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ORIGINAL ARTICLE

Inhibitory effects of probiotic *Bacillus coagulans* against MCF7 breast cancer cells

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ABSTRACT

Background and Objectives: Secondary metabolites in the supernatants of probiotic microorganisms have shown anticancer effects. The present study was aimed to investigate the cytotoxicity of *Bacillus coagulans* supernatants and their role in apoptosis induction in MCF7 cancer cells.

Materials and Methods: The inhibition of MCF7 cancer cells by *Bacillus coagulans* supernatants was assessed by MTT assay at three exposure times of 24, 48, and 72 h. Apoptosis induction was explored by flow cytometry while the expression levels of *bax*, *caspase 3*, *caspase 9*, and *bcl2* were examined by real-time PCR and compared with normal HFF cells.

Results: *Bacillus coagulans* supernatants exhibited inhibitory effects on MCF7 cells in a concentration-dependent and time-dependent manner; while lower cytotoxic effects were observed in normal HFF cells. The increase in the expression of *bax*, *caspase 3*, and *caspase 9* genes and the decrease in the anti-apoptotic gene of *bcl2*, along with the flow cytometry results, confirmed the induction of apoptosis in the cancer cells.

Conclusion: Regarding the cytotoxic influence of *Bacillus coagulans* supernatants against breast cancer cells, this bacterium can be considered as a potential candidate for a novel therapeutic strategy with lower side effects which of course requires further investigations.

Keywords: Apoptosis; *Bacillus coagulans*; Breast cancer; Probiotic; Supernatant

INTRODUCTION

After cardiovascular diseases, cancer is the second leading cause of death throughout the Globe (1). The rates of cancer incidence and mortality were reported 48.4 and 57.3%, respectively. In developing countries, the aging of the population and the increase in cancer risk factors have enhanced the cancer-related mortality rate (2). Studies in different parts of the world have reported lung, breast, colorectal, and prostatic cancers as the four main cancers with a high rate of incidence. Based on the report of the international cancer research agency, breast cancer is the second cause of cancer-related death after lung cancer. About 2.3 million people were diagnosed with breast cancer in 2020 which caused 685000 deaths. One out of four common cancers among women is breast cancer (3). Breast cancer has exhibited an ascending trend accounting for 25% of all cancer cases (4). Roushandel et al. reported a rise in the incidence of breast and colon cancer in Iran (5). Current treatments of breast cancer include chemotherapy, radiotherapy, hormone therapy, and surgery. These methods can remove the tumor tissue to some extent,

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but they also damage the normal tissues (6). Moreover, tumor relapse and resistance against the drugs are also reported during cancer treatment (7, 8). In this regard, the development of a novel drug delivery method with complementary drugs based on the plant and bacterial extracts could help in decreasing the complications of the anti-cancer drugs due to their lower side effects (9, 10).

Various types of probiotics have been investigated in the past decades. In 2001, the united nation (UN) and world health organization (WHO) presented the following definition for probiotics which were extensively accepted by the industrial and scientific communities: probiotics are living microorganisms whose sufficient administration will bring health benefits for the host (11). Probiotic organisms are classified into two groups: bacterial strains producing lactic acid or non-lactic acid and fungi. *Lactobacillus, Lactococcus, Bifidobacterium,* and *Enterococcus* are among the common probiotic bacteria (12). Regarding the extensive function of lactic acid (LA), LA-producing probiotic bacteria are of great importance (13).

Studies have shown that regardless of this definition, the dead cellular mass and metabolic products of probiotics could exhibit similar or even higher biological activities compared to alive cells (14, 15). Numerous studies have addressed the products derived from probiotic bacteria, including cell culture supernatant (16), exo-polysaccharide extract (17), bacterial wall components (18), and heat-killed bacteria (19, 20). Functional properties of the probiotic products include cytotoxicity and inhibition of cancer cell proliferation by inducing apoptosis and ultimately cell death (21).

