Probiotics and Antimicrobial Proteins (2023) 15:880–888
https://doi.org/10.1007/s12602-021-09899-w

In Vivo Investigation of Supportive Immunotherapeutic Combination of *Bifidobacterium infantis* 35624 and Doxorubicin in Murine Breast Cancer

Meltem Akbaba[1] · Gökhan Gurur Gökmen[2] · Duygu Kışla[2] · Ayşe Nalbantsoy[1]

Accepted: 23 December 2021 / Published online: 3 February 2022

Abstract

The aim of the study is to investigate the anti-tumor effect of *Bifidobacterium infantis* 35624 in a xenograft model in BALB/c mice injected with 4T1 cells as a support for chemotherapeutic treatments of doxorubicin in vivo. The MTT assay was used to determine the cytotoxicity of doxorubicin against cancer cells, and apoptosis was analyzed by using flow cytometry. 4T1 cells (2×10^4 cells/mouse) were injected to BALB/c mice, and mice were fed with/without gavage *B. infantis* milk (10^8 CFU/mL) for 14 days and treated with doxorubicin on 5th and 10th days. The weights of the mice were recorded during the study, and the tumor sizes were measured by caliper at the 14th day. CD8 + T cell response was analyzed by using flow cytometer, and the results were compared to control and tumor control groups. The IC_{50} value for doxorubicin on 4T1 cell lines was determined as 0.053 ± 0.012 µg/mL. The apoptotic effect of doxorubicin at IC_{50} concentration was determined as 82.3% of cells to late apoptosis, 3.6% of cells to pro-apoptosis, and 6.2% of cells to necrosis. The treatment of doxorubicin, *B. infantis* milk, and the combination of them inhibited the tumor volumes by 55.50%, 40.69%, and 75.95%, respectively. *B. infantis* administration significantly enhanced the PHA-induced splenocyte proliferation ($P<0.05$). It was shown that IFN-γ was effective in tumor growth and regression of metastasis. Consequently, the combination of *B. infantis* milk and doxorubicin showed the best anti-tumor effect.

Keywords *Bifidobacterium infantis* · Doxorubicin · Breast cancer · Immunotherapy

Introduction

Cancer cells are formed as a result of the change of normal cells over time, and the cells are constantly divided in the processes of growth and development. During these periods, various mutations could be accumulated in DNA. As a result of these mutations, cells gradually turn into cancer cells which have a genotype quite different from the original form [1, 2]. Approximately 85% of cancer formations are observed in epithelial cells and called as carcinoma. Those originating from mesoderm cells (such as bone, muscle) are sarcoma, and glandular tissue cancers (breast cancer) are defined as adenocarcinomas [3–5]. Extrinsic factors such as smoking and excessive weight and intrinsic factors such as genetic mutations, hormones, and immune system could increase the risk of cancer development [3, 6]. Ten million deaths due to cancer were reported in 2020, and 2.26 million of breast cancer, 2.21 million of lung cancer, and 1.93 million colon and rectum cancer were declared as the most common cancer cases [7]. Additionally, deaths because of the breast cancer were stated to increase by 14% from 2008 to 2020 [8].

Medication, radiotherapy, chemotherapy, and surgical operations are primarily and widely methods used in cancer treatments. As an alternatively to this clinical methods, a novel treatment method might be developed to stimulate specifically the patients' immune cells that destroy the tumor cells [9]. A lot of various supportive treatments have a big potential to activate the immune system, to treat cancer, to alleviate the symptoms associated with side effects of

✉ Duygu Kışla
duygu.kisla@ege.edu.tr

✉ Ayşe Nalbantsoy
analbantsoy@gmail.com; ayse.nalbantsoy@ege.edu.tr

[1] Department of Bioengineering, Faculty of Engineering, Ege University, İzmir, Turkey

[2] Department of Food Engineering, Faculty of Engineering, Ege University, İzmir, Turkey

current cancer treatments, and to increase the quality of life and perception in breast cancer patients. Immunotherapeutic applications could be classified as immune checkpoint inhibitors, T-cell transfer therapy, monoclonal antibodies, treatment vaccines, and immune system modulators [10].

The Food and Agriculture Organization defined the term of *probiotics* as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [11]. Probiotics have a lot of functional characteristics, but some of them have directly effect on human health such as modulation effect on intestinal microflora, production of antimicrobials, antagonistic effect, and competition to pathogens for nutrients and/or other growth factors [12]. It was clearly shown in many studies that probiotics could reduce the tumor growth and inhibit the cancer related to their immunomodulatory effect, anti-pathogenic, and anti-inflammatory activities [13]. *Bifidobacteria*, which is Gram-positive, anaerobic and naturally present in the dominant colonic microbiota, represent up to 25% of the cultivable fecal bacteria in adults and 80% in infants [14, 15]. *Bifidobacterium infantis* 35624 used in this study was isolated and identified from a gastrointestinal tissue about 20 years ago [16].

Doxorubicin, commercially called as Adriamycin, is commonly used in breast cancer treatments, and it has a complex antineoplastic activity mechanism such as directly affecting cell membrane, causing DNA damage by interacting with DNA, apoptosis by topoisomerase-II inactivation [17]. The maximum tolerable dose of doxorubicin was determined as 75 mg/m^2, and it has some undesirable side effects, which limited the use of doxorubicin, such as cardiac toxicity, bone marrow, and immune system suppression [18–20]. Therefore, doxorubicin therapy is used in various combinations, and new compounds, such as ICRF-187 and quercetin, that could block cardiac toxicity are investigated [3].

