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Original Article

Preventive effects of microvesicles isolated from *Bifidobacterium bifidum* on 4T1-induced breast cancer in BALB/c mice

Farzaneh Nazari[1], Parvaneh Jafari[1*], Bizhan Nomanpour[2], Kambiz Varmira[3], Farshid Raissi[2]

1. Microbiology Department, Science Faculty, Islamic Azad University , Arak Branch, Arak, Iran
2. School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
3. Research Center of Oils and Fats, Kermanshah University of Medical sciences, Kermanshah, Iran

ABSTRACT

Introduction: Breast cancer is one of the important causes of mortality among women. Many studies have focused on the development of natural health products for prevention and treatment of breast cancer. This study investigated the effects of *Bifidobacterium bifidum* and its microvesicles (MVs) on 4T1-induced breast cancer in BALB/c mice.

Methods: Sixty female BALB/c mice were divided into 5 groups: (1) negative control, (2) positive control, (3) doxorubicin, (4) probiotic and (5) MVs (isolated from *B. bifidum*). The mice in groups 4 and 5 were pretreated with *B. bifidum* and MVs for 4 weeks, respectively. Tumor was induced by subcutaneous administration of 4T1 cell culture. After tumor development, mice in group 3 were treated with doxorubicin while other groups were treated as before. The mice were sacrificed after 3 weeks, breast tumors were removed and histologically examined. Also, P53 and Ki67 protein were evaluated by immunohistochemistry staining, and P53 gene expression was assessed by RT-PCR.

Results: Pretreatment with MVs and probiotic reduced mortality and tumor growth rate. MVs and Probiotic prevented the weight loss and suppressed tumor cell proliferation which was indicated by Ki-67 and p53. The expression of p53 confirmed these results. Despite the malignancy in the breast tissue, necrosis of cancer cells occurred in the mice treated with MVs.

Conclusion: MVs of *B. bifidum* effectively inhibited breast cancer development in mice and suppressed tumor cell proliferation. Probiotic microorganisms and their metabolites can be used as a functional food to prevent breast cancer.

Keywords:
Triple negative breast cancer
Probiotic
Bifidobacterium bifidum
Microvesicles
Ki67
p53

Introduction

Breast cancer is one of the leading causes of mortality among women with increasing prevalence. This disease is followed by invasion steps in the lungs, bone and liver and is accompanied by changes in gene expression (Martin et al., 2013; van Zijl et al., 2011). The specific receptors inside and outside the breast cells receive messages from estrogen and progesterone that induce the growth and control performance of them. Disturbance in the production of these hormones and expression of their receptors are the most important risk factors for breast cancer, especially in postmenopausal women

* Corresponding author: Parvaneh Jafari, p-jafari@iau-arak.ac.ir
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(Tian et al., 2018).

Breast cancer is classified by 3 immunohistochemistry tumor markers, including estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2. About 10-20% of cancers are triple negative breast cancer (TNBC: none of the above receptors are expressed) (Kumar and Aggarwal, 2016; Pope, 2019). TNBC is more invasive with higher recurrence and metastasis than other types (Parise and Caggiano, 2018). Invasive ductal histology, high histological grade and high mitotic index are seen in more than 90% of TNBCs, with necrotic zone and pushing order and significant lymphocytic infiltrate. This kind of cancer is not responsive to conventional tumor treatments such as endocrine therapy and targeted therapy. Thus, chemotherapy with drugs such as doxorubicin and surgery are appropriate treatment modalities for it (John et al., 2018; Kaur et al., 2012).

Despite remarkable advancements in the treatment of breast cancer, resistance to anti-cancer drugs is still a big concern (Bao et al., 2011). In this regard, use of functional foods, especially probiotics is highly increasing due to their benefits in the prevention and treatment of cancers especially for TNCB (Shahidi et al., 2018). Probiotics are living microorganisms that exert their health effects if used in adequate amounts. The effects of probiotics on intestinal cancers have been proven. It seems that their oral intake can be effective in the treatment of non-intestinal tumors including breast cancer (Aragon et al., 2015). The effect mechanisms of probiotics, especially on the immune regulation process, have been attractive to researchers (van Baarlen et al., 2013). The exact contribution of probiotics to immunomodulation and neoplasia formation has not fully understood. The immunoregulation of probiotics are mediated through the modulation of anti-tumor immune responses by several mechanisms. Production of microvesicles (MVs) is one of the communication mechanisms between probiotics and immune system (McBroom and Kuehn, 2007). MVs formed by direct outward budding of plasma membrane and their size range from 100 nm up to 1μm in diameter. The uniqueness of MVs is that they have ability to package active cargo include proteins, nucleic acids and lipids. The concentration of proteins in MVs can be 100-fold higher compare to the cell lysate (Kaparakis et al., 2010; Kulp and Kuehn, 2010). This active cargo is responsible for intracellular and extracellular communication and involved in intra-kingdom communication and immune system regulation (Aragon et al., 2015).

This study aimed to investigate the preventive effects of *Bifidobacterium bifidum* and MVs in breast cancer in mice. *B. bifidum as a probiotic* is regarded as safe and exists in the mammalian digestive system (Aragon et al., 2015; Little et al., 2003). This bacterium is commonly used for its health promoting features and preventing of gastrointestinal cancers. We induced triple negative breast cancer in mice with injection of 4T1 cell line. Preventing effect of MVs and probiotic were investigated by immune-histological study and investigation P53 and Ki-67 expression as cancer marker genes. P53 (guardian of the genome) is one of the most significant suppressor genes and Ki-67 is extensively used for rating tumors and its level increases during the cell proliferation (Liu et al., 2017; Pappas et al., 2017).