Mitochondrial apoptosis pathway genes are driven by the BCL2 family, which includes two groups of genes. One group is anti-apoptotic genes, including the BCL2 gene, and the other group is apoptotic genes, which include BAX. When a stimuli such as probiotics leads the cell to apoptosis, it actually activates apoptotic proteins, so cytochrome c is released from the mitochondria, it activates Caspase-9, followed by Caspase-3, and finally fragmented DNA and causes cell death (22, 23).

Bacillus coagulans is a Gram-positive, aerobic to optionally anaerobic, non-pathogenic, catalase-positive, and oxidase-negative bacterium that produces spores capable of producing lactic acid similar to *Lactobacillus* and *Bifidobacterium*. One of the features of *Bacillus coagulants* is its spores formation with high temperature and pH resistance which can be used in the production of valuable food products (24, 25). No research has addressed the use of *Bacillus coagulans* supernatant and its cytotoxic effects and apoptosis on the MCF7 cell line. Thus, this study is aimed to examine the therapeutic effect of *Bacillus coagulans* supernatant including its cell growth inhibition and apoptosis induction against MCF7 breast cancer cells.

MATERIALS AND METHODS

Culturing and storage of bacteria. This study was performed experimentally in Parand Branch of Islmic Azad University during 2020-2021. *Bacillus coagulans* bacteria (GBI-30, 6068) was purchased from the American company Ganaeden in the form of leophilized. Briefly, leophilized bacterial powder was first incubated in Tryptic Soy Broth (TSB) and heat-treated for 10 min at 80°C until spores germination. The TSB medium was then incubated in a shaker incubator at 30°C at 150 rpm for 24 h. The purity of bacteria was explored by their culturing in TSA under the same conditions. Then, the biochemical characteristics of the bacteria such as its Gram type, sugars fermentation ability (sucrose, maltose, trehalose, glucose, and mannitol), catalase test, oxidase test, and mobility were determined (10, 26). To preserve the bacterium, an 18-hour culture of the bacterium was performed in De Man Rogosa and Sharpe (MRS) broth medium followed by incubation at 30°C. Then 25% glycerol was added and aliquoted in 1.5 ml Eppendorf tubes. The Eppendorf tubes were cooled and stored at -70ºC (American Thermo) (22).

Preparation of bacterium-free culture supernatant. The bacterial stock was first added to 50 mL MRS followed by 18 hours of incubation under the same conditions. 10% of the overnight culture was inoculated into an MRS broth medium and incubated for 72 hours for the complete production of metabolites. To collect the bacterial culture supernatant, centrifugation was performed at 6000 rpm (Germany Hettich,) at room temperature for 20 min. The supernatant was passed through a 0.2-micron filter to ensure no presence of bacteria followed by drying by a freezer dryer (Germany Alpha 1-2 LD Plus, Christ,) and storing in the refrigerator. At the time of use, 1

mg lyophilized powder was dissolved in 1 mL sterile distilled water. pH was adjusted to 7.3 using NaOH (MERCK, Germany) (27-29).

Cell culture. MCF7 (Michigan Cancer Foundation-7) breast adenocarcinoma cancer cell line and normal HFF (Human foreskin fibroblasts) cell line were purchased from the National Center for Genetic and Biological Resources of Iran. The cells were cultured in 25-cm^2 filtered flasks containing 10 mL RPMI 1640 and DMEM medium (Gibco, UK) along with 10% bovine fetal serum (FBS) (Gibco, USA) and penicillin (100 units per mL) and streptomycin (100 µg/mL) (Sigma, USA) at 37°C in an incubator with 95% humidity and 5% CO_2 for 24 hours. After 80% growth, the cells were washed with PBS. For passage, they were separated from the flask by trypsin/EDTA solution (0.25%; Gibco UK) and added to a fresh medium followed by incubation (Memmert, Germany). Cells were counted with a new bar slide (hemocytometer). The percentage of living cells was also determined by Trypan Blue staining (MERCK, Germany) (27, 30, 31).

