Inhibitory effects of postbiotic consisting sonication-killed *Bifidobacterium bifidum* on experimental triple negative breast neoplasm in mice: a preliminary study

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ABSTRACT

**Background and Objectives:** Breast cancer is the second leading cause of death and one of the most common malignancies among women in the world. The aim of this study was to investigate the preventive effects of postbiotic consisting of sonicated *Bifidobacterium bifidum* cells on triple negative breast cancer.  

**Materials and Methods:** Thirty-six female BALB/c mice aged 5-7 weeks were randomly divided into 3 groups (n=12): Ctrl-, healthy mice; Ctrl+, mice with breast cancer with no treatment; and Postbiotic, mice with orally gavage postbiotic before and after 4T1 cell line transplantation. Cancer progress and the effects of postbiotic were assayed by histological, immunohistochemical and gene expression quantification.  

**Results:** The histological results showed that administration postbiotic consisting of *B. bifidum* significantly decreased carcinogenesis in terms of tumor incidence, multiplicity and volume. The tumor progress was suppressed by oral intake of *B. bifidum* as showed by p53 and Ki-67 expression. Furthermore, Oral intake of postbiotic resulted in extended survival of mice and inhibited sever weight loss.  

**Conclusion:** Pretreatment with sonication killed *B. bifidum*, as a postbiotic, inhibited breast cancer progress and malignancy.

**Keywords:** Triple negative breast neoplasm; *Bifidobacterium bifidum*; Ki67 antigen; Tumor suppressor protein p53

INTRODUCTION

Breast cancer is the most common cancer and second leading cause of mortality among women worldwide. Triple negative breast cancer (TNBC) is a phenotype which accounts for 10-15% of all breast cancer. TNBS has higher prevalence among younger women under age 40 years. In TNBC, cancer cells do not express estrogen and progesterone hormones and don’t make too much of HER2 protein (1-5). These properties cause that TNBC does not respond to hormonal or trastuzumab-based therapies. So, surgery
and or chemotherapy are the only treatments available for these patients (6).

Most chemotherapy medications cause adverse effects such as loss of appetite, nausea, fatigue, weight and hair loss (6, 7). Many studies conducted on breast cancer patients show the reduced immune response such as enhanced delayed susceptibility, reduced proliferation of immune cells, and reduced production of cytokines (8-10). Despite dramatic therapeutic advancements, resistance to conventional chemotherapy has turned into a major challenge for TNBC treatment (6). Like other cancers, nutrition is an important factor which play a pivotal role in preventing and treatment of TNBC. Functional food, especially probiotics, have anti-cancer effects and are considered as a new modality (11, 12).

Based on the FAO/WHO definition “probiotic are live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Several in vitro and in vivo studies shows that some probiotic strains suppress breast cancer progression. They exert effects through different mechanisms including modulation of immune system, and anti-oxidative and anti-proliferative properties. Probiotics’ metabolites are associated with anti-cancer properties which are characterized as “postbiotic”.

A postbiotic is defined as a “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”. The probiotics secreted metabolites contain short chain fatty acids (SCFAs), organic acids, bacteriocins, and enzymes, which promote health status of the consumer. They show anti-cancer, anti-inflammatory, anti-oxidant, and anti-proliferative activities which are attributed to bioactive compound (13, 14).

MATERIALS AND METHODS

Bacterial culture and postbiotic preparation. B. bifidum ATCC: 29521 used in survey had been purchased from Center of Scientific and Industrial Research and its probiotic properties were evaluated in previous studies and cultured on DeMan-Rogosa-Sharpe (MRS) broth (Merck, Germany) supplemented with 0.5 g/l L-cysteine. The culture was incubated at 37°C for 48 h in the atmosphere containing 5% carbon dioxide. The bacterial growth was calculated by preparation of serial dilution and pour plate method in MRS agar. Then, the culture was centrifuged at 6000 rpm at 4°C for 30 min to remove the supernatant. The washed cells suspended in same volume of sterile phosphate-buffered solution (PBS) and killed by sonication (Hilscher, Germany) for 5 min while cooling on ice. The sonication-killed probiotic used as postbiotic. The prepared postbiotic was aliquot in standard Eppendorf tubes and stored at -20°C before use.