Material and methods

Bacterial culture and preparation of freeze-dried bacteria and MVs

B. bifidum ATCC 29521 was purchased from Iranian Research Organization of Science and Technology and its safety and efficacy were assayed based on WHO guidelines (Ganguly et al., 2011). The bacterium was cultured in DeMan-Rogosa-Sharpe (MRS) broth (Merck, Germany) supplemented with 0.5 g/l L-cysteine. Incubation was performed in an atmosphere containing 5% CO_2 at 37°C for 48h. Bacterial cells were isolated by centrifugation at 4000g at 4°C for 30min. The cells were washed three times with phosphate-buffered saline (PBS) before resuspended in cryo-protectant solution for freeze drying (11% skim milk supplemented with 2% monosodium glutamate). The viable cell count was enumerated in freeze dried powder in MRS agar.

MVs were isolated from isolated active cell by centrifugation at 70000g and 4°C under 20 mbar for 1h (Beckman Coulter, Optima L-90K). Isolated MVs resuspended in sterile PBS buffer corresponding in 10% of volume of initial medium culture. MVs were quantified by reference to the number of viable bacteria in the culture and also standardized by protein content (8mg/ml). The uniform suspension aliquoted in microtubes and kept at -20°C (Klimentová and Stulík 2015).

Cell line culture

The 4T1 cell line was purchased from Pasteur Institute

of Iran (NCBI: C604). The cells were cultured in RPMI-1640 (Biosera, UK) supplemented with 10% heat inactivated fetal bovine serum (Gibco, Grand Island, US), 10 mmol/l Glutamate, 100 units/ml penicillin and 100 µg/ml streptomycin (Biosera, UK cells) (Baliga et al., 2005; DuPre et al., 2007; Tao et al., 2008). The cultured medium was incubated for 48h at 37°C with 95% humidity and 5% CO_2. The spent medium was discarded and the cells washed with PBS. Then, 2ml of 0.25% trypsin/ 0.03% EDTA solution was added to the plate and swirled to cover the entire plate. Next, the plate was incubated at 37°C for 2min. The fresh RPMI medium was added to stop the trypsinization and the cells were suspended. The cells were centrifuged and washed with RPMI medium and resuspended in the same medium. The Cells' number was determined using a hemocytometer (Jadidi-Niaragh et al., 2017).

Animal feeding procedure and treatments

A total of 60 female BALB/c mice (5 weeks old; 20±2g weight) was purchased from Tehran Pasteur Institute (DuPre et al., 2007; Tao et al., 2008). All animals had *ad libitum* access to water and food and were kept at 23±2°C in 12/12 dark/light cycle with controlled humidity of 55±10%.After 14 days of adaption, animals were randomly divided into 5 groups: (1) healthy negative control, (2) positive cancer control, (3) doxorubicin, (4) probiotic and (5) MVs.

The mice in groups 1 to 3 were orally gavaged with 0.1ml normal saline, while groups 4 and 5 received 0.1 ml *B. bifidum* suspension (1×10^9 Colony Forming Unit (CFUs)/ml) and MVs (8mg protein/ml), respectively for 4 weeks (Aragon et al., 2015). After pretreatment, 0.1ml of 4T1 cell culture (5×10^5 CFUs/ml) was administered subcutaneously to the upper right mammary gland of the all groups except for the negative control group. The pretreatment trend was continued the same for more 5 weeks, except for group 3 that was intraperitoneally injected with doxorubicin (5mg/kg, Sigma-Aldrich), 3 times a week (Bao et al., 2011; Jadidi-Niaragh et al., 2017; Pommier et al., 2010). It must be mentioned that the tumors with dimension at least 0.09±0.02 cm^2 were considered as developed cancer.

Body weight gain was recorded for each mouse in all of the groups every week. At the end of the experiments the mice were scarified and blood, tumor, long and liver tissue was harvested for histological, immune-histological and gene expression assay. 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.IAU.ARAK.REC.1398.001).

Tissue preparation for histological and immuno-histological assessment

A part of the tissue samples were fixed in formalin and after preparation of paraffin blocks, 5µm sections of them were prepared (Jadidi-Niaragh et al., 2017). Tissue slides were stained by hematoxylin & eosin (H&E) and the morphology of the tissues were examined microscopically (Cardiff et al., 2014).

Immunohistochemical staining of the breast cancer tissues was performed for p53 (DAKO, Teb Azma) and Ki67 proteins according to the manufacturer's instructions. Briefly, the sections on silanized slides were treated at 56°C for 2h and the paraffin was removed by xylene. The tissues were hydrated in descending concentrations of ethanol and treated in 0.01M citrate buffer (pH: 6.0). The peroxide activity of tissue was stopped by adding 3% hydrogen peroxide for 15min and slides were incubated in tris-buffered solution (pH 7.6) for 15min. Finally, the sections were separately stained with p53 (clone DO-7, DAKO, dilution 1:50) and Ki-67 (Cell Marque, Rocklin, dilution 1:200) specific monoclonal mouse anti-human antibodies. After incubation at room temperature, the post-primary antibody was added. The staining was completed by adding DAB (3, 3'-diaminobenzidine). The levels of p53 and Ki-67 proteins were scored after counterstaining of slides with hematoxylin 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.