MTT assay. MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma Aldrich, Germany) was employed to measure the antiproliferative activity of bacterial culture supernatant. This test relies on the reduction of tetrazolium yellow crystals by the enzyme mitochondrial succinate dehydrogenase and the formation of insoluble purple formazan crystals. 5000 cells were cultured in each well of the 96-well plate and placed in a CO_2 incubator at 37°C for 24 h. Cells were treated with various dilutions of bacterial supernatants 1-10, and 15 mg/mL. The plates were then incubated for 24, 48, and 72 h at 37°C and 5% CO_2. Untreated cells were considered as negative controls. Then, the culture medium inside the wells was carefully removed and replaced by a fresh medium. 20 µl MTT (5 mg/mL) was added in the dark under incubation for 4 h. The supernatants were then discarded and the formazan crystals produced by the living cells were dissolved in 200 µl DMSO (dimethyl sulfoxide). Cell plates were read at 570 nm by ELISA (Germany Bio Tek, Synergy / HTX) and the cell viability was calculated by the following equation based on the graphs:

Viability (%)=(optical absorption of controls/optical absorption of treated cells) × 100

IC_{50} refers to the concentration leading to 50% growth inhibition of cancer cells. IC_{50} was calculated and each test was performed in triplicates (31, 32).

Detection of apoptosis induction by Annexin V-FITC. Apoptosis detection was carried out according to Annexin V-FITC kit (MabTag GmbH, Germany) protocol. The MCF7 cells (1×10^5 cell/well) were treated with IC_{50} value of supernatant of *Bacillus coagulans* for 48 h. The cells were then washed with PBS, harvested and centrifuged. The binding buffer (90 µL) was added to the cell pellet. Then, 5 µL of Annexin V dye was added and incubated for 10 min at room temperature. The cells were then washed with binding solution and then 5 µL of PI dye was added to the cells. Finally, the analysis was performed by flow cytometry (Biocompare, USA). For the results analysis according to the grouping, lower left square represents healthy cells with Annexin-$FITC^-$, and PI^-, lower right square represents early apoptosis with Annexin-FITC+ and PI^-, right upper square represents late apoptosis with Annexin-$FITC^+$ and PI^+, and left upper square indicates necrotic cells with Annexin-$FITC^-$ and PI^+ (33).

Real-time PCR for gene expression. The expressions of *bax, bcl2, caspase 3,* and *caspase 9* genes were analyzed by real-time PCR (Step One Plus, Applied Biosystems, US) to assess the influence of the *Bacillus coagulans* culture supernatant on the apoptosis activation in MCF7. First, 1×10^5 MCF7 and HFF cells were separately seeded in the wells of six-well plates. They were then treated with IC_{50} of the bacterial culture supernatant for 48 h in an incubator at 37°C. The untreated cells were considered as negative controls. The quantity of extracted RNA was assessed by Nanodrop (NanoDrop One, Thermo Scientific, USA) while the quality of the obtained RNA was examined by gel electrophoresis. The obtained RNA was then employed for the synthesis of cDNA (28).

The Revert AidTM Kit (Thermo Scientific Fermentas) was utilized for cDNA preparation. Briefly, 0.5 µg extracted RNA and 1 µL oligo dT primer were poured into an RNAse-free microtube and its volume was increased to 12 µL with water containing diethylpyrocarbonate (DEPC). It was then incubated for 5 min at 70°C. After adding 4 µL 5 × reaction buffer, 0.5 µL RNasin, and 2 µL dNTP mixture, the incubation was continued for 5 min at 37°C. Then, 1 µL M-MuLV Reverse Transcriptase enzyme was added and the samples were incubated at 42°C for 60 min.

The mixture was placed on a thermomix (Germany Analytikjena, TMix Thermalmixer) at 42°C for 60 min. Then, the transcription enzyme was inactivated using a thermomix device at 70°C for 10 min and the reaction product was stored at -20°C (19).

The SYBER Green technique was utilized to assess the expression of target genes in MCF7 cancer cells using β-actin gene as the internal control. In summary, 12.5 µL master mix, 1 µL cDNA, 0.5 µL forward primer, 0.5 µL reverse primer, and 9.5 µL DEPC were added (33).

Real-time PCR was employed with the following temperature-time program. An initial denaturation cycle for 10 min at 95°C, followed by 40 cycles composed of denaturation at 95°C for 20 s, annealing at 55 for 40 s, and DNA strand synthesis at 72°C for 40 s. The relative expression of genes was calculated by $2^{-\Delta\Delta ct}$ method.

