Antiproliferative Potential of *Bacillus coagulans* Supernatant on SKBR3 Breast Cancer Cell Line

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**ABSTRACT**

**Background and Aim:** Breast cancer is one of the most common types of cancer among Iranian women. To date, the usual cancer treatments have not been entirely effective. Therefore, creating anticancer products is of great importance. The aim of this study was to evaluate the cytotoxic, anticancer, and induction effects of *Bacillus coagulans* probiotic bacterial supernatant on SKBR3 cells.

**Materials and Methods:** The anticancer potential and cytotoxic effect of different concentrations of probiotic bacterial supernatants (1, 2, 3, 4, 5, 6 and 7 mg/mL) were evaluated on SKBR3 cells for 24, 48, and 72 h by MTT technique. QRT-RCR was used to assess the expression of *bax*, *bcl2*, *casp3*, and *casp9* genes, and flow cytometry was used to evaluate apoptosis in cancer cells.

**Results:** The inhibitory effect of dose- and time-dependent *B. coagulans* supernatant showed that the supernatant of this probiotic bacterium had a cytotoxic effect on SKBR3 cancer cells. On the other hand, analysis of flow cytometry results and increased expression of *bax*, *casp3*, and *casp9* pro-apoptotic genes and decreased *bcl2* expression in cancer cells showed induction of apoptosis.

**Conclusion:** The anticancer and cytotoxic effect of *B. coagulans* probiotic bacterial supernatant on SKBR3 cancer cells shows that with further research, this probiotic bacterium can be used as a new strategy for the possible treatment of breast cancer.

**Keywords:** Apoptosis, *Bacillus coagulation*, Breast cancer, Probiotic, SKBR3 cancer cells, Supernatant

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1. Introduction

Probiotics are non-pathogenic living microorganisms considered essential in medical science due to their beneficial effects on the host body regarding the prevention and treatment of many chronic diseases, including cancer (1, 2). The intestinal microbial flora plays a protective role against various diseases. An imbalance in the intestinal microbiome can lead to anti-inflammatory and immune reactions and the onset of pathogenic processes (3). Research has shown that some types of probiotics can participate in the prevention and treatment of cancer, primarily by inducing apoptosis, modulating the intestinal microbiota, and creating an immune response (4). The anticancer effects of probiotics are significant by preventing the conversion of procarcinogen to a carcinogen, binding and inactivating mitogen compounds, reducing the growth of carcinogenic bacteria, reducing mitogen uptake, inducing apoptosis, and enhancing immune function against cancer cells (5). Induction of apoptosis is a critical way to reduce cell proliferation to prevent cancer cell development and increase cancer.
treatments’ effectiveness. Increasing the expression ratio of pro-apoptotic to anti-apoptotic genes (bax/bcl2) and activating the caspase cascade are essential in the onset and execution of apoptosis (6).

Today, studied extensively in various fields, probiotic microorganisms belong mainly to bacterial genera such as Lactobacillus and Bifidobacterium, which produce lactic acid. However, other lactic acid-producing bacterial species have also been noticed by researchers (7). Several Bacillus species have recently been reported as new probiotics (8). Bacillus coagulans is a gram-positive, facultative anaerobic, non-pathogenic, catalase-positive, and negative oxidase bacterium and produces endospores. This bacterium can produce lactic acid, similar to Lactobacillus and Bifidobacterium, and since it produces spores, it is resistant to heat and pH. It can resist and survive while colliding with the stomach and bile acid. Madempudi et al. studied the effects of cytotoxicity, antiproliferative, and induction of apoptosis of this bacterium on human colon cancer cell line (COLO 205), cervical cancer (Hela), and chronic myeloid leukemia (CML) (KS62) compared to the normal cell line of the human embryo (HEK 293) (9, 10). Recent studies have shown that in addition to live probiotic bacteria, metabolic products of living probiotic bacteria, including bacterial cell-free supernatants (11, 12), inactive bacteria (heat-killed bacteria) (13), extracts of exo-polysaccharides (14, 15), bacterial cell wall components (16), cell extracts (17) and other cellular components have potential therapeutic, anti-tumor and anticancer activities and were also able to stimulate the immune system. According to previous studies, the benefits of metabolites released in the supernatant of probiotic bacteria can be anti-inflammatory, cytotoxic, antioxidant, and increasing the function of the immune system against cancer cells, as well as ease of use, stability, and longevity of these metabolic products compared to probiotic bacteria (18, 19).