Quantitative real-time PCR for P53

Total RNA was taken from tumor tissues using a universal purification kit (EURX Poland) according to the manufacturer's instructions. PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan) was used for cDNA synthesis from 1µg RNA. Quantitative RT-PCR was performed by rotor gene 6000 corbette detection system using SYBR Premix Ex Taq (Takara Bio). The thermal cycle conditions included primary activation for 5min

TABLE 1: The mean±SD of mice weight gain, Breast tumor weight, P53 and Ki67 protein level in tumor tissues, and relative expression of P53 in tumor tissues.

Groups	Ctrl -	Ctrl +	Doxorubicin	Probiotic	Microvesicles
Weight gain (g)	7.100 ± 0.360^{a}	-2.917 ± 0.312^{c}	-4.450 ± 0.369^{d}	4.000 ± 0.375^{b}	3.750 ± 0.217^{b}
Tumor Weight (g)	NS*	1.730 ± 0.027^{a}	0.1503 ± 0.057^{c}	0.7097 ± 0.125^{b}	0.2810 ± 0.070^{c}
P53 protein score in tumor tissue	NS	2.400 ± 0.245^{b}	3.600 ± 0.244^{a}	2.600 ± 0.243^{b}	2.800 ± 0.200^{ab}
Ki67 protein score in tumor tissue	NS	3.833 ± 0.167^{a}	2.000 ± 0.258^{b}	1.833 ± 0.307^{b}	1.500 ± 0.224^{b}
Relative gene expression of p53 in tumor Tissue	2.455 ± 0.282^{b}	0.866 ± 0.063^{c}	5.567 ± 0.154^{a}	2.336 ± 0.261^{b}	3.118 ± 0.212^{b}

* Not assayed. Different superscripts letters in rows indicates statistically significant differences.

TABLE 2: Cytological grading of breast carcinoma

Score	Grading ductal carcinoma	Necrosis/apoptosis of Acinar Ductal Carcinoma	Acini Necrosis/apoptosis to Carcinoma area	Atypical Mitosis	Cellular polymorphism
Ctrl +	2.667 ± 0.211^{a}	0.8333 ± 0.167^{c}	1.083 ± 0.083^{b}	2.750 ± 0.171^{b}	2.750 ± 0.171^{a}
DOX	2.657 ± 0.212^{a}	2.833 ± 0.168^{a}	3.708 ± 0.187^{a}	1.500 ± 0.227^{a}	1.333 ± 0.211^{b}
Probiotic	2.500 ± 0.224^{a}	1.500 ± 0.225^{b}	1.167 ± 0.168^{b}	2.833 ± 0.162^{b}	2.667 ± 0.215^{a}
MVs	2.500 ± 0.347^{a}	2.667 ± 0.212^{a}	3.500 ± 0.343^{a}	1.833 ± 0.307^{a}	1.667 ± 0.215^{b}

Ductal carcinoma of breast include grade I (+), grade II(++), grade III (+++). Necrosis of invasive acinar adenocarcinoma include mild (+), moderate (++), and sever (+++). The ratio of necrotic to tumore area include >50% (+++), 20%<X<50% (++), and <20% (+). Atipical mitosis include mild (+), moderate (++), and sever (+++). Cells Poleomorphisms include mild (+), moderate (++), and sever (+++). Different superscripts letters in column indicates statistically significant differences.

at 95°C, 45 cycles at 72°C for 10s and final extension at 72°C for 15s. Gapdh, was used as a housekeeping gene (internal control). The sequence of forward and reverse primers was as follows: *Gapdh*: 5'- AGAACATCATCCCTGCATCCAC-3' and 5'- GTCAGATCCACGACGGACACA-3'; *p53*: 5'- GTACCTTATGAGCCACCCGA-3' and 5'- AGAAGGTTCCCACTGGAGTC-5'.

The specificity of PCR was measured by melting curve analysis electrophoresing in 2% agarose gel and mRNA level was normalized by Gapdh expression and measured by ΔCT value (ΔCT = CT Gapdh – Ct target mRNA). ΔCT was determined by subtracting ΔCT calibrator from the sample. The normalized value of the expression levels relative to calibrator was determined by $R=2^{-\Delta Ct}$.

Statistical analysis

Statistical analysis was performed by GraphPad-Prism software 6 by the post hoc of two-way ANOVA and t test of data at $P<0.05$.

Results

Preventing weight reduction and tumor growth via pretreatment with probiotic and MVs

To study the preventive and therapeutic effects, mice were pretreated by *B. bifidum* and MVs for 4 weeks before tumor induction. Breast cancer was induced by transplantation of the 4T1 cell line. Development of cancer was obvious in all mice after 5-11 days. After 5 weeks, tumor development caused a significant weight reduction in the positive control group by 141.08%, but weight reduction was worst in the doxorubicin group (168.62%). Interestingly, the use of the *B.bifidum* and MVs inhibited severe weight decrease and even the weight increased in these groups compared to the positive control group (229.13 and 228.56%, respectively; Table 1). Our results showed development of tumors caused sever mortality. Surprisingly, the mortality rate in the doxorubicin treated group was the highest. Pretreatment with the probiotic and its metabolites (MVs) decreased the rate of death significantly.

