, the review of the changing expression of these genes can be considered as a therapeutic or diagnostic target in cancer studies.

Based on the above data, there has been much progress in the assessment of probiotics effects on cell apoptosis and proliferation pathways, which may be the basis for the use of probiotics to cope with cancer and induce apoptosis of cancer cells shortly. Considering the impact of microflora on the reduction of cancers, it is important to produce new types of probiotic products that prevent cancers and have no harmful effects on healthy cells. Studies have shown that probiotic bacteria

belonging to a single genus may have different cellular compositions and various probiotic effects in different geographic regions. Therefore, this study aimed to investigate cytotoxic effects of native probiotic *Lactobacillus paracasei* (IBRC_10784) extract on apoptosis of pancreatic adenocarcinoma (AsPC-1) cells and to analyze the expression of bax, bcl2, and DPC4 apoptotic genes.

Materials and Methods

Purification of *Lactobacillus Paracasei* Colonies: In this study, the standard IBRC_10784 strain of *Lactobacillus paracasei* was obtained in lyophilized from the microbial collection of Iranian Biological Resource Center (IBRC, No. 80, West Hoveizeh St, North Sohrevardi Ave, Tehran, Iran) and cultured in MSR (de Man, Rogosa and Sharpe, cat.No. 30912, Biomaxima company of Poland) broth specific medium. Then, the grown bacteria were cultured on MRS agar medium to ensure the purity of colonies.

Culture of ASPc-1 Cell Line: The pancreatic adenocarcinoma cell line (ASPc-1) was purchased from the cell bank of IBRC. RP

MI1640 (Roswell Park Memorial Institute, cat. No. LM-R1637/500, Biosera company of United Kingdom) culture medium was used as the basal medium, to which penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively) and fetal bovine serum (Gibco, Gibco™, cat. No. 11573397, thermo fisher company of South American) at the final concentration of 10% were added to prepare the complete medium. RPMI1640 medium containing Hepes was purchased as a sterile liquid from Biosera Company and the cells were incubated in 5% CO_2 incubator at 37 °C.

Determination of Surviving in ASPC-1 Cell Line: MTT assay was employed to evaluate the effect of culture supernatant solution on the survival of ASPC-1 cell line. The effects of various concentrations of bacterial cell-free supernatants on the conversion of the MTT assay in cells after 24 hours compared with control groups are presented 98, 97, 96, 93, 90, 88,82,75,68 and 59 mg/ml to ASPC-1 cell line. After this time, 20 µl of MTT dye solution was added to each well and the incubation continued for 4 hours. Then, the supernatant was removed and 100 µl of DMSO was added to each well. After pipetting, the absorbance was read at 590 nm wavelength using ELISA Reader, and cell viability was calculated by the following formula: Absorption of treated cells divided by that of treated cells×100 =Cell survival rate. Half-maximal inhibitory concentration (IC50) was also calculated.

Quantitative Real-time PCR: Total RNA of treated cells was extracted using an RNA extension kit (RNX-plus, Guanidine/phenol solution for total RNA isolation from homogenized sample, CinnaGen Co.) according to instructions and its concentration which was measured by nanodrop.

cDNA molecules were generated using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). The reaction mixture contained 4 µl reaction buffer (×5), 1 µg RNA, 20 Mm of random six-nucleotide primer, 2 µl oligo dt primer, 2 µl deoxynucleotide triphosphate mixture (10 mM), 0.5 µl Rnase enzyme inhibitor, 1 µl reverse transcriptase enzyme, and 10 µl double distilled water. The temperature program was as follows: 94 °C for 3 minutes, 60 °C for 1 minute for annealing, 72 °C for 1 minute for extension (inactivation of reverse transcriptase), and 72 °C for 5 minutes for the final extension. The primers targeted bax, bcl2, and DPC4 genes and Beta actin gene were used as the internal control. The sequence of forward and reverse primers of bax gene was 5'-GAGCTGCAGAGGATGATTCG-3', and 5'-AAGTTGCCGTCAGAAAACACG -3'

consecutively. The forward and reverse primer sequences of Bcl2 target gene were 5'-ATTGGGAAGTTTCAAATCACG -3' and 5'-CAGTCTACTTCCTCTGTGATGTGT-3' consecutively, and 5'-TTGCTTCAGGGTTTCATCCAG-3' forward and 5'-TGGAAACAGTGAAAAACAACAG -3' reverse for DPC4 gene. Also 5'-TCCTCCTGAGCGCAAGTAC -3' forward and 5'-CCTGCTTGCTGATCCACATCT -3' reverse for Beta actin gene. The binding temperature of these genes was considered to be 60 °C. BLAST (http://www.ncbi.nlm.nih.gov/blast) was used to ensure the sequence of primers and lack of their binding to other nonspecific sequences in other parts of the genome. For measuring the expression levels of bax, bcl2, and DPC4 genes, the authors used Real-Time PCR. This reaction was performed by using Bioneer device (South Korea) as follows: 95 °C for 1 minute for hot start, 95 °C for 15 seconds for the initiation, 60 °C for 60 seconds for annealing, 72 °C for 1 minute for extension in 35 cycles. And then analysis of gene expression was performed using REST(Representational State Transfer) software and P<0.05 was considered as the significance level.