The aim of the present study is to investigate the potential use of the combination of doxorubicin and *Bifidobacterium infantis* milk as a supportive treatment of breast cancer using mouse xenograft breast cancer model.

Material and Methods

Probiotic Bacteria

Bifidobacterium infantis 35624 was isolated from Align Probiotic Supplement (Procter & Gamble, Cincinnati, OH, USA). Briefly, 10 g of Align was diluted in 90 mL of 0.1% sterile peptone water (Merck, Darmstadt, Germany) and homogenized. Ten-fold serial dilutions were performed until a dilution of 10^{-7} was obtained. Appropriate dilutions were spread plated on De Man, Rogosa, and Sharpe (MRS: Merck, Darmstadt, Germany, pH 5.7 ± 0.2) agar supplemented with 0.05% L-cysteine (Sigma-Aldrich, Missouri, USA) and incubated in an anaerobic jar at 37 °C for 48 h. After incubation, Gram staining was performed to the colonies, and the probiotic *B. infantis* 35624 was observed under microscope [21, 22].

Cell Culture

The 4T1 (murine breast cancer cells) cells were kindly provided by Prof. Michael Kershaw (Cancer Immunology Program, Peter MacCallum Cancer Center, Victoria, Australia) [23] and cultured in Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (Gibco, Waltham, MA, USA) including 10% heat-inactivated fetal bovine serum (FBS, Gibco, Waltham, MA, USA), supplemented with 100 U/mL penicillin and 1 µg/mL streptomycin. The cells were incubated in a 95% humidified atmosphere with 5% CO_2 at 37 °C. Cells that actively grow in the logarithmic phase were used in the studies.

BALB/c Mice

Xenograft tumor mouse model was developed with 25 female BALB/c mice which were purchased from Uludag University, Faculty of Medicine Experimental Animal Breeding Application and Research Center, Bursa, Turkey. The Local Ethics Review Committee for Animal Experimentation of Ege University (number 2017–021) approved the experimental protocol.

Production of *B. infantis* Milk

In order to obtain the working culture, 1 mL of *B. infantis* 35624 was inoculated to 9 mL of 10% skimmed milk powder (Pınar Süt Mamulleri Sanayi A.Ş., Izmir, Turkey) sterilized at 115 °C for 10 min and incubated at 37 °C for 48 h. One milliliter of *B. infantis* 35624 culture was then incubated with 9 mL of sterilized 10% skimmed milk powder again at 37 °C for 48 h. Ten milliliter of working culture was inoculated to 90 mL of UHT milk (1.5% fat; Arkadaşım Süt, Kipa, Izmir, Turkey), homogenized, and 3 mL volumes of it was dispensed into sterile vials. The vials were then incubated at 37 °C for 48 h, and *B. infantis* milk was stored at 4 °C for future use [24].

The enumeration of viable *B. infantis* 35624 cells was determined on 2nd and 12th days of storage at 4 °C. Briefly, 1 mL of *B. infantis* milk was homogenized with 9 mL of 0.1% peptone water, and ten-fold serial dilutions were prepared. One milliliter of appropriate dilutions was inoculated in MRS agar supplemented with 0.05% of L-cysteine by pour plate method and incubated in an anaerobic jar at 37 °C for 48 h. After incubation, the colony counts were performed [25].

The Cytotoxicity of Doxorubicin on 4T1 Cells

The cytotoxic effect of doxorubicin was determined by using MTT [3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide)] (2.5 mg/mL; Acros Organics, NJ, USA) [26]. Briefly, 4T1 cells were cultivated for 24 h in 96-well microplates with an initial concentration of 5×10^5 cells/well in a 95% humidified atmosphere with 5% CO_2 at 37℃. Then, the cells were treated with different concentrations of doxorubicin prepared in serum-free DMEM/F-12 (10, 1, 0.1, and 0.01 µg/mL), followed by incubation for 48 h at 37℃. A UV–visible spectrophotometer (Thermo Multiskan Spectrum) was used to measure the optical density of the dissolved material in dimethyl sulfoxide at $\lambda = 570$ nm (reference filter, $\lambda = 620$ nm). The viability (%) was determined by the following formula:

$$\%\text{Viable cells} = \left[(\text{absorbance of treated})/(\text{absorbance of control})\right] \times 100$$

Apoptosis Assay

Analysis of apoptosis was performed according to the modified method by Du et al. [18]. 5×10^5 cells/mL of cells were seeded into 6-well plate and cultivated at 37 °C for 24 h. Doxorubicin treatment was performed to the cells at the concentrations of IC_{50} except control groups. After incubation at 37 °C for 48 h, the trypsinization was performed, and cells were washed with phosphate-buffered saline (PBS; Gibco, Waltham, MA, USA, pH 7.4) for 3 times. Cells were resuspended with 100 µL of binding buffer (BD, NJ, USA), and 5 µL Annexin V conjugated with FITC (BD, NJ, USA) and 5 µL of propidium iodide (PI) (BD, NJ, USA) were added into the tube for incubation at room temperature for 15 min in the dark. The cells were detected by using flow cytometry (Accuri C5 model, BD, NJ, USA).

In Vivo Studies

Formation of Xenograft Tumor Model by Using 4T1 Cells

4T1 cells were cultured in DMEM/F12 (10% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine). A final concentration of 2×10^4 4T1 tumor cells (mixed with Matrigel in 1:1 dilution) were injected intraperitoneally (IP) into the left flank of each mouse on 0th day of the experiment in a total volume of 100 µL [27]. Tumor growth was measured in mm by using a caliper and recorded as mean volumes (width × height × length). The measurement of tumor volume was performed only after sacrificing of mice. The average volume of tumor was calculated by using the following equation:

$$V = a \times \left(b^2/2\right),$$

where V is the volume of tumor (mm^3), a is width (mm), and b is length (mm).