Histological analysis of tissues

After sacrificing the mice at the end of the experiment, breast, lung and liver tissues were removed. The breast tissue was accurately weighed in all the groups. As shown in Table 1, doxorubicin efficiently inhibited tumor progress, so its weight was reduced up to 91.31%. Surprisingly, the *B. bifidum* and MVs effectively reduced tumor weight by 58.98% and 835.36%, respectively.

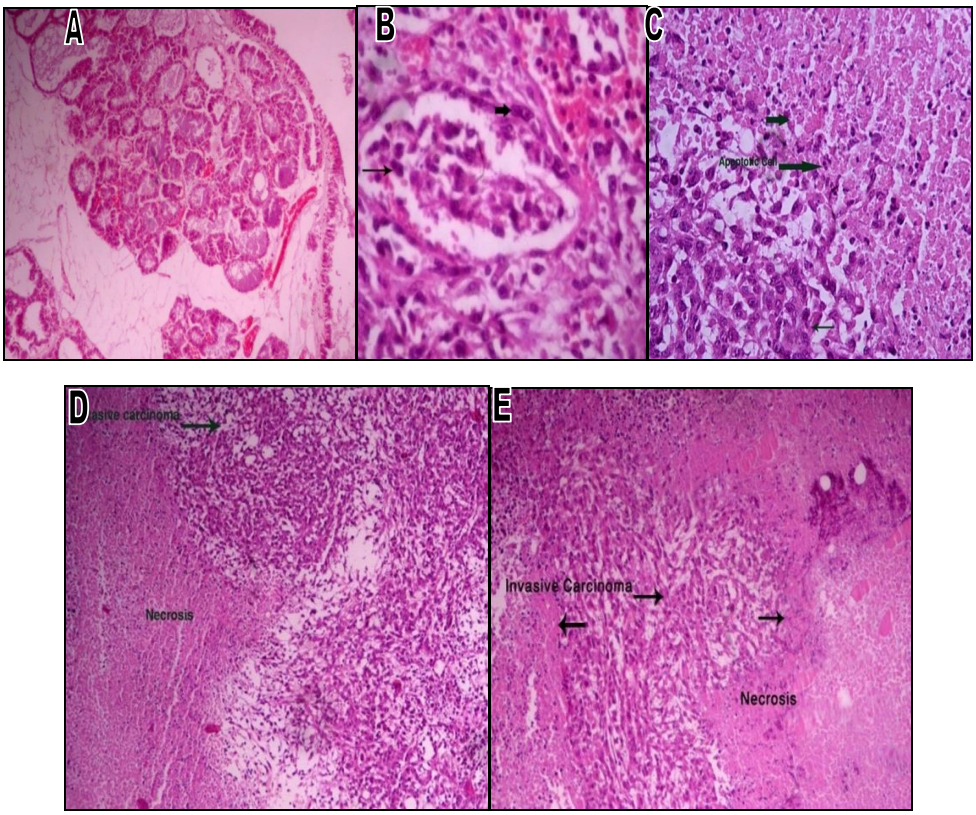

FIGURE 1. Histopathological representative of mammary gland and tumors developed in Balb/c mice after 4T1 breast carcinoma cell line transplantation. Mammry gland slides were stained with H&E. (A) Healthy negaive control group with normal Acini 10X, (B) Invading local blood vessles by breast tummor cells in positive control group, cancer cell metastasis to blood vessels (thick arrow) and their presence inside blood vessele (thin arrow) with severe bleeding and imflammatroy cells infiltation 40X, (C) Extensive parenchymal cell tumor necrosis in doxroubicin treated mice 40X, the tip of the arrow represents the apoptotic cell, (D) Moderate parenchymal cell tumor necrosis in probiotic treated mice 20X, breast carcinoma area (arrow) (E) Moderate parenchymal cell tumor necrosis in microvescle treated mice 20X, the tip of the arrow indicates the area between the necrotic and tumor areas.

The tumor weight in the MVs group was decreased compare to the probiotic group (p value=0.0406), but showed no significant difference with the doxorubicin group (P=0.223). This finding indicted the effectivity of this pretreatment.

Tissue sections from breast were stained and comparative scoring of the tissues was performed after H&E staining (Table 2 and Figure 1). As it expected, no malignancy was observed in breast tissue of the negative control group. The results indicated that administration of 4T1 cell line resulted in an invasive ductal carcinoma with highly severe grade III polymorphism and pleomorphism. Cell polarity in tumor tissues was disrupted and cell proliferation occurred in various directions. In the doxorubicin group, great necrosis/apoptosis of the tumor cells was clearly obvious. Administration of this drug induced more than 50% cell necrosis/apoptosis in tumor tissue. The same rate of cell death was observed with pretreatment with MVs, too. While using of *B. bifidum* selectively induced about 20% of the tumor cells necrosis/apoptosis.

The pathology of lung tissue in all experimental groups (except for negative control) followed a similar pattern. The results indicated infiltration of inflammatory cells, especially neutrophils and mononuclear cells in the lung. Lymphocytes and macrophage infiltration as well as interstitial pneumonia were observed with no trace of tumor cell metastasis. The inflammatory exudate was not seen in the pulmonary alveoli, bronchi, and bronchioles (Figure 2).

The histological analysis of the liver showed that infiltration of neutrophils and macrophages (Kupffer cells) occurred in the sinusoids and portal space, and surroundings of the central vein in all treated groups. Liver damage was observed and scored as necrosis at the presence of megalocytes in all groups, which indicated histological damage (Table 3 and Figure 3).

