

# Research Article:

## Lactobacillus Acidophilus Cytotoxicity Effect and Apoptosis in Human Bladder Carcinoma Cells: An *In Vitro* Study



Faeghe Taheri<sup>1</sup> , Elham Moazamian<sup>1</sup> , Mehdi Mahdavi<sup>2,3,4\*</sup>

1. Department of Microbiology, College of Sciences, Agriculture and Modern Technology, Shiraz Branch, Islamic Azad University, Shiraz, Iran.
2. Recombinant Vaccine Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
3. Immunotherapy Group, Institute of Pharmaceutical Science (TIPS), Tehran University of Medical Science, Tehran, Iran.
4. Departments of Immunology, Pasteur Institute of Iran, Tehran, Iran.



**Citation** Taheri F, Moazamian E, Mahdavi M. Lactobacillus Acidophilus Cytotoxicity Effect and Apoptosis in Human Bladder Carcinoma Cells: An *In Vitro* Study. Immunoregulation. 2020; 3(2):127-134. <http://dx.doi.org/10.32598/immunoregulation.3.2.3>

<http://dx.doi.org/10.32598/immunoregulation.3.2.3>



### Article info:

**Received:** 23 Feb 2020

**Accepted:** 26 May 2020

**Available Online:** 01 Jan 2021

### Keywords:

Probiotic bacteria,  
Apoptosis, Bladder cancer

## ABSTRACT

**Introduction:** Anticancer effects of Lactobacillus acidophilus, as a probiotic bacterium, have been indicated in several studies. There are common therapeutic options for bladder cancer treatment; however, their side effects and recurrence of disease are considerable. Therefore, complementary medication is essential and it must be safe and effective. In the present research, we assessed the anti-tumor activity of Lactobacillus acidophilus (LB) on the bladder cancer cell line by evaluating the apoptosis mechanism.

**Materials and Methods:** EJ138 bladder cancer cell line was provided and the cytotoxicity level of LB was evaluated with various concentrations after 24, 48, and 72 h. To evaluate the apoptosis effect, the gene expression of Bax, BCL2, and TNF- $\alpha$  was assessed using real-time PCR.

**Results:** The optimum concentration of the cytotoxicity level was 12.5  $\mu$ g/mL at 24, 48, and 72 h. Bax expression was upregulated in tumor cells treated with LB alone and the mixture of LB and BCG (LB/BCG) as compared to the control group after 24 and 48 h. Also, the mRNA level of TNF- $\alpha$  increased in treated cells with LB and LB/BCG compared to the control group. BCL2 expression did not show a significant difference between experimental groups versus the control group.

**Conclusion:** Our findings showed that LB has a synergistic effect with BCG through increasing apoptotic gene expression and TNF- $\alpha$  cytokine. Besides, at 48 h treatment, the apoptosis effect is more than 24 h. Thus, it seems that the mode of action of LB is time-dependent.

### \* Corresponding Author:

Mehdi Mahdavi, PhD.

**Address:** Departments of Immunology, Pasteur Institute of Iran, Tehran, Iran.

**Phone:** +98 (21) 64112840

**E-mail:** m\_mahdavi@pasteur.ac.ir ; mahdavivac@gmail.com

## Introduction

**B**ladder cancer is the 13 common cause of mortality and chemotherapy, radiotherapy, and BCG therapy are the common treatments for this cancer [1, 2]. Tumor cells have multiple mechanisms for evading from immune responses and as a result, they can grow and proliferate [3]. Although there are various therapeutic options for patients with bladder cancer, these patients experienced some adversary complications during their treatment such as recurrence of cancer, vomiting, diarrhea, fatigue, etc.

Therefore, alternative or complementary medications are important with their least deleterious effects. In this regard, probiotics have beneficial effects for the host such as producing preventive compounds, inhibiting competition against pathogenic agents for chemical materials, stimulating connection places, regulating immune system activity, and improving intestine microbial balance [4, 5]. Moreover, probiotics are known not only as growth inducer but also stimulator for the immune system to prevent many infectious diseases [6]. Today, Lactobacillus, and Bifidobacterium are often used as probiotics and also most of them are recognized harmless [7].

The Lactobacillus bacteria have the basil and cocobasil shape. They are Gram-positive; non-spore forming; catalase, oxidase, and indole negative. Also, they belong to the Lactobacillacea family. Lactobacillus strains potentiate the intestine mucosal barrier which could keep and promote the immune status and decrease bacteria movement. Along with this, they have a role in treating intestinal inflammatory diseases and Inflammatory Bowel Syndrome (IBS). Now, cancer common therapeutics such as surgery, radiotherapy, and chemotherapy could kill the tumor and also normal cells. Therefore, adversary effects would be considered in these patients [8].