Administration by Gavage

Three different formulations were performed for administration of mice. Group 1 (K1) was a control group, no tumor and no administration; group 2 (K2) was a tumor control group (untreated group), no administration; group 3 (D), doxorubicin injected intraperitoneally at the concentration of 2.5 mg/kg mouse; group 4 (B), administered with 100 µL of *B. infantis* milk by gavage; and group 5 (D + B), doxorubicin injected intraperitoneally at the concentration of 2.5 mg/kg mouse and administered with 100 µL of *B. infantis* milk. Administration was performed once a day during 14 days with 100 µL of *B. infantis* milk, and doxorubicin treatments were performed on 5th and 10th days. Experimental groups consisted of 5 mice each, and all mice were weighed on 0, 5, and 15th days. The study was terminated on day 15, and mice were sacrificed by cervical dislocation. Tumor and spleen samples were collected for performing further assays.

Splenocyte Proliferation Assay

Splenocyte proliferation assays were performed according to the modified method by Nalbantsoy et al. [28]. Briefly, single splenocyte suspensions were obtained by gently mincing and grinding spleen fragments through steel mesh in complete RPMI-1640 medium (10% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, 0.005 mM β-mercaptoethanol). After centrifugation ($450 \times g$ at 4 °C for 7 min), the pelleted cells were washed three times. At last, the cells were resuspended in 2 mL RPMI-1640 complete medium, and cell numbers were recorded with a hemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. An aliquot of 100 µL of splenocytes at 5×10^6 cells/mL was seeded into each well of a 96-well flat bottom microtiter plate. After that, splenocytes were treated with 100 µL of inactivated *B. infantis* 35624 cell suspension (final concentration 10^7 CFU/mL) and phytohemagglutinin (PHA; Sigma-Aldrich, Darmstadt, Germany; final concentration 5 µg/mL). After preincubation for 68 h at 37 °C in a humidified 5% CO2 incubator, 50.0 µL of MTT solution (2.5 mg/mL) was added into each well. The plate was incubated at 37 °C for 4 h and centrifuged ($1400 \times g$, 5 min) to remove the untransformed MTT carefully by pipetting. Then to each well, a total of 200 µL DMSO was added to fully dissolve the colored material. The absorbance at 570 nm with a 630 nm reference was measured on a ELISA reader (Versa Max, Molecular Devices, USA). The stimulation index (SI) was calculated based on the following

formula: SI = the absorbance value for mitogen-stimulated cultures/the absorbance value for non-stimulated culture. Each experiment was performed in triplicate.

In order to obtain the inactivated *B. infantis* 35624, the washed and resuspended cell suspension (10^7 CFU/mL) was homogenized with RPMI-1640 and filled to sterile petri dishes. Then, petri dishes were kept twice under UV-C (15 W, Philips, Amsterdam, Netherland), and an aliquot of suspension was plated on MRS agar to confirm that there was no live *B. infantis* 35624 [29].

Measurement of Total T Cells and IFN-ɣ Secreting CD8 + T Cells in Spleen Cell Culture

Splenocytes of sacrificed mice were isolated, and cells from each individual mouse belonging to same group were collected in a pool. An aliquot of 5×10^5 cells/mL from each group was treated with inactivated *B. infantis* 35624 suspension and incubated at 37 °C for 72 h. Cells were collected and centrifuged at 1200 rpm for 5 min and 50 µL (1:1000) FIT-C labeled anti-mouse CD3 antibody (BD, NJ, USA) was added. Samples were incubated at 4 °C for 30 min in dark. After incubation, cells were centrifuged and washed with 250 µL PBS containing 3% FBS. PerCP-labeled anti-mouse CD8 antibody was added to cells and further incubated for 30 min at 4 °C in dark. Cells were then washed; 100 µL fixation/permeabilization solution (BD, New Jersey, USA) was added to the cells and incubated for 30 min at 4 °C at dark. After incubation, cells were washed, and PE-labeled anti-mouse IFN-ɣ antibody (BD, NJ, USA) was added to cells followed by incubation at 4 °C for 30 min in dark. An aliquot of 20 µL perm wash (1:10) was added to the cells, and cells were washed twice. After washing steps, cells were analyzed by flow cytometry (Accuri C5 model, BD, NJ, USA) [30].

Statistical Analysis

The study was organized in triplicates and presented as mean ± standard error of mean (SEM) of samples. GraphPad Prism 7.0 software (San Diego, USA) was used to calculate the IC_{50} values and analyze variance (standard deviation calculation). The data were statistically analyzed using one-way ANOVA, followed by Bonferroni's multiple comparison test. The significance level was set to 0.05 for one-way ANOVA and 0.001 for Tukey's multiple comparison test.

Results

Enumeration of *Bifidobacterium infantis* 35624

The enumeration of *B. infantis* 35624 was found as 7×10^8 CFU/mL on the 2nd day and 2.8×10^8 CFU/mL on 12th day of storage at + 4 °C.

The Cytotoxic Effect of Doxorubicin on 4T1 Cells

MTT method was used to calculate the cytotoxicity of doxorubicin. The IC_{50} value for 4T1 cells was also measured as 0.053 ± 0.012 µg/mL according to percentage of 4T1 cell viability (Fig. 1).