Results

The effects of different concentrations of bacterial cells on the conversion of the MTT assay in cells after 24 hours in comparison with the untreated control cells, which were read using ELISA Reader, are illustrated in Fig. 1.

Based on MTT results, the concentration of supernatant of Lactobacillus culture killing 50% of ASPC-1 cells (IC50) was equal to 116.75 ± SEM in three renewal and treated groups. This level is considered statistically significant when P< 0.005 (Figure1).

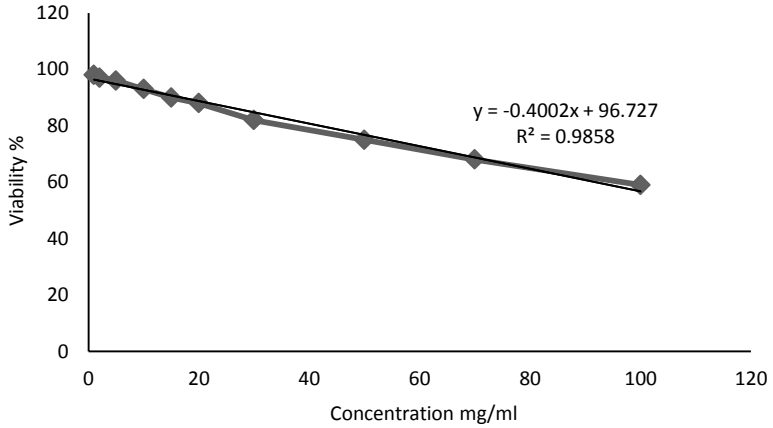

Fig. 1- Survival Rate of ASPC-1 after Treatment by Different Concentrations of Cell Extract, and IC5.0 Calculation of *Lactobacillus Paracasei* Culture Supernatant on ASPC-1 Cells

Expression analysis of Bcl2, Bax, and DPC4 apoptotic genes in ASPC-1 cells treated with IC50 concentration of cell extracts was performed after 24 hours using Real-Time PCR.

The difference in the threshold cycle among the studied samples (i.e. ASPC-1 cells treated with cell extracts) was calculated and the expression rate was determined using ΔΔCt formula relative to control groups (ASPC-1 cells Not treated with cell extracts) as illustrated in Fig. 2. The comparison of bax, bcl-2 and DPC4 sample groups with control groups are presented in Table1.

Table 1- The Effects of Bacterial Cell-free Supernatants on the Growth of Three Genes using MTT Assay

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
Beta actin	REF	0.96	1.000				
bax	TRG	0.98	1.869	1.063 - 2.852	0.873 - 3.426	0.271	
bcl-2	TRG	0.94	0.219	0.129 - 0.310	0.121 - 0.331	0.072	
DPC4	TRG	0.96	2.671	1.713 - 3.937	1.395 - 5.429	0.048	UP

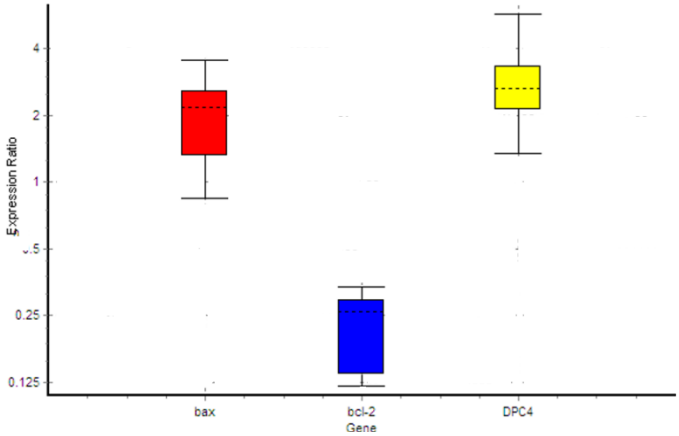

Fig. 2- The Expression of the Studied Genes in ASPC-1 Cell Treatment with *Lactobacillus Paracasei* Culture Supernatant and Control Groups

Discussion and Conclusion

Since the unchecked proliferation of cells and their resistance to programmed death are major features of malignant cells, any factor causing apoptosis in cancer cells could be regarded as an anticancer agent (22). During tumorization, additional growth signals are generated and the cells become resistant to natural growth-inhibitory signals. These abnormalities lead to the stimulation of programmed cell death (apoptosis) to inactivate the process in some ways (23). Food combinations and their relationship with the health of individuals have attracted the attention of many scientists. Probiotics, which are non-pathogenic microorganisms present in the digestive system of people, have beneficial effects on the host. The consumption of probiotics leads to the production of a wide range of fermentation products such as high concentrations of short-chain fatty acids (24). Among the probiotics, bacteria of Lactobacillus family such as Lactobacillus acidophilus, Lactobacillus casei, and Lactobacillus delbrueckii are the most important components of the normal flora of humans and animals (25). Lactobacillus casei ATCC 393 is a facultative non-pathogenic anaerobic bacterium and probiotic strain used in fermented dairy products and functional foods (26).