Acid lactic bacteria can prevent colon cancer with certain mechanisms such as alteration of metabolic activities of intestine microflora, alteration of colon chemicals, binding to carcinogens and destroying them, changing of intestine microflora (both quantity and quality), inhibiting carcinogenic compounds like ammonia, secondary bile acids, producing anticancer substances, and potentiating host immunity [9].

Some studies demonstrated that probiotics intake could increase natural killer cell activity and immunoglobulin status. Moreover, probiotics have anticancer performance through apoptosis induction. Therefore, probiotic

bacteria can be considered as a therapeutic agent without any side effects [10]. Probiotics can reduce fecal enzyme activity, prevent intestine harmful bacteria, and decrease solution bile acid. Additionally, secreted metabolites by probiotics could affect epithelial cells which result in decreased proliferation of cancerous cells [11].

Several studies showed that the simultaneous use of two probiotics could have a synergism effect. For the first time, we evaluated the synergism effect of Lactobacillus acidophilus (LB) and BCG on the immune system and the cytotoxic effects on bladder tumor cells and gene profiles which were involved in the apoptosis mechanism.

## Materials and Methods

### Probiotic bacteria culture

A standard strain of Lactobacillus acidophilus (PTCC:1643) were prepared from the microbial collection of the Scientific-Industrial Research organization of Iran. Initially, lyophilized cells were cultured in MRS broth for 24 h. After centrifuging, the supernatant was discarded and the bacteria residual was frozen at  $-70^{\circ}\text{C}$  and refrozen at  $50^{\circ}\text{C}$  for five times. Finally, bacterial cells were sonicated and a pellet of bacteria was provided for cytotoxicity assay with various concentrations of the cells.

### Cell lines culture

The present study used the EJ138 cell line which was provided by Pasteur Institute in Tehran. The cellular suspension was transferred into RPMI-1640 medium containing 10% FBS and some antibiotics such as streptomycin ( $100\ \mu\text{g/mL}$ ) and penicillin ( $1000\ \text{U/mL}$ ).

### Cytotoxicity assay

The MTT assay was performed to evaluate the cytotoxicity effect of the extraction of LB on the EJ-138 cell line. At first,  $100\ \mu\text{L}$  suspension of bacteria was cultured in RPMI-1640 medium with 10% FBS serum. Then, the cells were incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 24 h. Diluted extracts with various concentrations of each extraction were added to segregated wells in triplicate. After incubation for 24, 48, and 72 h,  $20\ \mu\text{L}$  of MTT solution was dispensed to each well and incubated for 4 h again. Finally, the supernatant was discarded and  $100\ \mu\text{L}$  of DMSO was added to each well and the plates were read by an ELISA reader in 570 wavelengths.

Gene expression assay

Cellular RNA was isolated by total RNX plus kit (CinnaGen, Iran) according to the manufacturer’s instructions. Then, cDNA was synthesized by using a cDNA synthesis kit (Yekta Tajhiz, Iran) according to the manufacturer’s instructions. In the present study, the mRNA level of BCL2 and Bax was evaluated in cancerous cells and also TNF-α gene expression was measured by real-time PCR. In addition to primers of BCL2 and Bax genes, one more pair of primers were designed for the β-actin gene as a housekeeping gene. Along with, Master Mix (Bioneer) primers of β-actin, BCL2, and Bax genes were used duplicate, and also cDNA TNF-α gene expression was measured. Finally, the quality and quantity of extracted RNAs were evaluated by Nanodrop and electrophoresis machine.