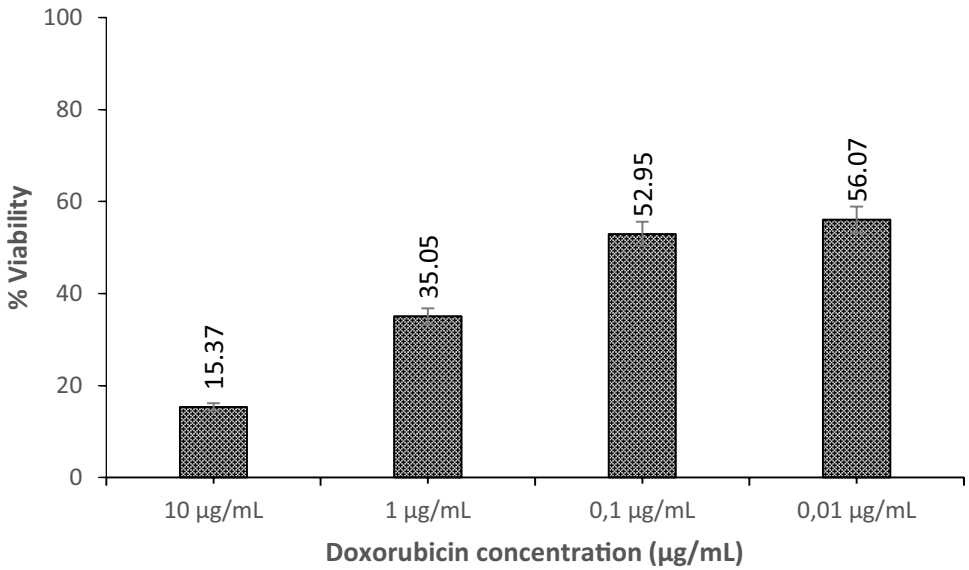

Fig. 1 The cytotoxic effect of doxorubicin on 4T1 cell line

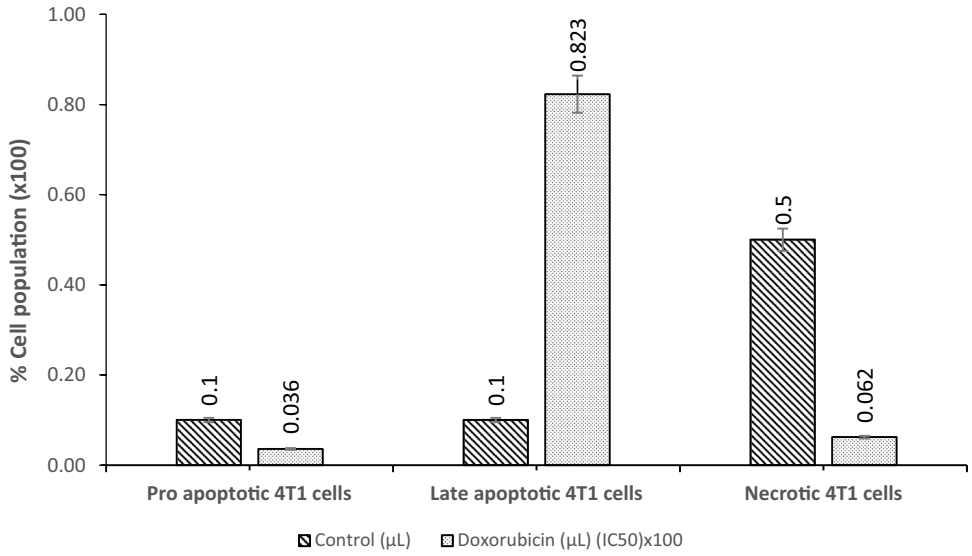

Fig. 2 The apoptotic effect of doxorubicin on 4T1 cell line

Apoptotic Effect of Doxorubicin on 4T1 Cells

0.053 ± 0.012 µg/mL (IC_{50} value) of doxorubicin was treated to 4T1 cells for determination of the apoptosis. The flow cytometer was used to analyze the apoptotic effect of doxorubicin on 4T1 cells. The late apoptotic (82.3%), pro apoptotic (3.6%), and necrotic (6.2%) 4T1 cells are shown as percentages in Fig. 2.

In Vivo Studies

The average weight of mice groups (K1, K2, D, B, and D + B) and the ratio of the average tumor volumes to it were shown in Table 1 and Fig. 3, respectively. No death was observed because of the toxic effect as given in Table 1. Any metastases was not observed during study; however, tumor formations were determined only at the sites where 4T1 cells were injected. The highest average weight loss (2.72 g) was observed in K2 group, while 0.58 g of average weight gain was observed in D + B group. The tumor formation was thought to be effective in weight loss. On the other hand, doxorubicin treatments and apoptotic effect of doxorubicin on 4T1 cells might increase the average weight.

Table 1 The average weight of Balb/c mice

Group	Average weight (g)			Number of death due to toxic effect
	Day 0	5th day	15th day	
K1	21.60	20.50	22.10	0
K2	26.26	24.54	23.54	0
D	23.33	23.35	23.55	0
B	23.08	24.57	25.40	0
D + B	25.22	25.78	24.64	0

The average tumor volumes were determined as 182.9, 81.39, 108.5, and 58 mm^3 in K2, D, B, and D + B groups, respectively. The average tumor volumes were statistically significant in all experiment groups ($P < 0.001$). D + B group showed the best tumor suppression ($P < 0.01$) based on the ratio of the tumor volumes to weight of mice. The D ($P < 0.05$) and B ($P < 0.05$) groups also showed a statistically significant suppressive effect on tumor growth following D + B.