Statistical analysis. The experiments were performed in triplicates. Statistical analysis of data was based on ANOVA (Analysis of Variance One-Way) as implemented in SPSS software (version 22) and Graphpad Prism software 8.0.2. In all analyses, the significant level was considered P <0.05. The data were recorded as mean ± standard deviation (SD) 3.

RESULTS

Cytotoxic activities of *Bacillus coagulans* supernatant against MCF7 breast cancer cell and HFF normal cell lines. The results indicated a decline in the proliferation ability and viability of the cells by increasing the concentration and prolonging the treatment time of the cancer cells with bacterial supernatant (Fig. 1). The IC50 value of supernatant against cancer cells was 7.4, 3.7, and 1 mg/mL at exposure times of 24, 48, and 72 h, respectively.

Comparison of cytotoxic effects of *Bacillus coagulans* supernatant on HFF normal cells and MCF7 cancer cells after 48 h are presented in Fig. 2. The IC_{50} value for normal HFF cells at 48 h was approximately 6 mg/mL. The results showed that the IC_{50} concentration of supernatant (for cancer cells) inhibited growth in normal cells by 23%; suggesting a lower cytotoxic effect of *Bacillus coagulans* supernatant on normal HFF cells.

A significant high cell viability was observed in HFF healthy cells compared to MCF7 cancer cells following treatment with supernatant (for 1-7 mg/mL concentration). The results indicated the lower cytotoxicity of supernatant on HFF normal cells.

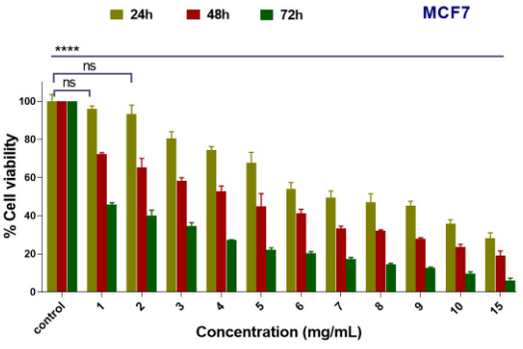

Fig. 1. Cytotoxic effects of *Bacillus coagulans* supernatant against MCF7 breast cancer cells. Untreated MCF7 cancer cells were considered as a control group. Results are reported as viability in comparison with control group (ns: non significant, p ≤ 0.0001: ****).

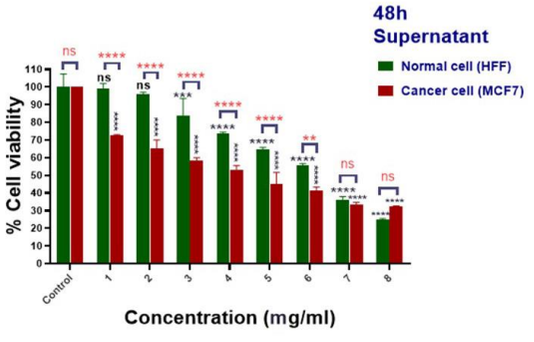

Fig. 2. Comparison of cytotoxic effects of *Bacillus coagulans* supernatant on HFF normal cells and MCF7 cancer cells after 48 h. The stars at the top of the chart show a statistically significant difference (ns: non significant, p ≤ 0.001:***, p ≤ 0.0001:****) Data are shown as mean ± SD.

The significant level between control group and treated HFF cells with *Bacillus coagulans* supernatant has presented with black asterisk (*). The significant level between MCF7 cancer cells and HFF normal cells has presented with red asterisk (*).

Untreated MCF7 cancer cells and HFF normal cells were considered as a control group.

Apoptosis induction. Flow cytometry technique based on the Annexin V-FITC kit protocol was employed to evaluate apoptosis. As shown in Fig. 3, a strong apoptosis induction can be seen in MCF7 cells after 48 hours of treatment with bacterial supernatant

as compared to the control group. Early apoptotic cells were identified by Annexin V-FITC adsorption, while late apoptotic cells were detected by adsorption of both PI and Annexin V-FITC. Necrotic cells were also determined by PI adsorption. 33.50 ± 2.63% early apoptosis, 16.47 ± 2.43% late apoptosis, and 0.27 ± 0.25% necrosis were induced in cancer cells (Fig. 3b, 3d).