Statistical analysis

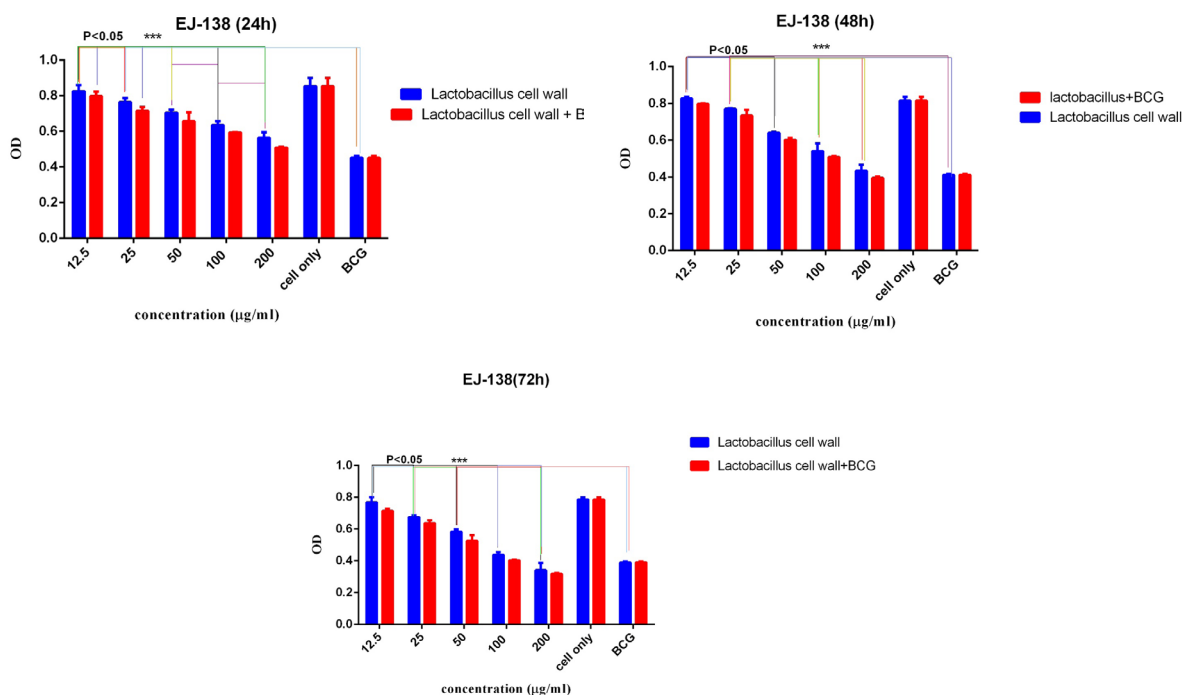
Data analysis was carried out with GraphPad Prism and the results were evaluated by One-way ANOVA test. Also, target genes expression between control and treated samples were measured and compared by the Tukey post-hoc test. The data analysis of real-time PCR was performed by threshold cycle and its formula is  $2^{-\Delta\Delta Ct}$ .

Results

Cytotoxicity

We evaluated the cytotoxicity effect of LB with various concentrations of this bacterium (12.5, 25, 50, 100, and 200 μg/mL) after 24 and 48 h incubation. After 24, 48, and 72 h, the treated cell line with 12.5 μg/mL of LB extract showed a significant increase compared to treated cells with the concentrations of 50, 100, and 200 μg/mL (P<0.0001). Also, treatment with 12.5 μg/mL caused a significant increase in cytotoxicity levels as compared to the treated cells with BCG (P<0.0001). Besides, there was a higher cytotoxicity effect in tumor cells treated with 25 μg/mL of the probiotic bacteria than with 100 and 200 μg/mL (P<0.0189).

The cancerous cells treated with 25 μg/mL of LB showed a remarkable increase in cytotoxicity effect compared to 100 and 200 μg/mL concentration of LB/BCG (P<0.0001) (Figure 1a,b). There were no significant differences between other concentrations versus each other. As such, 12.5 μg/mL was the best optimum concentration (IC50) and had the most cytotoxicity effect on EJ138 tumor cells of bladder cancer.



**Figure 1.** Cytotoxicity effect of Lactobacillus acidophilus on EJ138 tumor cells of bladder cancer A, B, C: After 24 h, 48 h, and 72 h treatment, respectively. The optimum cytotoxicity level has been obtained at a concentration of 12.5 μg/mL. There was a higher cytotoxicity effect in tumor cells treated with 12.5 μg/mL of the probiotic bacterium than other concentrations (P<0.0001).

**Gene expression**

**TNF- $\alpha$ :** Results of TNF- $\alpha$  expression showed a significant upregulation in treated cells with LB compared to LB/BCG after 24 h treatment ( $P<0.0472$ ). A significant increase in the expression of TNF- $\alpha$  was observed between the cells treated with LB/BCG at 24 h compared to the cells treated with LB and the control group ( $P<0.005$ ). There was a significant upregulation in TNF- $\alpha$  expression in cells treated with LB/BCG in comparison to the control group at 48 h treatment ( $P<0.0001$ ). Also, the experimental group treated with BCG showed a significant elevation in the mRNA level of TNF- $\alpha$  compared to the control group after 48 h treatment ( $P<0.0098$ ) (Figure 2).

