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# Evaluation of Cell Growth Inhibition of *Bifidobacterium Bifidum* Cell-free Supernatant Extract on 4T1 Tumor Cell Lineage

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## ABSTRACT

**Background:** Cancer is amongst the leading causes of death all over the world. Breast cancer is responsible for the largest number of deaths among women. Several studies confirm that *Bifidobacterium bifidum* as a probiotic significantly inhibits breast cancer development. The present study aimed to investigate the effect of *B. bifidum* supernatant on the cell growth inhibition of the breast cancer 4T1 cell line in vitro.

**Materials and Methods:** The present experimental work was conducted at Mazandaran University of Medical Sciences, Sari City, Iran. *B. bifidum* was cultured in the de Man Rogosa, and Sharpe broth at 37° C for 72 h anaerobically and the *B. bifidum* Supernatant (BS) was prepared by the freezing-thawing procedure. The cell growth inhibition of the probiotic strain was assessed using the MTT assay through breast cancer (4T1) cell line.

**Results:** The results showed that the supernatant extracted from *B. bifidum* strain had good antiproliferative effects against 4T1 cancer cell line, compared with the control group. The inhibitory effects are enhanced by passing the time.

**Conclusion:** *B. bifidum* supernatant could be a potential probiotic candidate for the treatment of breast cancer. However, further in vitro and in vivo studies are required to support our initial findings.

## Introduction

Nowadays, cancer has one of the highest morbidity and mortality rates worldwide [1]. Breast cancer is the most prevalent malignancy. Despite recent progress in its management, breast cancer has remained the second leading cause of cancer deaths in young women in the world [2]. Judging from population-based data in developing countries, the collective contingency for breast cancer occurrence in people aged 15-79 years in IR Iran has risen since the past 30 years. In 2009, the Iranian Cancer Registry Report (ICCR) reported that 8616 females were diagnosed with breast cancer [3].

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Chemotherapy can be an effective and significant method of treating breast cancer [4]. However, it is commonly accompanied by many side effects for all the dividing cells regardless of the nature of cells (malignant or non-malignant). Thus, new treatments should be designed for cancer that targets explicitly tumor cells [5, 6]. The use of bacteria in cancer immunotherapy dates back to more than 150 years ago [7]. Since then, researchers have tried to understand the bacterial mechanisms that may influence oncogenesis. Posited mechanisms include deleterious alterations in physiological host processes such as inflammation, antigen-driven lymph proliferation, and induction of hormones that increase epithelial cell proliferation [8].

Bifidobacteria as the main group of microbiota, has been demonstrated to exert antitumor effects via various mechanisms [9]. Several studies showed that bifidobacteria had cytotoxic effects on tumor cells. Bifidobacterium increases immune response and inhibits many tumor growths in vivo, such as liver cancer, breast cancer, etc. [10]. Also, the bacterium is capable of controlling the overgrowth of pathogens and modulating systemic inflammation, cell proliferation, and apoptosis. The antitumor activity of these bacteria is attributed to the activation of an immune response against tumor antigens.

Several research studies have been conducted to identify pure metabolites and other components of the microbial cells that might have antitumor activity [11-13]. For example, the identification of secondary metabolites (epothilones A and B) of the myxobacterium *Sorangium cellulosum*, which has cytotoxicity on various cancer cells both in vitro and in vivo [14]. The present study aimed to investigate the effect of *B. bifidum* supernatant on the cell growth inhibition of the breast cancer 4T1 cell line in vitro.

## Materials and Methods

### Preparation of supernatants from *B. bifidum:*

The probiotic strain tested in this experimental work was purchased from the Pasteur Institute of Iran, Tehran, IR Iran. *B. bifidum* strain (ATCC: 29521) was grown in de Man Rogosa and Sharpe (MRS) broth with 0.5% L-cysteine supplement (Merck; pH 6.5) at 37º C for 24–48 h under anaerobic conditions. After 48 h, the suspension was centrifuged at 2000 g (for 20 min at 4º C) until the bacterial count reached 2x109 CFU/mL, via the pour plate technique. The *B. bifidum* Supernatant (BS) was filtered through a 0.2 mm membrane filter to remove the remaining bacteria and debris [15].