Normal cells showed 6.00 ± 1.292% early apoptosis, 13.02 ± 0.62%, late apoptosis, and 0.26 ± 0.46 necrosis which was lower than the cancer cells (Fig. 3c, 3d). The high apoptosis levels were detected in cancer cells compared to the healthy cells following treatment with bacterial supernatant (Fig. 3d) ($p < 0.0001$).

Gene expression analysis. Expression of *bax*, *baspase 3*, and *caspase 9* genes was significantly enhanced in MCF7 cancer cells treated with IC_{50} concentration of bacterial supernatant as compared to the control group, while *bcl2* expression showed a significant reduction. The expression ratio of *bax/bcl2* represents the overall estimate of apoptosis induction, this ratio showed a significant increase over 48 h in MCF7 cancer cells compared to control cells (Fig. 4). Gene expression levels of *caspase 3*, *caspase 9*, and *bax/bcl2* ratio were not significant in HFF normal cells compared to the control group. Also, the expression of proapoptotic genes including *bax*, *caspase 3*,

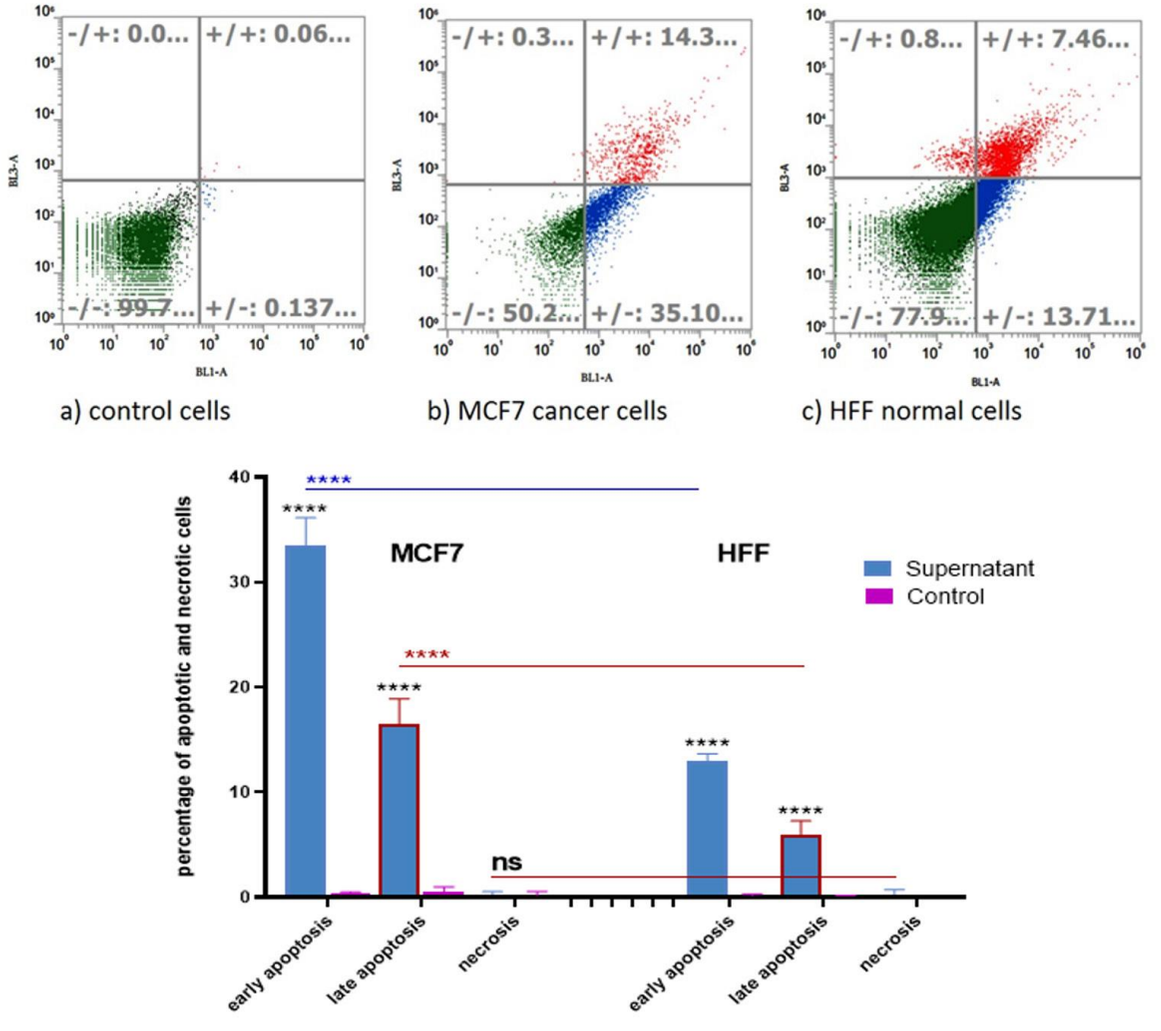

Fig. 3. Flow cytometry plots for apoptosis detection in control group (untreated MCF7 cancer cells) (a), MCF7 cancer cells treated with *Bacillus coagulans* supernatant (b) HFF normal cells treated with *Bacillus coagulans* supernatant (c) after 48 h. Lower left square: viable cells (A^-/PI^-), lower right square: early apoptotic cells (A^+/PI^-), right upper square: late apoptotic cells (A^+/PI^+), left upper square: necrotic cells (A^-/PI^+). d) Flow cytometric statistical analysis in cancer cells (MCF7) and normal cells (HFF) relative to the control of each cell line and relative to each other ($P < 0.0001$****, ns: non significant).