TABLE 3: Cytological grading of liver

Score	Infiltration of Lymphocyte and Macrophages in liver	
	Portal space	**Sinusoidal endothelial cells**
Ctrl +	0.334 ± 0.211^{b}	0.333 ± 0.211^{c}
DOX	2.833 ± 0.167^{a}	2.833 ± 0.167^{a}
Probiotic	2.833 ± 0.166^{a}	2.167 ± 0.167^{a}
MVs	2.333 ± 0.210^{a}	1.167 ± 0.166^{b}

Infiltration of mono nuclear cells (lymphocytes and macrophages) in portal triad and sinusoidal cells of liver include, Mild (+), Moderate (++), and sever (+++). Different superscripts letters in column indicates statistically significant differences.

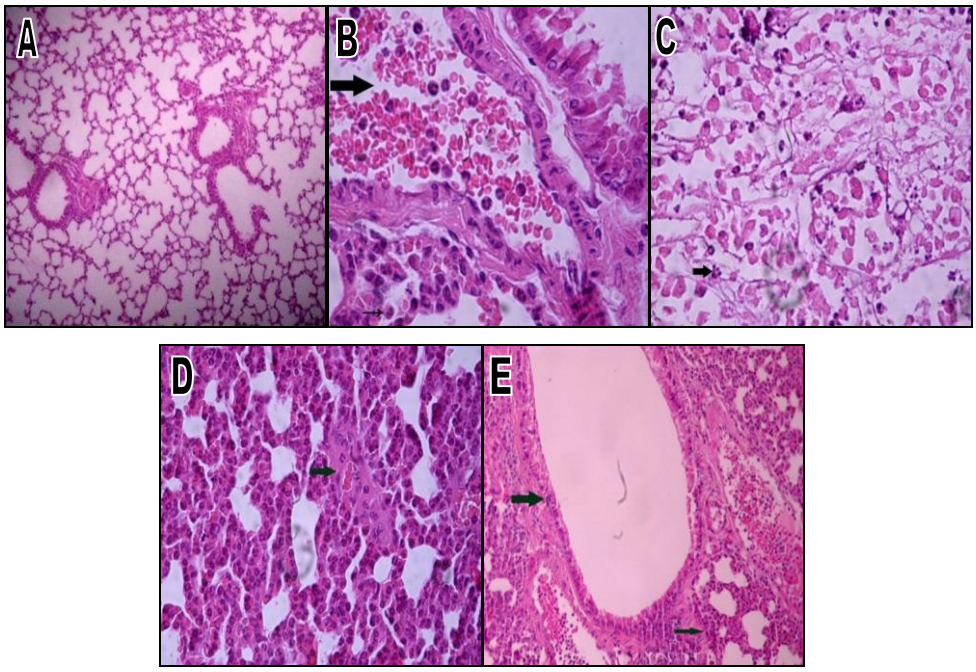

FIGURE 2. Histopathological representative of lung in Balb/c mice after 4T1 breast carcinoma cell line transplantation. Lung slides were stained with H&E. (A) Healthy negaive control group with normal bronchial airways and aloveolar ducts and aleveolar sacs 10X, (B) Normal lung parenchyma in positive control group 40X, pulmonary vascular hypermia with a marked presence of neutrophil cells in the inner duct of the artery (thick arrow) and around the artery (thin arrow) (C) Arrow show extensive cell necrosis and imflammatroy cells infiltation in doxroubicin treated mice 10X, (D) Focal accumulation of imflammatroy cells and fibrinous exudate around a capillary (arrow) in probiotic treated mice 40X, (E) Infiltration of inflammatory cells in bronchial biopsy (thin arrow) cross-section of pulmonary bronchioles (thick arrow) in microvescle treated mice 20X.

Immuno-histological analysis of tumor markers

Ki-67 as a cell proliferation antigen and p53 as a tumor suppressor protein were assayed in this study. The levels of these proteins in sections were determined by immunohistochemistry staining and scored from +1 to +4 (<10 +, 10 to 30% ++, 31 to 50% +++, and >50% ++++; Table 1). Results showed that p53 protein in the doxorubicin group increased by 50% compared to the positive control group (Figure 4). This increased level cause tumor growth suppression, but could be one of the side effects of this medication which result in cardiomyopathy. This protein increased by 8.33% and 16.67% in the probiotic and MVs groups with no significant difference with the positive-control group.

Figure 5 shows immunohistochemistry staining of Ki-67 protein as dark color in the nucleus of the cancer cells. The Cell proliferation significantly repressed in all treated groups. The Ki-67 protein level in the doxorubicin group was decreased by 47.82%. This medication is a DNA intercalator that terminates cell proliferation by inhibiting topoisomerase II. Surprisingly, cell proliferation in the probiotic and MVs groups was suppressed,