Splenocyte Proliferation Assay

The effects of *B. infantis* milk and doxorubicin on PHA- and inactive *B. infantis* 35624-stimulated splenocyte proliferation are shown in Fig. 4. The stimulation index (SI) was used to state the degree of proliferation. The highest SI values were observed in group B and D + B for PHA-stimulated and inactivated *B. infantis*-stimulated cells, respectively ($P < 0.001$). On the other hand, the lowest SI values were observed in K1 group for both stimulated cells.

Measurement of Total T Cells and IFN-ɣ Secreting CD8 + T Cells in Spleen Cell Culture

The percentage of total T cells and the percentage of IFN-ɣ secreting CD8 + T cells were given in Figs. S1 and S2, respectively. The percentages of T cells and CD8 + T cells were also measured by analyzing in flow cytometry by CD3 staining and given in Table 2. The highest and the lowest percentages of total T cells were observed in the K1 and B groups, respectively. On the other hand, the percentage of total CD8 + T cells secreting IFN-ɣ was the lowest in K1

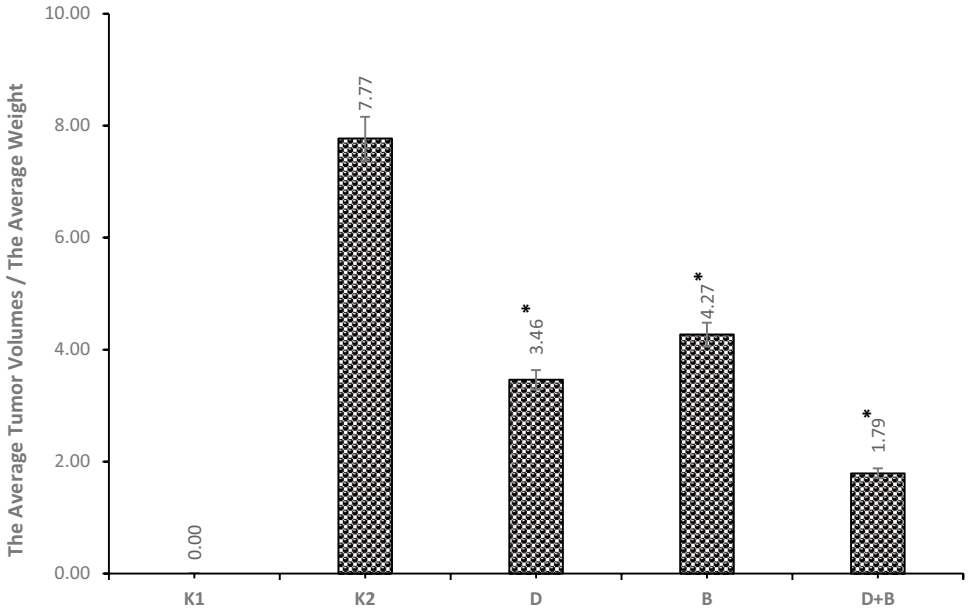

Fig. 3 The ratio of the average tumor volumes to average weight of the mice groups was compared with the data of the K2 ($n=5$) (*$P<0.5$)

group, while it was the highest in group D + B. The obtained data showed that the other cellular mechanism might be inferred to stimulate in the last phase of the treatment for suppressing the tumor growth.

Discussions

It is specified that most commercial probiotic products consist of *Lactobacillus* and *Bifidobacterium* species [31]. The probiotic bacteria content of milk was declared as 10^7 CFU/mL in the Fermented Milks and Lactic Acid Beverages Association in Japan, while the Swiss Food Regulation as well as the Standard FIL/IDF requires that such products contain more than 10^6 CFU/mL [32]. Jaworska et al. [24] stated that the viable bacteria counts in probiotic products should be 10^6–10^8 CFU/mL during storage periods. It was also reported that 10^7 CFU/mL of *B. infantis* was clinically effective in probiotic products [33]. The viable probiotic counts of *B. infantis* milk used in the study during storage was clearly observed to be similar to the studies in the literature.

The cytotoxic effect of doxorubicin was found as 0.17 µmol/L [26], 2.65 µM [34], and 0.2 µM [35] in different studies. McCarthy et al. [35] stated that the 4T1 cells might gain a resistance against doxorubicin over time. However doxorubicin, which is an anthracycline antibiotic, is used successfully in various cancer treatments [18]. Guo