Cells and culture conditions. The 4T1 cancer cells were obtained from the cell bank of Pasteur Institute of Iran, with epithelial morphology, NCBI:C604, viability of 90%, and 1.8 × 10^6 CFUs/ml. After isolation of culture medium, it was washed with PBS to remove the remaining cells, added to the flask containing 0.25% trypsin-0.03% EDTA solution and incubated for 2 min at 37°C. Subsequently, the separation of cells from the surface of culture medial was observed using an invert microscope. Then, 2 ml RPMI-1640 (Bio sera, UK) was added to terminate trypsinization and the solution was peptized well. RPMI-1640 culture medium, 1% Pen-Strep (100 units/mL penicillin,100 μg/mL streptomycin, 10 mmol/L L-glutamate (Bio sera, UK), 10% inactivated bovine serum (Gibco, Grand Island, USA), and cell suspension were added to a new flask, mixed well, and incubated at 37°C, with 5% CO₂, and 95% humidity (15).

Animals and tumor challenge. A total of 36 BALB/c mice with an age range of 5-7 weeks and weight of 20 ± 2 g were prepared from Pasteur Institute of Iran. Animals were kept at 23 ± 2°C in 12/12 dark/light cycle with controlled humidity of 55 ± 10% in a quiet area. During the experiments the animals had freely access to standard food and water (16, 17). The protocols developed by animal welfare committee of Kermanshah University of Medical Sciences were implemented in this study.

After 14 days of adaptation, the mice were randomly classified into 3 groups including healthy mice (Ctrl-), 4T1-induced breast cancer (Ctrl+); and probiotic treated (Postbiotic). After adaptation period, the mice in Ctrl- and Ctrl + groups were orally gavaged with 0.1 ml of Phosphate-buffered saline (PBS) for 4 weeks while Postbiotic group received 0.1 ml of postbiotic daily. To induce breast cancer, 100 μl of 4T1 cells (5 × 10^5 cells/ml) were injected into the upper right mammary gland of the mice in Ctrl+ and Postbiotic groups (6, 15) at the end of week four. The treatments continued for another 5 weeks and then the
mice were sacrificed and blood sample were collected from the heart directly.

The breast tumor, liver, and lung tissues of the mice were removed and weighed precisely. The effects of postbiotic on treatment were evaluated by Immunohistochemical assay, histological analysis and relative gene expression level.

All interventions performed in accordance with the Guide for Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources approved in Arak university (ID: IR.JAU.ARAK.REC.1398.001).

**Tumor growth analysis.** Tumor growth was measured with caliper. The volume of tumors (mm³) was calculated via the following equation: \( \text{Tumor Volume} = \text{length} \times \text{width}^2 \times 0.52 \) (18).

**Body weight analysis.** Body weight was determined at the first and at the end of the experiment using a digital balance weighed.

**Histological analysis of tissue samples.** For histological analysis the removed tissues (breast tumor, liver and lung) were fixed in 10% formalin. Paraffin blocks were cut into 5 μm sections and staining of tissues were performed using hematoxylin and eosin (H&E) method (15). The Slides were inspected under a Nikon microscope (Nikon Instruments, Inc., Tokyo, Japan). Histological analysis of experimental groups was done and scored by a pathologist.

**Immunohistochemical assay of p53 and Ki-67.** The Immunohistochemical staining was done with polyclonal antibody against p53 (DAKO, TEB Azma No: K5007) and ki67 polyclonal antibody (DAKO, TEB AZMA No: k5007) according to the manufacturer’s instructions. The samples were fixed in 10% formalin for 12 h in the environment. The samples (5 μm) were dehydrated with xylene and a descending series of ethanol solution (100,100,95 and 80%). Antigen retrieval was done in 10mmol/L sodium citrate solution (pH: 6) at 100°C for 15 min and then samples were cooled for 30 min. The slides were incubated with 5% goat serum (Ori Gene Technologies, Inc.) for 15 min in the environment to block non-specific binding. In the following, the slides were incubated with antibodies against p53 and ki67 (ready to used) at 4°C overnight. Then, the slides were incubated with biotinylated secondary antibody (ready to used) at 37°C for 30 min then stained with 3,3-diaminobenzidine (DAB No. K5007: DAKO) in the environment for 10 second and slides counterstaining were done by hematoxylin. Slides were analyzed using an optic microscope. The expression of p53 and Ki-67 proteins was scored based on the number of stained cells and staining intensity with a specific antibody of the protein (19). Scoring was performed by two independent individuals on 5 random sections of each tissue, as follows:

1=≤33% cells stained at low intensity; 2=between 33 and 66% of cells stained at low intensity; 3=≥66% of cells stained at low intensity; 4=≥66% of cells stained at high intensity.

**Evaluation of p53 expression by quantitative real-time PCR.** Total RNA was extracted from tumor tissue and blood by a Universal Purification Kit (EURX Poland) according to the manufacturer’s instructions. The RNA concentration and purity were determined by a spectrophotometer (Thermo Scientific Nano Drop TM 1000). cDNA synthesis from 1 μg of RNA was done by Prime Script RT reagent kit (Takara Bio, Otsuka, Japan) and cDNA concentration was measured spectrophotometrically.