and *caspase 9* increased significantly in MCF7 cancer cells compared to normal cells treated with bacterial supernatant.

The significant level between control group and treated cells (cancer cells and normal cells) with *Bacillus coagulans* supernatant has presented with black asterisk (*). The significant level between MCF7 cancer cells and HFF normal cells has presented with red asterisk (*).

Untreated MCF7 cancer cells and HFF normal cells were considered as a control group.

DISCUSSION

Common cancer therapies such as chemotherapy have different influences on various cancers and may cause diverse side effects such as damage to normal cells and development of drug resistance in cancer cells (34). According to WHO, breast cancer is the most common cancer type among women (3). Therefore, the development of anti-cancer agents with low side effects is of crucial importance for the recovery of patients. Based on previous reports, probiotic microorganisms could be a suitable candidate for treatment (35). In this regard, numerous studies have addressed the anti-cancer and cytotoxicity effects of LA-producing bacteria such as *Lactobacillus, Bifidobacterium* (36-38). In recent years, *Bacillus coagulans* has attracted the attention of researchers. In addition to producing lactic acid, this bacterium generates spores at a suitable growth temperature (35-50°C) and pH (5.5 to 6.5). Therefore, this bacterium is resistant to high temperatures and can withstand and grow under pH conditions of the stomach and intestine (24, 25).