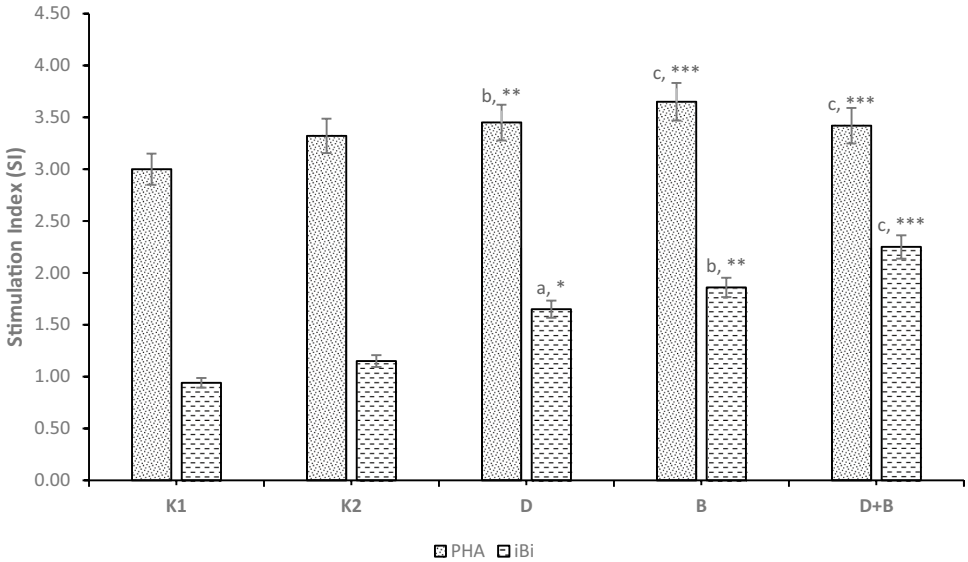

Fig. 4 The effects of *B. infantis* milk and doxorubicin on PHA- and inactivated *B. İnfantis* 35624-stimulated splenocyte proliferation in BALB/c mice ($n=5$). All data obtained from each mitogen were compared to K1 and K2 (a, b, and c indices show the comparison of the data to K1; *, **, and *** indices show the comparison of the data to K2) (a, *$P<0.05$; b, **$P<0.01$; c, ***$P<0.001$). (PHA, phytohemagglutinin-stimulated cells; iBi, inactivated *B. İnfantis* 35624-stimulated cells)

Table 2 The percentages of total T and CD8 + T cells in spleen cell

	K1	K2	D	B	D + B
% T cells	20.9	14.9	17.9	14.6	15.1
% CD8 + T cells	2.8	8.6	5.6	9.2	10.1

et al. [19] investigated the apoptotic effect of doxorubicin on 4T1 cells, and they found that 10 µg/mL of doxorubicin resulted 40.2% of 4T1 cells to late apoptosis. Gill et al. [36] investigated the average weight of mice administered with 10^8 CFU/mL of probiotic bacteria (*Lact. acidophilus* HN017, *Lact. rhamnosus* HN001, and *B. lactis* HN019) by gavage, and they reported that any changes in average weight could not observed. The average weight results in the study are similar to the studies in the literature.

duPre et al. [37] characterized the 4T1 tumor-infiltrating hematopoietic cells, particularly with respect to the cellular source or sources of IFN-ɣ in the tumor microenvironment, and they used C.129S7(B6)-Ifng^{tmlT}/J (IFN-ɣ$^{-/-}$) and BALB/cJ (IFN-ɣ$^{+/+}$) mice. The researchers reported that the tumor volumes were higher in IFN-ɣ-free mice. Maroof and Hasan [27] investigated the use of *Lact. acidophilus* as food supplement, and they administered the BALB/c mice with 2×10^8 CFU/mL of *Lact. acidophilus* for 15 days. They found that the secretion of IL-4 decreased while the secretion of IFN-ɣ increased. Similarly, Gill et al. [36] found an increase in the rate of CD4 T lymphocytes (40%) and CD8 T lymphocytes (14%). The secretion of IFN-ɣ also increased in mice administered with *Lact. acidophilus* HN017 and *Lact. rhamnosus* HN001, but there was no changes in mice administered with *B. lactis* HN019.

Many studies based on various combinations have been conducted over the years to increase the effectiveness of doxorubicin and reduce its side effects. Du et al. [18] studied the use of luteolin to increase the effectiveness of doxorubicin in breast cancer cells. Researchers found that luteolin did not have any effect alone on tumor growth delay, but the effectiveness and lesser toxicity of doxorubicin might be based on 4T1 and MCF-7 bearing mice. In another study, quercetin and doxorubicin combination was investigated [18]. It was found that the tumor growth and prolonged survival were suppressed by quercetin in BALB/c mice bearing 4T1 breast cancer, and quercetin importantly enhanced therapeutic effectiveness of doxorubicin and simultaneously reduced doxorubicin-induced toxic side effects. Putri et al. [34] reported that the combination of potassium pentagamavunon-0 (K PGV-0) and doxorubicin showed synergistic effect and decreased the viability of the breast cancer cells. The combination was also found to cause cell accumulation in G2/M phase, induction of apoptosis, and inhibition of the activity of MMP-9 which has an important role in extracellular matrix degradation.

Probiotics and probiotic food supplements are also studied in cancer treatments. It was reported by many studies that diets enriched with dairy products could inhibit the tumor growth in various cancer including breast cancer [38]. Some researchers argued that the anti-tumor effect of milk consumption was related to multimeric α-lactalbumin, but Biffi et al. [39] reported the efficacy of raw milk, and milk which sterilized at high temperature was different on tumor growth. They also declared that the fermented milk products contained non-bacterial substances which had immunosupportive and anti-tumor effect. Wang et al. [40] studied to evaluate the growth inhibitory and apoptosis-inducing activities of the cyclic lipopeptide (CLP) purified from *Bacillus subtilis* natto T-2 in human leukemia K562 cells with special emphasis on its mode of action. It was found that 32 µg/mL of CLP resulted 20.6% of K562 cells to apoptosis. Surfactin (27.3 µM), produced by *B. subtilis* natto, was also reported to cause 13.7% of MCF-7 cells [41].