Quantitative RT-PCR was assayed by the rotor gene 6000 covette detection system and SYBR Premix Ex Taq (Takara Bio). The thermal cycle included primary activation stages at 95°C for 10 min (with primer forward: 1 μl, reverse: 1 μl, cDNA: 5 μl, Master mix:(2X): 12.5 μl, ROX:0.1 μl, UNG:0.25 μl, DHW20:5.15 μl), then 95°C for 15 seconds repeated for 35 cycles, then 56°C for 15 seconds for annealing, then at 72°C for 30 seconds, and final extension at 72°C for 10 min (20). The sequences of forward and reverse primers were as follows:

**P53 gene**

F: 5’- GTACCTTATGAGCCACCAG-3’

R: 5’-AGAAAGGTTCCACTGAGTC-3’

**GAPDH**

F: 5’- AGAACATCATCCCTGCATCCAC-3’

R: 5’- GTGACATCCACGGGACACA-3’

PCR specificity was evaluated by melting curve analysis electrophoresing in 1.2% agarose gel. To normalize each sample, mRNA level was assessed by Gapdh expression using ΔCt value (ΔCt = Ct Gapdh – Ct target mRNA) and. The levels of expression relative to the calibrator were determined by R=2^((ΔCt)
**Statistical analysis.** GraphPad Prism software 6 (GraphPad Software Inc., CA, USA) was used for statistical analysis and drawing charts. All values were expressed as Mean ± Standard Deviation and analyzed by t-test and one-way ANOVA at p value <0.05.

**RESULTS**

**Sonication killed of B. bifidum as a postbiotic.** The bacterium was cultured in MRS broth supplemented with L-cysteine and incubated for 48 h. The viable cell count was 1.5 × 10⁸ CFUs/ml in a batch culture fermentation.

**Inhibition of tumor development by postbiotic.** Induction of breast tumor was obvious in all mice 5 to 11 days after injection of 4T1 murine breast cancer cells. The body weight of mice reduced significantly as the tumor progressed in Ctrl+ group compared to Ctrl- negative group (141.08% weight loss). Surprisingly, administration of postbiotic inhibited severe weight loss. As shown in Table 1, the body weight in Postbiotic group significantly increased compared with Ctrl + group (p value<0.0001).

The mice were sacrificed at the end of experiment and breast tumors were removed and the weight of then were precisely assayed. As expected, postbiotic administration effectively inhibited the tumor growth and by 78.50% (p<0.0001) (Table 1).

**Histological analysis of tissues.** The pathological analysis of the breast tissues section showed that 4T1 cell line induced invasive ductal carcinoma with very severe grade-III, and polymorphism in the Ctrl+ group. So that cell polarity disturbance due to high-grade tumor and cell proliferation in different directions as well as cell metastasis along with atypical mitosis were observed (Table 2). In the postbiotic group, necrosis rate was low grade, which indicated the efficacy of this metabolite in inhibiting the growth of cancer cells (Fig. 1).

The histological analysis of lung in Ctrl+ and postbiotic group showed a similar pattern. They indicating the infiltration of inflammatory (neutrophilic) and mononuclear cells (lymphocytes and macrophages) in interstitial pneumonia as well as no indication of inflammatory exudate in pulmonary alveoli, bronchi, and bronchioles (Fig. 2).

The pathological results of liver tissue showed necrotic groups and presence of megalocytes in Ctrl+ and Postbiotic groups. Infiltration of neutrophilic cells and macrophages (Kupffer cells) in the sinusoidal space, portal, and surroundings of central hepatic vein were obvious, too (Fig. 3).

**Immunohistochemical assay of p53 and Ki-67 proteins.** p53 is a tumor suppressor protein and Ki-67 protein is a cell proliferation antigen which are important markers for the classification of tumors. In this study, the levels of these proteins in breast tumor tissues were quantified by Immunohistochemistry (IHC) Staining. The expression of the above proteins was scored from +1 to +4 through microscopic observation (Table 3, Figs 4 and 5).

As shown in Fig. 4, the dark points in the nucleus and cytoplasm of the cells indicate p53 protein. The p53 protein level Postbiotic and Ctrl+ groups indicated no significant difference (p value=0.17).

Fig. 5. illustrates the results of IHC staining of Ki-67 as a dark point in the nucleus of cancer cells. The level of this biomarker is highly increased in active cancer cells (Table 3). Decreased level of this factor in the postbiotic treated group indicated that this metabolite significantly inhibited cell proliferation. The level of Ki67 in postbiotic treated group decreased by 56.51%.