**Bax gene:** Bax expression was increased remarkably in the experimental group treated with LB as compared to LB/BCG group after 24 and 48 h treatment ( $P<0.0039$ ). Results showed a significant elevation in Bax expression when cells were treated with LB/BCG compared to the control group ( $P<0.0001$ ). There was a significant upregulation in Bax expression in the group treated with LB and LB/BCG versus the control group ( $P<0.0225$ ,  $P<0.0001$ , respectively) (Figure 2).

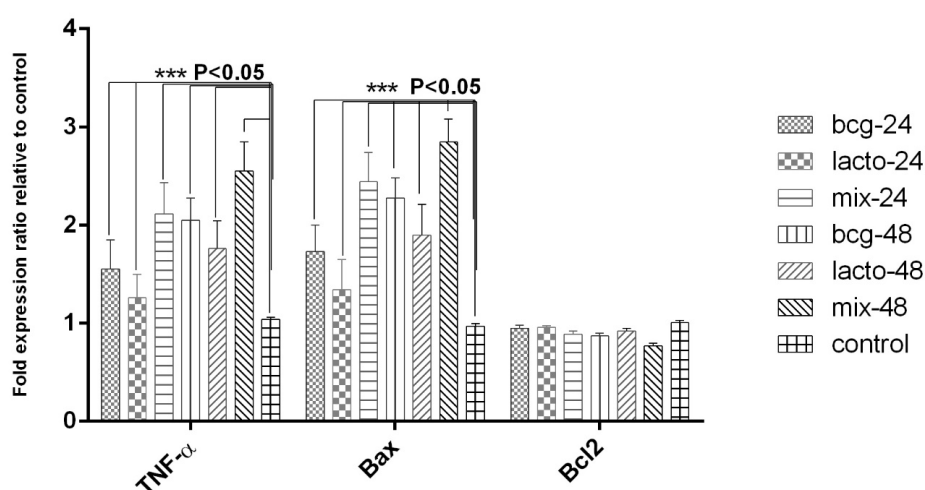
**BCL2:** Results of BCL-2 expression in the group treated with LB did not show a significant upregulation compared to the LB/BCG group after 24 and 48hrs treatment. Besides, in LB/BCG group, no significant difference was observed as compared to the control group after 24 and 48 h treatment ( $P>0.05$ ) (Figure 2).

**Bax/BCL2 ratio:** The result of the Bax/BCL2 ratio in the group treated with LB was significant at 24 h ( $P<0.05$ ). This ratio was significant after 48 h ( $P<0.0136$ ). There was a remarkable increase in the ratio of Bax/BCL2 in the group treated with LB/BCG after 24 and 48 h ( $P<0.0001$ ).

**Discussion**

Cancer is a serious problem and more efforts are needed to find novel therapeutic approaches. One of these cancers is Bladder cancer which 694 death was reported in 2017 among women [12]. Generally, chemotherapy, radiotherapy, and surgery are the common therapeutic ways that could damage the normal cells along with tumor cells. Additionally, BCG is also used for the treatment of bladder cancer. However, the reoccurrence often occurs after the treatment [13, 14]. In this regard, apoptosis induction is one of the important mechanisms to defeat cancer progression [15]. Therefore, we evaluated the effect of probiotic bacteria to stimulate the apoptosis mechanism in EJ138 bladder tumor cells. Furthermore, Lactobacillus are probiotic bacteria that could reinforce the immune system and are capable of shifting Th1 immune responses in infectious and cancer models [16].

In the present study, the best tumoricidal effect of LB on EJ138 cells has been achieved at 24, 48, and 72 h incubation time.



**Figure 2.** Gene expression assay

TNF- $\alpha$  expression in LB/BCG group showed 2.2 and 2.7 folds upregulation after 24 and 48 h treatment, respectively. Gene expression of Bax has the most increment (2.5 and 2.9 folds) in LB/BCG group after 24 ( $P=0.003$ ) and 48 h ( $P<0.0001$ ) treatment. Meanwhile, BCL2 expression did not show remarkable differences among experimental groups. The gene expression was assessed using the real-time PCR method by  $\Delta\Delta C_t$  formula.

Mahmudi et al. studied the *Bifidobacterium bifidum* effect on Caco II cancer cell line and showed that inhibitory percentage of *Bifidobacterium bifidum* supernatant on cancer cell was 55% to 82% that was related to 100  $\mu\text{L/ml}$  and 300  $\mu\text{L/ml}$  concentration after 24 and 72 h treatment, respectively [17]. Also, Dallal et al. reported the same results as ours [18]. Our results revealed that the effect of the probiotics is different in comparison to each other based on tumor cell type. Several studies have approved the antitumor effect of LB [19, 20].