The anti-tumor effect of substances produced by probiotic microorganisms was studied in many studies; however, the viable cells have also a significant role on tumor growth as shown in this study. Maroof et al. [42] reported that administration of *Lact. acidophilus* isolated from home-made yoghurt induced a significant decrease in tumor growth pattern ($P = 0.00$). The researchers also indicated that the oral administration of *Lact. acidophilus* was able to alter the cytokine production in tumor bearing mice into a Th1 protective pattern, favorable to anti-tumor immunity. Yazdi et al. [43] declared that administration of *Lact. plantarum* ATCC 8014 enriched with selenium nanoparticles increased the production of the pro-inflammatory cytokines IFN-ɣ, TNF-α, and IL-2 in spleen cell cultures. The significant increase was also observed in NK cell activity. Aragon et al. [44] analyzed the effect of milk fermented by the probiotic *Lact. casei* CRL 431 on a murine breast cancer model, and it was reported that probiotic administration delayed or blocked the tumor growth.

Conclusion

The administration of *B. infantis* 35624 was clearly shown to be effective on tumor growth on murine breast cancer model, and the combination of doxorubicin and *B. infantis* 35624 treatments showed the higher anti-tumor effect. Although the study was conducted on murine breast cancer models, it may also apply to different experimental animals and various cancer types. In vivo results of the present study could be developed comprehensively with additional analyses such as taking tumor sections and immunohistochemical

investigation and cytokine measurements by ELISA from the blood samples in further researches.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-021-09899-w.

Author Contribution Conception and design of the study: Meltem Akbaba, Duygu Kışla, and Ayşe Nalbantsoy. Acquisition of data: Meltem Akbaba, Duygu Kışla, and Ayşe Nalbantsoy. Analysis and interpretation of data: Meltem Akbaba, Gökhan Gurur Gökmen, Duygu Kışla, and Ayşe Nalbantsoy. Drafting or revising the manuscript: Gökhan Gurur Gökmen, Duygu Kışla, and Ayşe Nalbantsoy. All authors have approved the final article.

Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Compliance with Ethics Requirements BALB/c mice used in this study were purchased from Uludag University, Faculty of Medicine, Experimental Animal Breeding Application and Research Center. The experimental protocol was approved by the Local Ethics Review Committee for Animal Experimentation of Ege University (number 2017–021).

Conflict of Interest The authors declare no competing interests.

References

1. Baba AI, Catoi C (2007) Chapter 3: Tumor cell morphology. In: Baba AI (ed) Comparative Oncology. The Publishing House of the Romanian Academy, Romania
2. Weinberg RA (2014) Chapter 2: The nature of cancer. In: Weinberg R (ed) The Biology of Cancer, 2nd edn. GarlandScience, Taylor and Francis Group, LLC, pp 31–71
3. Pecorino L (2012) Molecular biology of cancer. Mechanisms, Targets and Therapeutics, 4th ed. Oxford University Press, United Kingdom
4. Thiery JP (2002) Epithelial-mesenchymal transition in tumour progression. Nat Rev Cancer 2:442–454. https://doi.org/10.1038/nrc822
5. Weinberg RA (2014) Chapter 16: The rational treatment of cancer. In: Weinberg R (ed) The Biology of Cancer, 2nd edn. GarlandScience, New York, pp 797–876
6. Doll R, Peto R (1981) The quantitative of cancer causes of cancer: estimates of avoidable risks in the United States Today. JNCI 66(6):1193–1308. https://doi.org/10.1093/jnci/66.6.1192
7. WHO (2021) Cancer. World Health Organisation Website. https://www.who.int/news-room/fact-sheets/detail/cancer. Accessed 2 May 2021
8. Anon (2017) Kanser. T C Sağlık Bakanlığı Website. http://kanser.gov.tr/. Accessed 29 June 2020
9. Abbas AK, Lichtman AH, Pillai S (2015) Temel immünoloji, immün sistemin işlevleri ve bozuklukları. Güneş Tıp Kitabevleri, Ankara
10. Anon (2019) Immunotherapy to treat cancer. National Cancer Institute Website. https://www.cancer.gov/about-cancer/treatment/types/immunotherapy. Accessed 2 May 2021
11. Food and Agriculture Organization (2006) Probiotics in food, food and nutrition paper. http://www.fao.org/3/a0512e/a0512e.pdf. Accessed 24 January 2021
12. Marteau P, de Vrese M, Cellier CJ, Schrezenmeir J (2001) Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nutr 73:430–436. https://doi.org/10.1093/ajcn/73.2.430s
13. Yu AQ, Li L (2016) The potential role of probiotics in cancer prevention and treatment. Nutr Cancer 68(4):535–544. https://doi.org/10.1080/01635581.2016.1158300
14. Leahy SC, Higgins DG, Fitzgerald GF, Sinderan VD (2005) Getting better with *Bifidobacteria*. J Appl Microbiol 98:1303–1315. https://doi.org/10.1111/j.1365-2672.2005.02600.x
15. Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C (2005) Review article: *Bifidobacteria* as probiotic agents-physiological effects and clinical benefit. Aliment Pharmacol Ther 22:495–512. https://doi.org/10.1111/j.1365-2036.2005.02615.x
16. Konieczna P, Akdis CA, Quigley EMM, Shanahan F, O'Mahony L (2012) Portrait of an immunoregulatory *Bifidobacterium*. Gut Microbes 3(3):261–266. https://doi.org/10.4161/gmic.20358
17. Pasqualini JR (2008) Breast cancer prognosis, treatment and prevention, 2nd edn. Taylor & Francis, Florida
18. Du G, Lin H, Wang M et al (2010) Quercetin greatly improved therapeutic index of doxorubicin against 4T1 breast cancer by its opposing effects on HIF-1α in tumor and normal cells. Cancer Chemother Pharmacol 65:277–287. https://doi.org/10.1007/s00280-009-1032-7
19. Guo Q, Li X, Yang Y, Wei J, Zhao Q, Luo F, Quian Z (2014) Enhanced 4T1 breast carcinoma anticancer activity by co-delivery of doxorubicin and curcumin with core-shell drug-carrier based on heparin modified poly (L-lactide) grafted polyethylenimine cationic nanoparticles. J Biomed Nanotechnol 10:227–237. https://doi.org/10.1166/jbn.2014.1785
20. Jones A, McAdam K (2003) Medical therapy of advanced disease. In: Rayter Z, Mansi J (ed) Medical therapy of breast cancer. Cambridge University Press, Cambridge, pp 283–308. https://doi.org/10.1017/CBO9780511545870.013
21. Collins EB, Hall BJ (1984) Growth of *Bifidobacteria* in milk and preparation of *Bifidobacterium infantis* for a dietary adjunct. J Dairy Sci 67(7):1376–1380. https://doi.org/10.3168/jds.S0022-0302(84)81451-4
22. Gregersen T (1978) Rapid method for distinction of Gram-negative from Gram-positive bacteria. Eur J Appl Microbiol 5:123–127. https://doi.org/10.1007/BF00498806
23. Kershaw MH, Jackson JT, Haynes NM, Teng MWL, Moeller M, Hayakawa Y, Street SE, Cameron R, Tanner JE, Trapani JA, Smyth MJ, Darcy PK (2004) Gene-engineered T cells as a superior adjuvant therapy for metastatic cancer. J Immunol 173(3):2143–2150. https://doi.org/10.4049/jimmunol.173.3.2143
24. Jaworska D, Neffe K, Kolozyn-Krajewska D, Dolatowski Z (2011) Survival during storage and sensory effect of potential probiotic lactic acid bacteria *Lactobacillus acidophilus* Bauer and *Lactobacillus casei* Bif3' / IV in dry fermented pork loins. Int J Food Sci Technol 46:2491–2497. https://doi.org/10.1111/j.1365-2621.2011.02772.x
25. Muramella T, Aryana KJ (2011) Some low homogenization pressures improve certain probiotic characteristics of yogurt culture bacteria and *Lactobacillus acidophilus* LA-K. J Dairy Sci 94:3725–3738. https://doi.org/10.3168/jds.2010-3737
26. Bao L, Haque A, Jackson K, Hazari S, Moroz K, Jetly R, Dash S (2011) Increased expression of P-glycoprotein is associated with doxorubicin chemoresistance in the metastatic 4T1 breast cancer model. Am J Pathol 178(2):838–852. https://doi.org/10.1016/j.ajpath.2010.10.029
27. Maroof H, Hassan ZM (2012) *Lactobacillus acidophilus* could modulate the immune response against breast cancer in murine model. J Clin Immunol 32(6):1353–1359. https://doi.org/10.1007/s10875-012-9708-x