### Cell culture:

Mouse breast cancer cell line, 4T1 was purchased from cellular bank of Pasteur Institute of Iran, and was maintained in RPMI 1640 (Gibco, Life Technology, MD) supplemented with 10% fetal bovine serum (Gibco, Life Technology, MD), 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma), and incubated at 37° C in 5% $CO_2$ with appropriate humidity [16].

### MTT test

The cell growth inhibition was measured with the MTT colorimetric assay (3-[4,5 dimethylthiazol2-yl]-1,5 diphenyltetrazolium bromide) kit (Sigma-Aldrich). In brief, 2×104 cells were seeded in 96-well plates (Sigma-Aldrich). The cells were then treated at 37° C for 24 h with the fresh RPMI medium. The culture medium was removed, and different concentrations of BS (0, 10, 20, 40, and 80 [v/v]) were added. The plates were then incubated for 48 h at 37º C in a humidified incubator with 5% $CO_2$. After incubation, cell viability was determined using the colorimetric MTT assay. In this regard, 20 µL of MTT stock was added to each well, and the plates were incubated for 4 h in the dark. The mixture was then discarded from the wells and 200 µL DMSO (dimethyl sulfoxide) was added to the cells (shook in a dark chamber for 15 minutes at room temperature) [17]. Finally, the optical density for each well was measured at 595 nm with ELISA plate reader.

### Statistical analysis:

The tests were performed in triplicate. Data analysis was carried out in SPSS V. 19. The obtained data were expressed as Mean±SD and analyzed using the paired sample t test. The P value of 0.05 or less was regarded as statistically significant.

## Results:

### Growth of probiotic strain in MRS Medium

According to compration between the optimum time for the growth of *B. bifidum* was 48 h. Colonies cultured under the microscope were observed as Gram-positive bacilli (Figure 1).

### Cell growth inhibition by MTT

The inhibitory effect of probiotic samples BS on the growth of 4T1 cell line was evaluated by MTT assay. As shown in Figure 2 (A to E), in comparison with the control group (not treated with supernatant BS), an increase

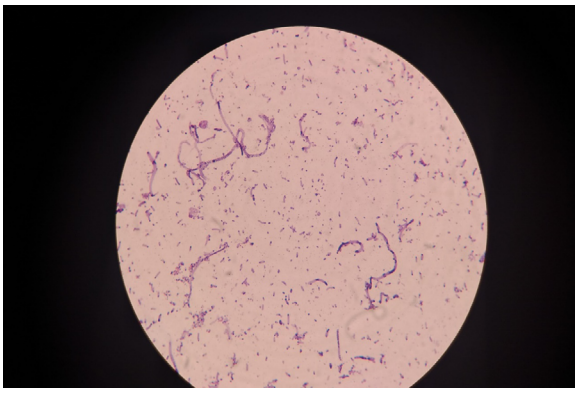

**Figure 1.** Gram-positive bacilli of *B. bifidum* in de Man Rogosa and Sharpe broth

in cell growth inhibition with BS started at 10 µL/mL concentration and reached its highest at 80 µL/mL. Our study showed that this cell growth inhibition role was dose-dependent (Table 1).

## Discussion

According to our study, BS has cell growth inhibition effects on the 4T1 cell line. This effect will increase with higher concentrations of BS. Several studies indicated the inhibitory and cytotoxicity effects of lactic acid bacteria strain on different cell lines [18]. Yuna et al. showed that B. adolescentis SPM0212 cell-free supernatant inhibited the growth of SW480, HT-29, and Caco-2 cells. The cell viability percentages with different doses of supernatant in 25, 50, 100, and 200 mg/mL were less than 50% in 72 h [19].