This experiment demonstrated that the mixture of LB and BCG has a boosting effect in tumor inhibition of the EJ138 cell line. Ghezelbash et al. showed that probiotic subscriptions significantly suppress tumor growth as compared to the control group. Potentiating the immune system is a hallmark of anticancer properties in *Lactobacillus* bacteria [21]. The result of cytotoxicity assay revealed that LB significantly decreases cell growth after 24 h treatment. In addition, 48 h treatment with LB and its mixture with BCG showed significant differences between treated and untreated cells.

Also after 72 h treatment with LB, the cell growth was confined significantly. The highest upregulation in Bax gene expression was observed in LB/BCG group as compared to the control group. The important point is that after 48 h treatment, there is a considerable upregulation in comparison to 24 h treatment. Thus, the antitumor effect increased after 48 h that shows a time-dependent manner for this bacterium. Besides, the anti-apoptotic effect of *Lactobacillus* is amplified in companion with BCG. Our results showed that the ratio of Bax/Bcl2 increased in LB/BCG group after 48 h treatment compared to 24 h treatment. Therefore, the potency of tumoricidal is enhanced by the time.

Likewise, Asghari et al. studied the cytotoxicity effect of *Lactobacillus casei* as a probiotic bacteria on HT-29 cancer cell line and evaluation of Bax and Bcl2 apoptotic genes expression. Their results demonstrated that Bax and Bcl2 gene expression increased after treatment with *Lactobacillus casei* [22]. The increment of TNF- $\alpha$  expression showed that LB and LB/BCG can increase necrosis in tumor cells of bladder cancer. In another study, Seow et al. showed that immunotherapy with *Lactobacillus rhamnosus* is successful in treating bladder cancer [23].

Although TNF- $\alpha$  is a double-edged sword in cancer progression and inhibition of tumor cells, its increment with the pro-apoptotic gene (Bax) may be a reason for suppressor effects of LB on bladder cancer.

Our results showed that LB/BCG group significantly upregulated TNF- $\alpha$  compared to LB. The highest mRNA level of TNF- $\alpha$  was related to LB/BCG after 24 and 48 h treatment. This finding indicated that anti-tumor effects on bladder tumor cells are augmented by the mixture of LB and BCG. Also, with increasing treatment time, we observed an increase in TNF- $\alpha$  expression. Likewise, Dallal et al. showed that LB has anti-tumor properties in mice with breast cancer [18]. Baldwin et al. showed that LB and *Lactobacillus casei* could increase apoptosis induction in the Ls513 carcinoma cell line and the bacteria could be adopted as adjuvant or chemotherapy in therapeutic approaches [9].

Several studies demonstrated that these probiotic bacteria can increase IFN- $\gamma$ , TNF- $\alpha$ , and type-I cytokines that are consistent with our scientific findings. Thus, probiotic bacteria may suppress and remove the tumor by boosting the immune system [9, 24]. Our obtained results indicated that LB has antitumor properties. Besides, we found that the anti-tumor activity of this probiotic bacterium is due to the mutual increment of TNF- $\alpha$  and Bax expression that amplify each other. The apoptosis of EJ138 tumor cells was promoted in treatment with LB and its mixture with BCG. The mode of action of the probiotic bacterium demonstrates a time-dependent manner. Increasing TNF- $\alpha$  and Bax stimulated the apoptosis mechanism in tumor cells of bladder cancer. In this study, there were financial constraints to perform experiments *in vitro* and evaluate protein concentrations or CD markers and other molecular assays.

## Conclusions

Our findings indicated that *Lactobacillus acidophilus* has antitumor properties. Therefore, based on our given results in this study, this probiotic bacterium had an anti-tumor effect on bladder cancer. On the other hand, the mode of action of LB is time-dependent. The mixture of LB and BCG has the synergism effect. The mixture of the probiotic bacterium cell wall and BCG increased the apoptosis mechanism synergistically through elevating the Bax pro-apoptotic gene and TNF- $\alpha$  inflammatory cytokine.

## Ethical Considerations

### Compliance with ethical guidelines

This is an *in vitro* study. All ethical principles are considered in this article. The participants were informed about the purpose of the research and its implementation stages.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Authors' contributions

Conceptualization: Mehdi Mahdavi; Methodology and writing – original draft: Faeghe Taheri; Data analysis: Elham Moazamian and All authors studied the manuscript.

## Conflicts of interest

The authors declared no conflict of interest.

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