It is generally believed that specific LAB strains can beneficially activate anticancer mechanisms, thereby regulating the host's immune response (27).

The present study investigated the apoptotic effects of *Lactobacillus paracasei* on ASPC-1 cancer cell line. Overall, the induction of programmed cell death is an approach to cancer treatment. The apoptotic pathway can involve the activation of pro-apoptotic events in the cell, which begins with the permeability of the mitochondrial membrane to Bax and Bak proteins, resulting in the release of cytochrome C and eventual activation of Caspase 9 followed by Caspase 3 (28). The proposed effects may be mediated at least in part due to soluble factors, for example, bioactive metabolites, which are secreted from live bacteria. Compared to live bacteria, high

concentrations of cell-free supernatants were needed to induce considerable growth inhibition in ASPC-1 cancer cell line. In a recent study, Cell-Free Supernatants (CFS) from two LAB strains, L.casei and L.rhamnosus GG, were shown to inhibit colon cancer cell invasion by influencing matrix metalloproteinase-9 (MMP-9) activity and levels of the tight junction protein zona occludens-1 (ZO-1) in cultured metastatic human colorectal carcinoma cells. In another study, L.gasseri SF1183 produced molecule(s) with the ability to interfere drastically with HCT116 cell proliferation and stimulate G1-phasearrest (29).

The mechanism of the anti-proliferative activity of Lactobacillus strains against cancer cells is still unclear. However, it may be attributed to the production of organic acids by Lactobacillus strains and/or their antioxidant activity. Lactobacillus strains that produce antioxidant compounds may have anti-carcinogenic effects against some types of cancers. Since the antioxidant activity of lactic acid bacteria is a new approach, there are not too many reports on the antioxidant activity of lactobacilli. Hence, Lactobacillus strains could be considered as good potential probiotics for human consumption due to their beneficial antioxidant and anticancer effects.

ASPC-1 is a cancer cell line derived from adenocarcinoma of the pancreas head. After culturing, this ascites cell line results in the production of copious mucin as well as a carcinoembryonic antigen (30). Mutation in SMAD4/DPC4 has been detected in approximately 50% of pancreatic adenocarcinoma cases, which indicates a pivotal role in the progression of pancreatic cancer. DPC4 is, in fact, a tumor suppressor gene, and the complete lack of its expression is observed in 17.46% of pancreatic tumors. Pancreatic cancer shows heterogeneous mutagenic characteristics with active mutations in more than 90% of DPC4 genes with an incidence of <5%.

In previous studies, the role of DPC4 gene in pancreatic cancer was investigated. DPC4 suppressor gene is an essential transcription factor in TGF-β pathway, which is frequently mutated and deleted in pancreatic cancer (31, 32). A noteworthy point is the changing expression of DPC4 gene, which increased 2.67 folds relative to the reference gene and indicated a significant change ($P<0.005$). The DPC4 tumor suppressor gene is an essential transcription factor in TGF-β pathway, which is regularly mutated and deleted in pancreatic cancer. Therefore, increasing the expression of DPC4 can be important in the suppression of pancreatic cancer.

In the present research, the supernatant of Lactobacillus paracasei culture managed to change the expression of the studied genes and increase the expression of bax gene by 1.86 times in comparison with the reference gene. Furthermore, the expression of bcl-2 gene was decreased by 4.56 fold compared to the reference gene. In the present study, the effects of lactobacilli casein on the pro-apoptotic BAX gene expression and the decrease in bcl-2 gene expression in pancreatic cancer cells and the induction of planned cell death were demonstrated. Shin et al. (2017) studied DPC4/SMAD4 genetic in resected pancreatic ductal adenocarcinoma. The results showed that the genetic status of DPC4 was associated with the overall survival with recurrence patterns and inactivation of the DPC4 gene was the predictor of metastatic recurrence. Additional studies in mice show that lactobacillus was among the quickest flora to recover in the gut after antibiotic therapy (33). Therefore, further investigation is required to determine mRNA expression profile of these genes, which could be an indication of its role in the prediction of cancer response to treatment.

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There were no relevant financial interests.

Conflict of Interests

The authors had no conflict of interest in this study.

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