**Table 1. Tumor and body weight of mice at the end of experiment**

| Groups/ Parameter | Ctrl - | Ctrl + | Postbiotic |
|-------------------|--------|--------|------------|
| Weight gain (g)   | 7.10 ± 0.36⁺, 2.92 ± 0.31⁺ | 3.67 ± 0.21⁺ |
| Tumor Weight (g)  | NS*    | 1.73 ± 0.03⁺, 0.37 ± 0.035⁺ |

* Not assayed
The data in a column with different letters are significantly different.

**P53 gene expression level in breast tumor and blood sample.** The changes of p53 gene expression level in the tumor tissue were quantified by QRT-PCR (Fig. 6). Our results showed that the P53 expression in Ctrl+ group was reduced significantly compare with the other groups (p value= 0.002). Interestingly, the result of p5 expression in blood were significantly different among positive control, negative control and postbiotic groups. The metastasis of 4T1 cancer cells to blood might not have occurred during the experiment (p value= 0.0087).
Table 2. Value of cytological finding of breast carcinoma (Mean ± SEM)

| Score     | Grading ductal carcinoma | Necrosis of Acinar Ductal Carcinoma | Acini Necrosis to Carcinoma area | Atypical Mitosis | Cellular polymorphism |
|-----------|--------------------------|-------------------------------------|---------------------------------|-----------------|----------------------|
| Ctrl +    | 2.67 ± 0.21a             | 0.83 ± 0.17b                        | 1.08 ± 0.08a                    | 2.75 ± 0.17a    | 2.75 ± 0.17a         |
| Postbiotic| 2.50 ± 0.22c             | 2.17 ± 0.16c                        | 2.25 ± 0.17c                    | 2.00 ± 0.26c    | 2.50 ± 0.18c         |

Value are presented as the mean ± SEM statistical differences between the postbiotic and Ctrl + groups (n=12)
The data in a column with different letters are significantly different.

**Fig. 1.** Histopathological representative of mammary gland and tumors developed in Balb/c mice after 4T1 breast carcinoma cell line transplantation. Mammary gland slides were stained with H&E. (A) Healthy Negative Control group with normal Acini (×40), (B) Malignant breast tumor in Positive Control Group shows ductal invasive carcinoma, with highly hyperchrome nucleous (×40) (C) Moderate Parenchymal cell tumor necrosis in postbiotic treated mice (×40).

**Fig. 2.** Histopathological Representative of lung in Balb/c mice after 4T1 breast carcinoma cell line transplantation. Lung slides were stained with H&E. (A) Healthy Negative Control group (×10), (B) Infiltration of inflammatory cells in Positive Control Group (×20), (C) Infiltration of inflammatory cells in bronchial biopsy in postbiotic treated mice (×20).

**DISCUSSION**

Extensive research has been done on the progression of breast cancer, but a full understanding of it still needs further study. Diet is one of the factors that cause cancer development and the relationship between cancer and diet is currently being studied (21).

Previous studies have shown that probiotics have important functional properties and increase resistance to diseases such as cancer by strengthening the immune system, and in particular play a key role in defense functions (22). It is clear the probiotic features of immunomodulation, cytokine production through murine spleen and Peyer’s patch (23). The stimulated lymphocytes move to distant sites such as
Table 3. P53 and Ki67 Protein level in tumor tissue

| Protein level in tumor tissue | Ctrl +       | Postbiotic |
|------------------------------|--------------|------------|
| P53                         | 2.40 ± 0.25* | 3.00 ± 0.32* |
| Ki67                        | 3.83 ± 0.17+ | 1.67 ± 0.21+ |

Protein expression <10 (score 1), 10 to 30% (score 2), 31 to 50% (score 3), and >50% (score 4)
The data in a raw with different letters are significantly different.

Fig. 3. Histopathological Representative of liver in Balb/c mice after 4T1 breast carcinoma cell line transplantation. Liver slides were stained with H&E. (A) Healthy Negative Control group with normal tissue (×10), (B) accumulation hepatic Kupffer cells in liver of Positive Control Group (×100), (C) Increased Kupffer cells level in liver of Postbiotic treated mice (×100).

Fig. 4. Immunohistochemical Staining of p53 protein (Dark purple point) in mammary gland of mice after Breast cancer induction with 4T1 cells. (A) Positive control with breast cancer, (B) Postbiotic treated mice.