Cancer is the first or second leading cause of death in older adults in most countries worldwide (20). Breast cancer is the first most common cancer (2.3 million people), the fifth leading cause of death (685,000) due to cancer worldwide, and the second most common cause of cancer death among women (20, 21). To treat cancer, depending on the patient’s age, genetics, and patient’s physical condition, various treatment methods, including chemotherapy, radiation therapy, hormone therapy, and surgery, are performed (22). However, besides killing cancerous tissue, these methods can cause damage to healthy tissue, tumor recurrence, and resistance of cancer cells to the drugs used during treatment. Therefore, studying the effect of probiotic bacteria or their metabolic products on inhibiting the growth of cancer cells without side effects can play an effective role in public health (23, 24). To date, several studies have been performed on the effects of different strains of probiotics on the prevention and treatment of cancers, including breast cancer, by inhibiting proliferation and inducing apoptosis (25). Evaluation of the efficacy of metabolites secreted by standard strains of Lactobacillus and Bifidobacterium on breast cancer cell lines (MCF-7) and gastric cancer (AGS) showed that metabolites could cause cancer cell death through the apoptosis pathway activation (26).

Reviewing the literature, no report was found on the use of B. coagulant supernatant and cytotoxic effects on SKBR3 cancer. Therefore, this study was performed due to the therapeutic effect of probiotic bacteria on inhibiting the growth of cancer cells, with the aim of investigating the therapeutic effect of B. coagulans supernatant, including anticancer effects and induction of apoptosis, against breast cancer cells.

2. Materials and Methods

Cell Culture

Bacillus coagulans bacterial supernatant (GBI-30, 6068) was prepared according to the protocol reported in the previous study (27). SKBR3 breast cancer cells (IBRC C10147) and HFF normal cells were purchased from Iran’s National Center for Genetic and Biological Resources. Cells were cultures in DMEM High and DMEM (Gibco, UK) culture medium with 10% bovine fetal serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Sigma, USA) at 37°C in a CO2 incubator with 95% relative humidity (RH) and 5% CO2 for 24 h. Then, by an inverted microscope, about 80% of the growth of cells inside the flasks and their health were examined and confirmed. Then they were washed using PBS. Trypsin-EDTA solution (0.25%) (Gibco, UK) was used for cell passage. Isolated cells were centrifuged, then the number and percentage of viable cells were determined by a slide neobar (hemocytometer) and Trypan Blue (Merck, Germany) (28).

MTT Assay

In each 96-well plate, 200 μL containing 5000 cancer cells were cultured and incubated for 24 h in a CO2 incubator with 5% CO2 and 95% relative humidity (RH) at 37°C. Cells were treated with different dilutions of bacterial supernatants 1, 2, 3, 4, 5, 6, and 7 mg/mL. The plates were then incubated for 24, 48, and 72 h, similarly to the previous conditions. After removing the medium inside the wells, a new medium was added, and 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at a concentration of 5 mg/mL (MTT) (Sigma Aldrich, Germany) was added in the dark and incubated for 4 h to form formazan...
crystals. Then, the surface of the wells was washed, and 200 μL of DMSO (dimethyl sulfoxide) was added and placed on the shaker for 30 min in the dark and read by ELISA (Bio Tek, Synergy/HTX, Germany) at 570 nm, and the cell viability percentage was calculated by the following formula, and the graphs were drawn. Untreated SKBR3 cells with culture supernatant bacteria were used as a negative control.

Percentage of cell viability = (mean absorbance of treated cells/ mean absorbance of control cells) x 100

This formula calculated inhibitory concentration (IC50) or the concentration of bacterial culture supernatant, which can inhibit the growth of 50% of cancer cells. All experiments were performed in three replications (29).

Detection of Apoptosis Induction by Annexin V-FITC/PI

Flow cytometry was used to quantify primary, early and late apoptotic and necrotic cells. Apoptosis was assessed according to the protocol of the Annexin V-FITC/PI kit (MabTag GmbH, Germany). In summary, in each 6-well plate, 1× 10^5 cells (SKBR3 and HFF) were cultured and, after 24 h, treated by IC50 of bacterial culture supernatant for 48 h, incubated at 37°C. Untreated cells were considered as negative controls. The cells were washed with PBS and centrifuged for 5 min. After adding 90 μL of binding buffer, 5 μL of Annexin-V solution was added and incubated in the dark for 10 min at room temperature. Then, 5 μL of PI was added. The resulting suspension was transferred to a flow cytometry tube and analyzed by flow cytometry (Thermo Fisher Scientific, USA) (27, 30).