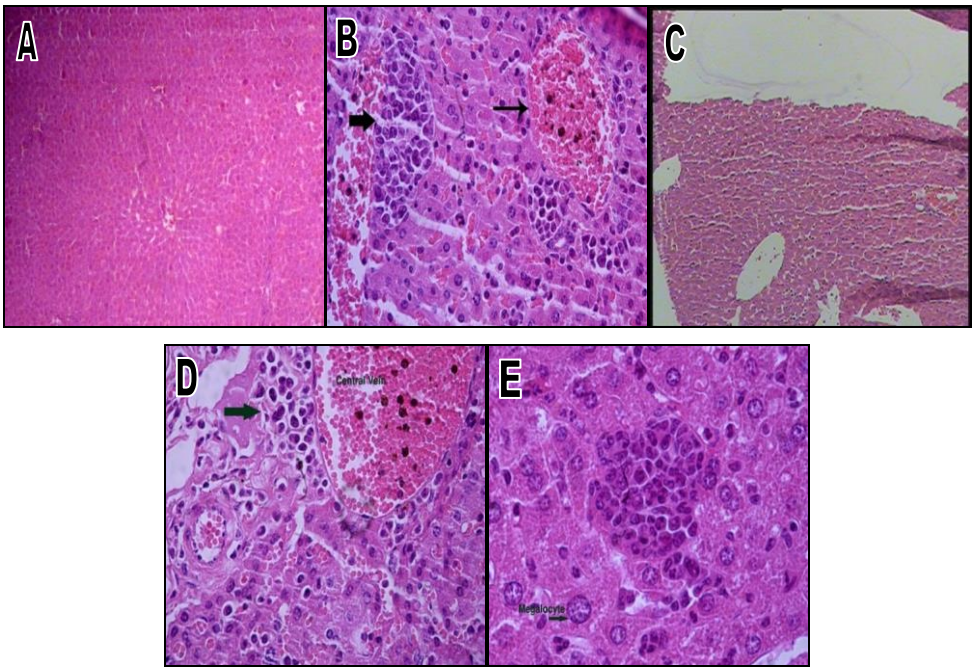

FIGURE 3. Histopathological representative increased Kupffer cells level in liver (thick arrow) of positive control group with hyperemia, hemorrhage and mild proliferative changes in sublobular central veins (thin arrow) 40X, (C) Increased Kupffer cells level in liver of doxorubinc treated mice with inflamaion of liver parenchyme 10X, (D) Accumulation of Kupffer cells around the central vein of the hepatic lobule 40X , (E) Focal infiltration of Kupffer cells (in the center) and presence of megalocytes (arrow) in liver of microvesicle treated of liver in Balb/c mice after 4T1 breast carcinoma cell line transplantation. liver slides were stained with H&E. (A) Healthy negaive control group with normal tissue 10X, (B) mice 100X.

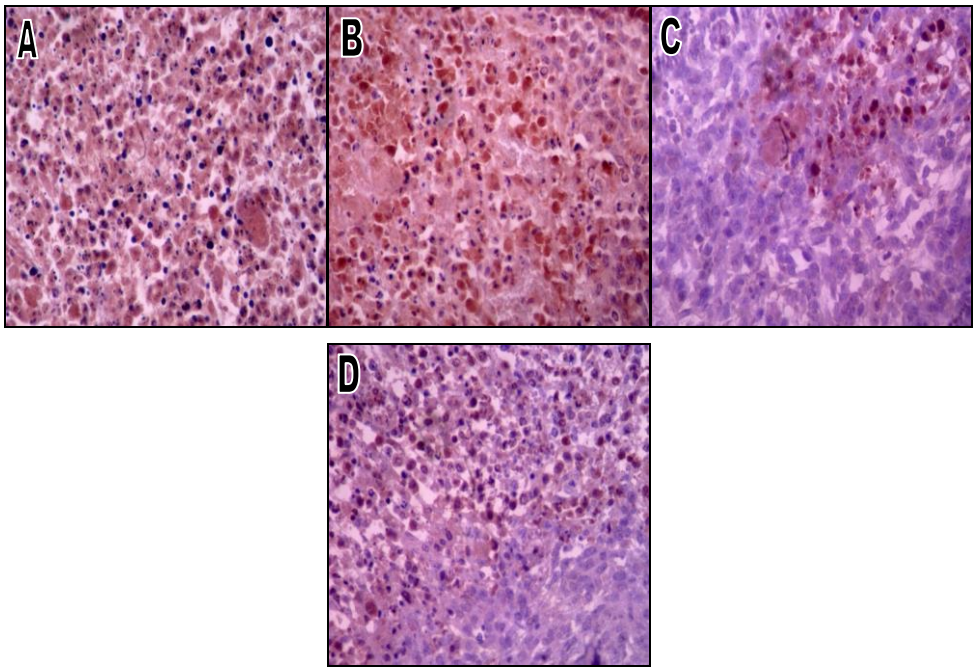

FIGURE 4. Immunohistological staining of p53 protein expression in mammary gland of mice after breast cancer induction with 4T1 cells and pretreatment with probiotic and microvesicles. (A) Positive control with breast cancer, (B) doxorubicin treated rats for 3 times a week after tumor induction, (C) probiotic pretreated rats for 3 weeks and its prevention effects, (D) microvesicles pretreated mice for 3 weeks and its prevention effects. The objective used was 40x.