Fig. 5. Immunohistochemical Staining of Ki-67 protein (Dark purple point) in mammary gland of mice after Breast cancer induction with 4T1 cells. (A) Positive control with breast cancer, (B) Postbiotic treated group.

Fig. 6. Relative expression of p53 in Tumor tissue of mice in different groups

P values were regarded as significantly different at *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001

The anti-tumor properties of sonication killed *B. bifidum* as a postbiotic.

The aim of this study was investigation the effects of sonication-killed *B. bifidum* on prevention and treatment of breast cancer. We want to formulate a postbiotic drink based on this bacterium. In such...
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products the problems of bacterial survival are not considered. These products can have a longer shelf life than the probiotics ones.

Postbiotics which also known as Metabiotics are metabolic products of probiotic bacteria which provide benefits to the host. The exact mechanisms of action for postbiotic is poorly understood. Some possible mechanisms in prevention and treatment of cancers are regulating immune responses, activating pro-apoptotic cell death pathways, increasing apoptosis and necrosis, and increasing tumor cell death via autophagy.

In this study the cytotoxic effects of postbiotic on prevention and treatment of 4T1 induced breast cancer in mice model was evaluated. The 4T1 mammary carcinoma is a transplantable tumor cell line which easily metastasize to liver, lung and other body organs. The 4T1 cells were injected to upper right mammary gland of mice for induction of triple negative breast cancer.

Our results showed that daily administration of postbiotic before and after tumor implantation significantly decreased mammary tumor growth in mice. Furthermore, oral intake of this postbiotic resulted in extended survival of mice. Therefore, it is clear that postbiotic components have impressive anti-proliferative attributes due to their potentiality in adjusting cell cycle, stimulating differentiation, and up-adjusting the pro-apoptotic pathways in cancer cells (25).

The histological analyses of tumor tissue in Ctrl+ group showed vivid disturbance in cell polarity, inducing invasive ductal carcinoma with very severe grade-III polymorphism and pleomorphism. In the postbiotic group necrosis of cancer cells was observed, indicating the clear efficacy of postbiotic in the inhibition of cancer cell growth. Inhibition of cell proliferation and promotion of apoptosis and necrosis, and 4T1 cell proliferation, migration and invasion by the postbiotic were obvious (21). The results of this study were in line with the findings of other studies that reported cytoplasmic extract of B. bifidum could inhibit the growth of cancer cells (26).

Evidence of cancer protective action of postbiotic was also provided by evaluation of p53 and ki67 protein level in tumor tissue. As expected, Ki-67 protein level which assayed by ICH method was lower in the postbiotic group. These results was accompanied by higher concentration of p53 protein in postbiotic group.

These results were confirmed by evaluation of p53 gene expression by quantitative RT-PCR method. This technique with higher accuracy proved the positive effect of postbiotic in cancer therapy.

One of the characteristic signs of cancer is severe weight loss, called cachexia which is due to loss of adipose tissues and skeletal muscles. The cancer patients experience elevated lipolysis of adipose tissues on the one hand and reduced synthesis of natural proteins and their increased degradation on the other hand (27, 28). In the present study, along with tumor progress significant weight loss was observed in mice.

CONCLUSION

Our results showed that administration of postbiotic not only inhibited weight loss but also significantly increased the weight of mice during the experiment. Administration of postbiotic attenuated the degree of tumor malignancy so inhibited sever weigh loss in animals (29, 30).

The prevalence of breast cancer among women is extensively on the rise despite current therapeutic methods. So, finding novel and safe preventive and therapeutic methods is highly important because of resistance to some chemical drugs. Use of functional foods, especially probiotics has been seriously taken into account because of preventive effects against various infectious diseases and cancers. The findings of the present research showed that administration of killed B. bifidum’s postbiotic significantly decreased carcinogenesis in terms of tumor incidence, multiplicity and volume.

In this study we investigated the postbiotic effects of Bifidobacterium bifidum in prevention and treatment of 4T1-induced TNBC in mice model. B. bifidum is a most common probiotic strain which can be found in the mammalian intestine. It is considered as safe because of long term traditional use in fermented dairy products and its GRAS (Generally Recognized As Safe) status (31, 32).

We assayed the value of p53 and Ki-67 as tumor progression markers. P53 is one of the most important tumor suppressor genes that plays a pivotal role in genomic stability (33, 34). Mutation in p53 gene accelerates tumor growth, increases angiogenesis, disrupts the apoptosis process, and induces treatment.
resistance (35-37). This gene encodes a phosphoprotein that regulates the cell cycle and can also be called a tumor suppressor (38, 39). Ki-67 plays an important role in mitosis and cellular interphase and whose cellular distribution dramatically changes during cell cycle progression (40).

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