Statistical Analysis

Statistical data analysis was performed by ANOVA (Analysis of Variance One-Way) and Paired-Samples T-test using GraphPad Prism 8.0.2 software. All experiments were performed in three times, and the results were analyzed in the form of Tables and bar graphs. In all analyses, the significant level of the tests was less than <0.05, and the data were presented as mean ± standard deviation (Mean±SD).

3. Results

**Bacillus coagulans** was confirmed according to diagnostic tests of The Prokaryotes book and the Bergey's Manual. This bacterium was cultured in MRS agar; after gram staining, it was examined under a microscope, and Gram-positive bacteria were detected. The results of catalase and oxidase tests and sugar fermentation tests were reported in Table 1 (31).

<table>
<thead>
<tr>
<th>Bacterium species</th>
<th>Spore</th>
<th>Gram staining</th>
<th>Sugar Fermentation</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. coagulans</em></td>
<td></td>
<td>+</td>
<td>Glucose, Maltose, Mannitol, Trehalose, Sucrose</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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**The Cytotoxic Effect of *B. coagulans* Supernatant on the Cell Viability of SKBR3 Cancer Cells**

MTT assay showed that with increasing concentration of supernatant (1, 2, 3, 4, 5, 6, and 7 mg/mL) and treatment time (24, 48, and 72 hours), the cell viability of SKBR3 cancer cells decreased significantly (P<0.0001). The IC50 at 24, 48, and 72 h was 5.72, 4.98, and 3.49 mg/mL, respectively. Time has shown a significant effect on inhibiting the proliferation of SKBR3 cancer cells treated with supernatant.

**Induction of Apoptosis in SKBR3 Cancer Cells**

Analysis of the regions in Figure 2 showed that 36.02 ± 3.52% and 11.01 ± 1.78% of early and late apoptosis were observed in cancer cells, respectively. Of cancer cells, 0.1 ± 0.00% developed necrosis, which is negligible. The extent of induced apoptosis is summarized in Table 2.
Table 2. Results of induction of apoptosis in SKBR3 cells after treatment with B. coagulans for 48 h.

<table>
<thead>
<tr>
<th></th>
<th>SKBR3 Control</th>
<th>IC50 (5 mg/mL)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>early apoptosis</td>
<td>0.47 ± 0.10</td>
<td>36.02 ± 3.52</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>late apoptosis</td>
<td>0.56 ± 0.32</td>
<td>11.01 ± 1.78</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>necrosis</td>
<td>0.50 ± 0.61</td>
<td>0.1 ± 0.00</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Figure 1. Survival percentage of SKBR3 cancer cells treated with different concentrations of B. coagulans culture supernatant at 24, 48, and 72 h compared to the control group (untreated SKBR3 cancer cells). Data are represented as mean ± SD of three replications.

(ns: non-significant, P ≤ 0.01: **, P ≤ 0.001: ***. P ≤ 0.0001: ****)

Figure 2. Flow cytometric diagrams of the effect of apoptotic supernatant of B. coagulans culture for 48 h on SKBR3 cancer cells. (a, b group of control cells (untreated SKBR3 cells), c, d) group of treated SKBR3 cancer cells. Lower left square: living cells (Annexin-FITC−/PI−), lower right square: early apoptotic cells (Annexin-FITC+ / PI−), the upper right square of late apoptotic cells (Annexin-FITC−/PI+), Upper left square of necrotic cells (Annexin-FITC+ / PI+)

Gene Expression Analysis
To evaluate the development of apoptosis in SKBR3 cancer cells, the expression levels of pro-apoptotic genes bax, bcl2, caspase 3, and caspase 9 in cells treated by IC50 of B. coagulans culture supernatant for 48 h were evaluated by Real-Time PCR. After RNA extraction, its quantity was evaluated by nanodrop, and its quality was assessed by electrophoresis (Figure 3).
Figure 3. Quality of RNA extracted from SKBR3 cells on 2% agarose gel. The two 28S rRNA and 18S rRNA bands are observable with suitable quality due to staining by Safe Stain on 2% agarose gel.