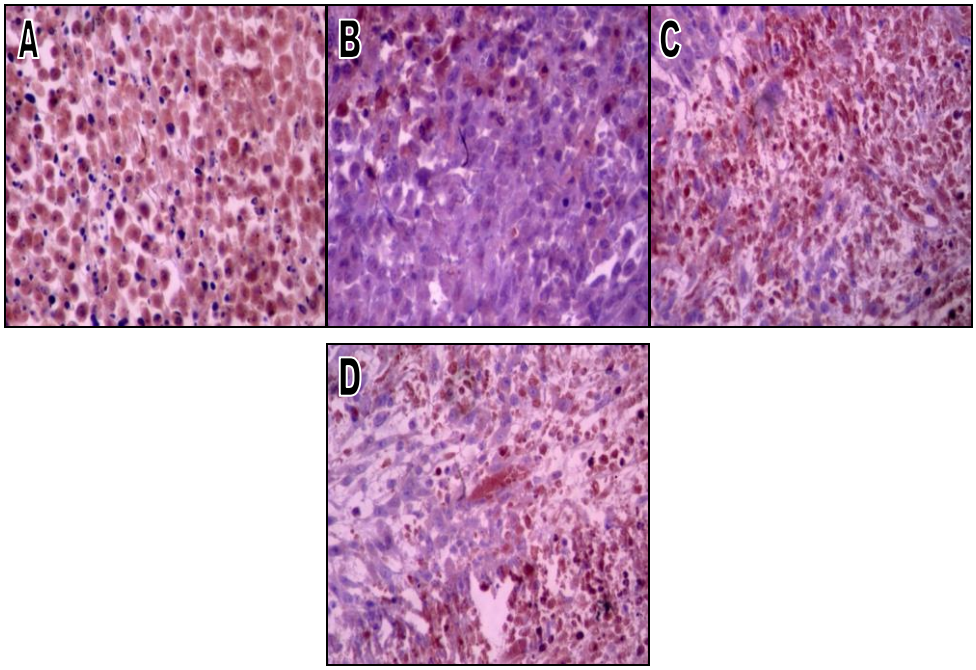

FIGURE 5. Immunohistological staining of Ki-67 protein expression in mammary gland of mice after breast cancer induction with 4T1 cells and pretreatment with probiotic and microvesicles. (A) Positive control with breast cancer, (B) doxorubicin treated rats for 3 times a week after tumor induction, (C) probiotic pretreated mice for 3 weeks and its prevention effects, (D) microvesicles pretreated mice for 3 weeks and their prevention effects.

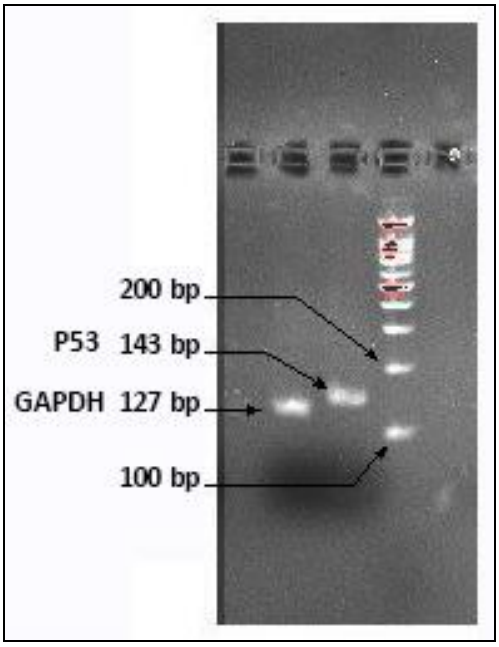

FIGURE 6. P53 gene expression. PCR product electrophoresis on amplification of p53 gene fragments and internal control of GAPDH on agarose gel.

too. In these groups, the expression of this protein decreased by 52.18% and 60.87%, respectively, compared to the positive control group, indicating no significant difference with doxorubicin group.

P53 gene expression in breast tumor based on quantitative RT- PCR analysis

The specificity of PCR was showed by electrophoresing in agarose gel (Figure 6). The genomic expression of p53 protein was analyzed in the tumor cells (Table

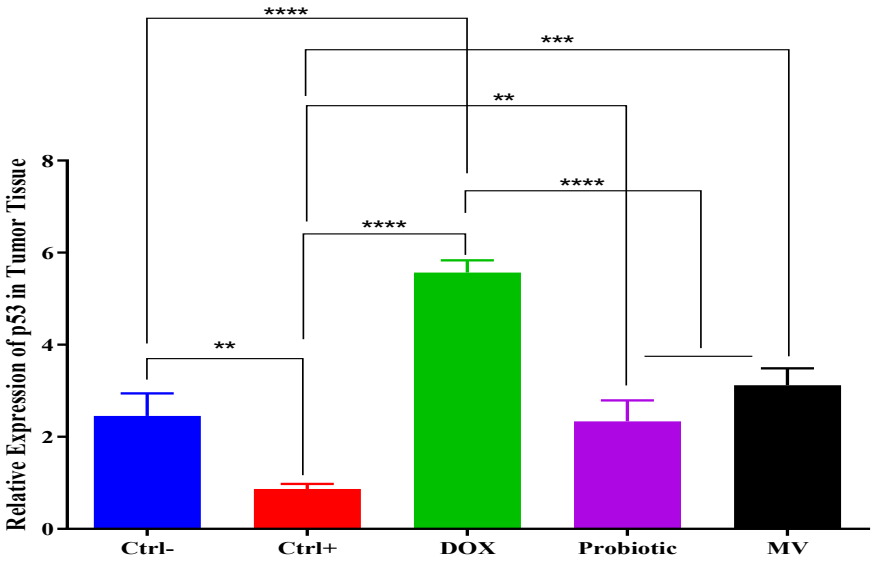

FIGURE 7. Relative expression of p53 gene in the tumor tissue of mice after breast cancer induction by 4T1 cell line. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$.