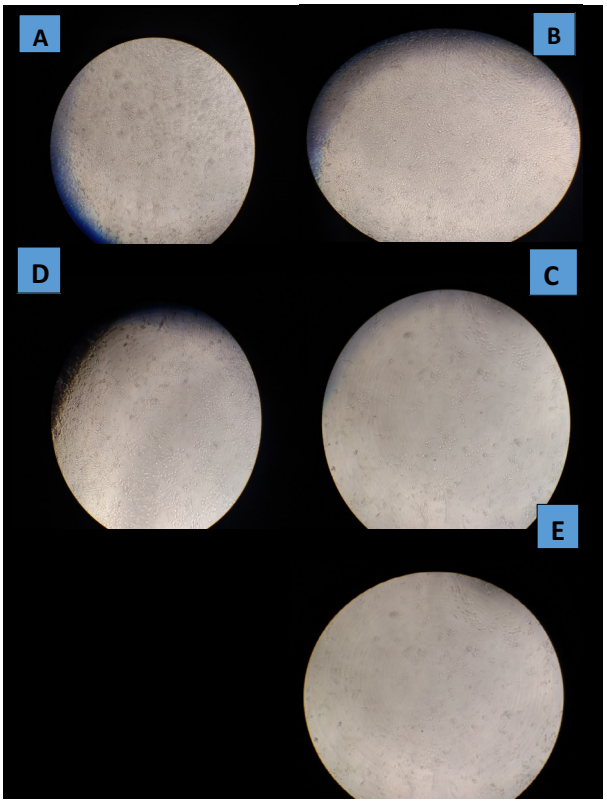


**Figure 2.** The microscopic image of cells treated with *B. bifidum* supernatant (BS) in different concentrations

A: The microscopic image of the growth of cancer cells in enriched medium (not treated with BS as the control group); B: The microscopic image of cells treated with BS (concentration: 10 µL); C: The microscopic image of cells treated with BS (concentration: 20 µL); D: The microscopic image of cells treated with BS (concentration: 40 µL); E: The microscopic image of cells treated with BS (concentration: 80 µL)

**Table 1.** The cell growth inhibition of probiotic samples BS on the 4T1 breast cancer cell line

| BS Concentrations (µL/mL) | SS | Mean±SD | P |
|---|---|---|---|
| 10 | 9.29 | 0.48667±0.09074 | 0.011 |
| 20 | 10.723 | 0.9±0.14526 | 0.009 |
| 40 | 30.41 | 1.7933±0.05897 | 0.001 |
| 80 | 45.240 | 2.582±0.09885 | 0.000 |

The supernatant extracted from the probiotic bacteria could significantly inhibit the growth of cancer cells. For example, in our study, the inhibitory effect of the BS against the 4T1 cell line was enhanced, with volume increasing. The findings in the present study are an agreement with the results of Bonyadi et al. [20]. The researchers proved that cytoplasmic extraction of the recovered lactobacilli had a significant anti-proliferative role on in vitro K562 cell lines. Also, in line with our findings, a study conducted by Ghoneum et al. in Taiwan showed supernatants made from microorganisms could inhibit the growth of most cancer cell lines such as breast, tongue, intestine, and blood [21]. In contrast with our findings, Thanomsub et al. reported that the chemical structure and biological activities of rhamnolipids produced by Pseudomonas aeruginosa had an anti-survival effect on human breast cancer cell lines [22].

Other studies revealed that *B. bifidum* supernatant inhibited the proliferation of Caco-2 cells. In this study, cell viability percentages with different doses of BS (10, 20, and 30 µL/mL) were less than 50% in 24, 48, and 72 h after treatment. Also, cytoplasmic extraction doses of 10 and 20 µL survived cells were less than 50% in 24 and 48 h. Zhao et al. reported that biosurfactants prepared from Pseudomonas aeruginosa had anti-proliferative activity against human breast cancer cells in minimal volume and concentration [23]. While in our study, this minimum inhibitory volume was found to have less inhibitory effect compared with the high volumes. Hence, especially at the early stages, BS may inhibit the growth of primary breast cancer cells. It is possible to speculate that bifidobacteria or other genera of probiotics could be used as an adjuvant treatment during anticancer chemotherapy. Our study indicates that BS inhibits cell growth of the 4T1 cell line. However, further in vitro and in vivo studies are needed to confirm this inhibitory or cytotoxicity effects.

## Ethical Considerations

### Compliance with ethical guidelines

The Ethics Committee of Mazandaran University of Medical Sciences, Sari Iran, approved the experiment (IR.MAZUMS.REC.1397.380).

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### Authors contribution's

Advise, conceptualization, study design: Mohammad Ahanjan, Saeid Abediankenari, Hamid Reza Goli; Experimental tests: Parisima Karami. Data analysis, preparing manuscript draft: Mehrdad Gholami, Parisima Karami.

### Conflict of interest

The authors declared no conflict of interest.

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