Exercising changes in the expression of different genes in SKBR3 cancer cells treated with IC₅₀ concentration of B. coagulans culture supernatant confirmed the results of inducing apoptosis by flow cytometry. The bax, casp3, and casp9 pro-apoptotic genes were up-regulated, and the anti-apoptotic bcl2 gene was down-regulated after 48 h (Figure 4). The expression ratio of bax to bcl2 genes increased over 48 h in treated SKBR3 cancer cells compared to control cells (Figure 4).

Figure 4. Expression of bax, casp3, casp9, and anti-apoptotic bcl2 pro-apoptotic genes in SKBR3 cells treated with IC₅₀ supernatant of Bacillus coagulans bacterial culture compared to the control group (untreated SKBR3 cancer cells), the data is presented as Mean ± SD of three dependent experiments is shown.

(P≤ 0.05: *, P≤ 0.001: ***, P≤ 0.0001: ****)

4. Discussion

Today, probiotic supplements or compounds are increasingly being produced. These compounds are bacterial metabolites with beneficial properties related to community health, including anticancer properties (32, 33). Studies have shown that probiotic bacteria have beneficial effects on inhibiting intestinal cancer by their location in the intestine. Also, they activate their protective properties against cancer by activating immune system cells and migrating them to more distant mucosa, including the mammary glands (34). One of the most common cancers in the world is breast cancer, the second leading cause of death among women, with an increasing incidence and mortality rate each year (20). Various methods are used to treat cancer, including chemotherapy, radiation therapy, hormone therapy and surgery. In all of these methods, in addition to killing cancer cells, some healthy cells are also destroyed, which is a side effect of chemotherapy (22). Therefore, new strategies are needed to prevent and treat breast cancer. In various studies, the effects of live and heat-killed probiotic bacteria (35) and their supernatant (11, 12) through direct induction of apoptosis on various cancer cells have been investigated. Currently, the most commonly used lactic acid-producing bacteria are Lactobacilli and Bifidobacteria. Still, some yeasts and other bacilli have also been used (36), including the bacterium B. coagulans, which produce not only lactic acid but also are resistant to temperature due to spore production’s high pH. They can resist and grow against the pH of the stomach and intestines (8, 9).

In the present study, the cytotoxic and anticancer effects and induction of apoptosis of Bacillus coagulans supernatant against SKBR3 breast cancer cells were evaluated due to the therapeutic effect of probiotic bacteria on inhibiting cancer cell growth. The results of this study showed that B. coagulans supernatant has a high potential to induce apoptosis and inhibit SKBR3 cell proliferation in a time and dose-dependent manner. The results of comparing the MTT test at 24, 48, and 72 h and in different concentrations (1, 2, 3, 4, 5, 6, and 7 mg/mL) on SKBR3 cancer cells showed that with increasing supernatant concentration and over time, the cell viability rate of SKBR3 cancer cells decreased significantly compared to the control group (P<0.0001) due to the increased cytotoxic concentration of supernatant on treated cancer cells.

On the other hand, the induction rate of apoptosis in treated cancer cells was estimated to be about 47%, indicating the strong apoptotic effect of bacterial culture supernatant on cancer cells. In addition, increased expression of bax, casp3, and casp9 pro-apoptotic genes and decreased expression of bcl2 anti-apoptotic gene in cancer cells treated for 48 h indicates strong apoptosis in cancer cells.

In the study by Madempudi et al., the bacterial supernatant of B. coagulans, based on MTT assay, reduced cell proliferation in human colon (COLO 205), cervical (HeLa), and chronic myeloid leukemia (K562) cell lines, respectively, compared with normal human
embryonic kidney cells (HEK 293T). Similarly, in this study, a decrease in cell proliferation was observed in the SKBR3 cell line compared with normal HFF cells. Bacterial supernatant was also effective in inducing apoptosis by increasing BAX protein expression, decreasing BCL2 protein, decreasing mitochondrial membrane potential, increasing cytochrome c release, and increasing caspase 3 activity in cancer cell lines, especially in intestinal cancer cells (10).