1). The p53 gene did not express in the 4T1 cell line, so its expression level reduced by 64.71% in the positive control group, while its expression in the doxorubicin group increased by 542.62%. The expression of p53 in probiotic and MVs groups increased by 169.65% and 259.92%, respectively, compared to the positive control group (Figure 7).

Discussion

It has been demonstrated that gut immunological homeostasis is stablished and maintained by the interaction between host immunity and gut microbiota. The immunomodulatory effects not only exert by direct interaction of microbiota and the components of immune system, but also by the microbial metabolites. It is proven that microbiota dysbiosis is implicated in a variety of disease including cancer. Looking forward, some probiotic strains and their metabolites have been proven to play an important role in immunomodulation. Recent studies have shown that probiotics can stimulate local immunity through the Peyer's patch. The stimulated lymphocytes B and T migrate to distal mucosal sites such as respiratory system, urogenital system, salivary and mammary glands, and improve immunity (Ranjbar et al., 2019).

The safety of probiotic is stablished but there is growing concern about administration of intact bacteria for certain vulnerable groups such as premature infants, immunocompromised patients and critically ill individuals. Therefore, using probiotic components and metabolites, especially extracellular vehicles are gaining importance in mentioned groups. Extracellular vehicles (e.g., MVs) are the key elements for bacteria-host interaction. These vesicles carry a vast variety of component such as proteins, nucleic caids and lipids which modulate different signaling pathways in host cells.

In this study we investigated the effects of *B. bifidum* and its secreted MVs in preventing of breast cancer. To the best of our knowledge, this is the first report about using of MVs to prevent this kind of cancer. We used 4T1 cell line for induction a triple negative breast cancer which can metastasize to the lung, liver, lymph nodes, brain and bone. Our results showed one-month pretreatment with *B. bifidum* and MVs suppressed the breast tumor as indicated by Ki-67 and p53 concentration. The Ki-67 marker is strongly associated with tumor cell proliferation and progression. The significant reduction of this marker in all treated groups, showed the effectiveness of probiotic strain and MVs compare with doxorubicin. The p53 is a guardian protein which suppress tumor cells proliferation. In our experiment, the p53 concentrations were not interpretable based on immune-histological assy. So, we decided to evaluate p53 gene expression by quantitative RT-PCR which is more accurate method. Our results showed that expression of this gene was significantly higher in the doxorubicin group than probiotic and MVs groups. The highly

elevated expression of p53 is one of the side effects of doxorubicin which causes cardiac impairment and cardiomyopathy (Doroshow et al., 1981).

Histological analysis showed an invasive breast ductal carcinoma with grade III polymorphism and pleomorphism in mice. Our results showed that doxorubicin induce extreme cells necrosis in cancer tissue. This commercial drug induces exogenous and endogenous signals which results in necrosis/apoptosis in tumor cells but could also result in resistant cells and treatment complication. Surprisingly, in the MVs group the necrosis/apoptosis area was equal to the doxorubicin group. The results of this study were in line with the findings of other studies that proved the interactions between extracellular vesicles and epithelial cells can regulate the mechanisms of cell division and induce apoptosis in cancer cells (Kunsmann et al., 2015; Li et al., 2015; Mondal et al., 2016). The higher efficacy of MVs than probiotic in preventing the tumor progress can be due its short-chain-fatty acids and protein component, which can easily be absorbed by intestinal cells (Kim et al., 2016). These active compounds can activate different signaling pathway, including those involved in antitumor responses.

Patients who suffer from cancer lose weight because of degeneration of fatty tissues and skeletal muscles, which is called cachexia. Several factors are involved in this problem, e.g., increased resting energy expenditure due to thermogenesis in skeletal muscles caused by expression of abnormal proteins and increased performance of the Cori cycle. In these patients, lipolysis of fatty tissues is highly increased and synthesis of normal proteins is reduced followed by their elevated disintegration. The proteolysis-inducing factor induced by tumor and TNF-α, angiotensin II and glucocorticoids induced in the host body can cause the atrophy of muscles (Fearon et al., 2013). Weight loss after chemotherapy is associated with side effects of the chemo drugs including doxorubicin which was obvious in this experiment too. Besides, long-term use of doxorubicin causes severe impairment of normal body cells, weakened immune system, anorexia, and nausea (Aad et al., 2012; Bao et al., 2011; Varna et al., 2009).

It has been proved that probiotics improve food conversion ratio and increase the weight of animals which was obvious in our experiment (Saber et al., 2017; Tacar et al., 2013). An interesting point was the similar effect of MVs, which inhibited weight loss as much as probiotic group. The exact mechanisms of preventing weigh loss are not clear, but it is proposed that MVs carry digestive enzyme which may help in food digestion. Besides, preventing of tumor development could be another explanation for preventing of weight loss.

Conclusion

Considering the increasing incidence of breast cancer in women, it is highly important to find new treatment and preventive methods. Functional foods, especially probiotics, has been taken into account in this regard. Probiotics show immunomodulatory effects and long-term consumption of them inhibit breast cancer formation and proliferation. But there is a great concern about the side effects of probiotics in some vulnerable groups.

Our results showed that MVs, as a non-replicative component of *B. bifidum*, can be used as a new therapeutic and preventive complement for breast cancer in animal model. Administration of MVs suppressed 4T1 induced breast cancer in animal model better than intact cells of probiotic.

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Conflict of interest

The authors declared no conflict of interest regarding publication of this paper.

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