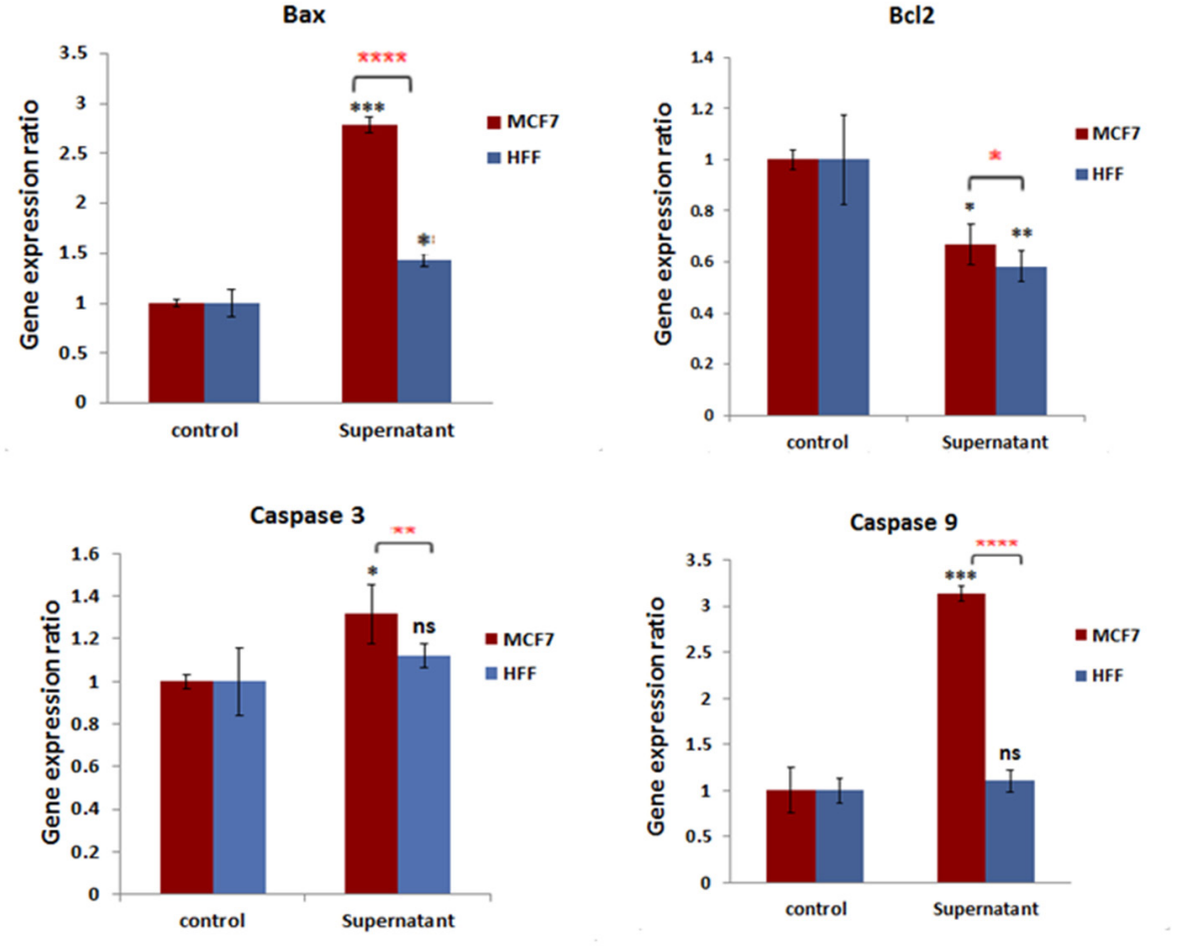

Fig. 4. Expression levels of *bax, bcl2, caspase-3, caspase-9* following treatment with *Bacillus coagulans* supernatant in MCF7 cancer cells and HFF normal cells after 48 h. (ns: non significant, $p \leq 0.05$:*, $p \leq 0.01$:**, $p \leq 0.001$:***, $p \leq 0.0001$:****) Data are shown as mean ± SD.

The survival rate of treated cancer cells and normal cells was compared to control cells (untreated). *Bacillus coagulans* supernatant exhibited an inhibitory effect against MCF7 cancer cells for 24, 48, and 72 h, compared to the control group. The antiproliferative activity of bacterial supernatant showed dependence on dose and treatment. Treatment of MCF-7 cells with concentrations of 1 and 2 mg/mL inhibited 30% and 40% of cancer cells after 48 h, while no decline was observed in the viability of normal cells under the same conditions. This reveals the anti-cancer activity of supernatant against cancer cells at lower cytotoxic effects on normal cells. A comparison of the IC_{50} value of cancer cells and normal cells also confirms this. Flow cytometry results for induction of apoptosis also revealed the ability of supernatant to induce 50% of apoptosis in cancer cells. Noteworthy, the necrosis due to supernatant treatment was negligible. Bacterial supernatant also induced lower apoptosis in HFF cells (2.5 fold less). This is one of the advantages of the supernatant as it managed to kill cancer cells at reduced side effects to the normal cells.