In this regard, Dehghani et al. showed that Lactobacillus rhamnosus supernatant, based on a time-dependent pattern (24, 48, and 72 h) and different concentrations of bacterial supernatant, has a high potential to inhibit HT-29 cancer cell proliferation and induce apoptosis. Thus, 99% inhibition of cancer cell proliferation was observed after 72 h at a 30 mg/mL concentration. According to this study, 7 mg/mL of B. coagulans bacterial supernatant in our study in 72 h inhibited the growth of about 90% of SKBR3 cells, which shows the strong effect of this supernatant compared to the study of Dehghani et al. Also, induction of apoptosis compared to the control group was investigated in 48 h, similar to this study. In both studies, strong apoptosis was induced after 48 h. L. rhamnosus supernatant and Bacillus coagulans supernatant (in this study) increased the expression of pro-apoptotic genes, including bax, casp3, and casp9, compared to the control group and decreased bcl2 gene expression (30).

The cytotoxic effect and induction of apoptosis of heat-killed bacterium Lactobacillus brevis (TD4) on HT-29 colon cancer cells and normal HEK-293 cells were investigated based on a time-dependent pattern. The results obtained by comparing the MTT test at 24, 48, and 72 h and different concentrations showed that heat-killed L. brevis had the highest cytotoxic effect of 1000 µg/mL dilution at 72 h compared with the control group. The control showed significant viability ($P<0.001$). The viability of HT-29 and HEK-293 cells was estimated to be 23% and 50%, respectively. The results of DNA extraction from the HT-29 cell line treated with different concentrations of TD4 bacteria also confirmed the induction of apoptosis in the HT-29 cell line (37).

The cytotoxic effects of Lactobacillus plantarum HBM-IAUF-1 and Lactococcus lactis HBM-IAUF-8 supernatant on SKBR3 breast cancer cells have been reported in a time- and dose-dependent manner. L. plantarum HBM-IAUF-1 and L. lactis HBM-IAUF-8 had the highest cytotoxic effect on SKBR3 cancer cells compared with the control group at 1000 and 500 µg/ml, respectively, at 72 hours after treatment (38).

The cytotoxic activity of different concentrations (5, 10, 15, 20 mg/mL) of supernatant of some gram-positive bacteria taken from the soil for different periods on PC-3 prostate cancer cells was examined. Among them, the Bacillus licheniformis strain, which had the best antimicrobial compounds, was selected for further investigation for survival and the effects of induction of apoptosis on PC-3 cell lines. MTS assay with an incubation time of 24, 48, and 72 hours of treated cells showed that survival was dose-dependent; increasing concentration could significantly reduce survival compared with the control group ($P<0.05$). The highest effect of cytotoxicity and apoptosis was observed on PC-3 cancer cells at a concentration of 20 mg/mL supernatant within 72 h after cell treatment (39).

Elmanama et al. studied the anticancer effects of extracellular metabolites (supernatants) of eight bacterial species (Escherichia coli, Staphylococcus aureus, Micrococcus, Pseudomonas aeruginosa, lactic acid bacteria, Klebsiella, and Proteus). They dealt with the HeLa cell line. Their results showed a time- and concentration-dependent variation between the eight bacterial species in inhibiting proliferation against HeLa cells. Pseudomonas and E. coli with phage had significant anticancer activity compared to the others, with an inhibitory effect of 63% and 86% at a 1000 µg/mL concentration. The IC50 for these two bacteria was estimated to be 301 and 1395 micrograms per deciliter in 24 h, respectively. Proteus and Micrococcus showed less inhibitory effects, and S. aureus even increased HeLa cell proliferation at low concentrations (40).

5. Conclusion

One of the critical features of anticancer drugs is their high effect on suppressing cancer cells with low side effects on normal cells. Studies to date have shown that metabolites produced by probiotic bacteria are effective in human health and could be used as a new treatment for cancer. The results obtained in this study, with the results obtained on other human cell lines, indicate that bacterial supernatants, which contain metabolites secreted by bacteria, can be a suitable model for anticancer tests. It seems that B. coagulans supernatant can induce apoptosis in cancer cells by increasing the bax pro-apoptotic gene expression and decreasing the anti-apoptotic bcl2 gene expression. In the future, further studies on the cytotoxic and apoptotic effects of B. coagulans biomass, its various components, and heat-killed species on various cancer cells and laboratory animals may be proposed to determine the effective metabolite on cancer cells and take steps to create the proper medication that has less of an adverse effect on healthy cells.
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None.

Conflict of Interest

The authors declare that they have no conflict of interest.

Reference


Cytotoxicity Activity of Bacillus coagulans Supernatant