To investigate the molecular pathway of apoptosis induction, the expression levels of *bax, bcl2, caspase 3,* and *caspase 9* genes were investigated in MCF7 cancer cells. In general, *Bacillus coagulant* supernatant increased the expression of *bax, caspase3,* and *caspase9* genes over 48 h, while the Bcl2 gene exhibited a significant decrement. As *caspase* is an apoptotic gene, treatment of cancer cells with the bacterial supernatant activated apoptosis by increasing the expression of *caspase 3* and *caspase 9* genes. Gene expression studies also confirmed the flow cytometry results indicating the apoptosis induction in cancer cells.

Reports on probiotic bacteria are promising. Probiotic bacteria can play a key role in controlling cancer cells by affecting the expression of genes involved in the internal and external pathways of apoptosis. In these pathways, the mitochondrial membrane becomes permeable, leading to the release of cytochrome c, hence, first activating *caspase 9* and then Caspase 3. Bax and Bak proteins are among the factors that promote mitochondrial membrane permeability. On the other hand, Bcl2 family proteins prevent the accumulation of Bax and Bak proteins and thus cause anti-apoptotic activity (32).

In line with the present study, other studies have also reported the inhibitory effects of bacterial supernatant and its anti-cancer activity.

The cell-free supernatant of *Pediococcus,* a probiotic bacterium isolated from traditional dairy products, significantly declined the viability of MCF7 cells and led to the death of 85% of cancer cells. This bacterial supernatant contains organic acids and exopolysaccharides. Western blot technique results also revealed an increase in apoptosis of cancer cells by the enhancement in Bax protein expression and decline of Bcl2 protein expression (10).

Dehghani et al. indicated that the supernatants of *L. rhamnosus* can induce apoptosis and inhibit the proliferation of HT-29 cells in a time-dependent and dose-dependent manner. Stronger apoptotic effects were observed in 48 h as compared with 72 h. Similar to this research, an increase was observed in the expression of the genes involved in the intrinsic mitochondrial pathway while the Bcl2 gene exhibited a decline (33).

Name et al. stated that the supernatants of *Enterococcus* lactis IW5 (isolated from the human intestine) are resistant to low pH values and high bile salt concentrations. This supernatant managed to inhibit the growth of various cancer cells including MCF7, Hela, AGS, HT-29, and Caco-2; it, however, exhibited no toxicity against Hs-74 normal cells (39).

Reports have indicated that *Bacillus coagulans Unique IS2* is heat resistant due to its spores and can therefore be used in a variety of foods. The resistance of this bacterium in low pH environments such as stomach acids has caused it to enter the large intestine and survive. In this study, the anti-cancer effects of this bacterium against Hela, Colo 205 and K562 cell lines were proven, but did not inhibit the proliferation of normal HEK 293T cells (40).

Reports have demonstrated that *Bacillus polyfermenticus KU3,* which was isolated from a fermented vegetable-based Korean food, has spore and showed resistance against artificial stomach juice. It also survived in the bile acids for 24 h. The supernatant of this bacterium exhibited anticancer effects against HeLa and LoVo; but did not inhibit the proliferation of normal MRC-5 cells (41).

Antiproliferative effects of supernatants of *L. rhamnosus* and *L. crispatus* have been also reported on HeLa cells (29).

As *Bacillus coagulans* exhibited anticancer and apoptotic effects on the MCF7 cell line, this bacterium could be a promising candidate for the treatment and control of breast cancer cell lines.

CONCLUSION

Metabolites produced by probiotic bacteria can affect human health. They seem to be a promising solution for cancer treatment. The supernatant of the bacterial cultures contains bacterium-secreted metabolites that can serve as a proper model for anticancer tests. In this study, the supernatants of *Bacillus coagulans* showed a significant cytotoxic effect on the MCF7 cell line with lower cytotoxicity on the normal cell line. It seems that by upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes, *Bacillus coagulans* can induce apoptosis in cancer cells through the mitochondrial pathways. This bacterium can be also considered as a potential strategy to inhibit the activities of cancer cells.

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