28. Nalbantsoy A, Nesil T, Erden S, Çalış İ, Bedir E (2011) Adjuvant effects of Astragalus saponins macrophyllosaponin B and Astragaloside VII. J Ethnopharmacol 134(3):897–903. https://doi.org/10.1016/j.jep.2011.01.054
29. Nalbantsoy A, Özverel CS, Kışla D (2020) A co-culture study to determine the supportive role of probiotics on immune system against cancer cells. Food and Health 6(4):287–298. https://doi.org/10.3153/FH20028
30. Özverel CS, Uyanikgil Y, Karaboz İ, Nalbantsoy A (2020) Investigation of the combination of anti-PD-L1 mAb with HER2/neu-loaded dendritic cells and QS-21 saponin adjuvant: effect against HER2 positive breast cancer in mice. Immunopharmacol Immunotoxicol 42(4):346–357. https://doi.org/10.1080/08923973.2020.1775644
31. Neffe-Skocinska K, Rzepkowska A, Szydlowska A, Kolozyn-Krajewska D (2018) Chapter 3 – Trends and possibilities of the use of probiotics in food production. In: Alternative and replacement foods. Academic Press, pp 65–94. https://doi.org/10.1016/B978-0-12-811446-9.00003-4
32. Sendra E, Fayos P, Lario Y, Fernandez-Lopez J, Sayas-Barbera E, Perez-Alvarez JA (2008) Incorporation of citrus fibers in fermented milk containin probiotic bacteria. Food Microbiol 25:13–21. https://doi.org/10.1016/j.fm.2007.09.003
33. Lyseng-Williamson KA (2017) *Bifidobacterium infantis* 35624 as a probiotic dietary supplement: a profile of its use. Drugs Ther Perspect 33(8):368–374. https://doi.org/10.1007/s40267-017-0423-9
34. Putri H, Jenie RI, Handayani S, Kastian FR, Meiyanto E (2016) Combination of potassium pentagamavunon-0 and doxorubicin induces apoptosis and cell cycle arrest and inhibits metastasis in breast cancer cells. Asian Pac J Cancer Prev 17:2683–2688. https://doi.org/10.7314/APJCP.2016.17.5.2683
35. McCarthy M, Auda G, Agrawal S, Taylor A, Backstrom Z, Mondal D, Moroz K, Dash S (2014) In vivo anticancer synergy mechanism of doxorubicin and verapamil combination treatment is impaired in Balb/c mice with metastatic breast cancer. Exp Mol Pathol 97:6–15. https://doi.org/10.1016/j.yexmp.2014.04.013
36. Gill HSK, Rutherfurd J, Prasad J, Gopal PK (2000) Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). Br J Nutr 83:167–176. https://doi.org/10.1017/s0007114500000210
37. duPre SA, Redelman D, Hunter KW (2008) Microenvironment of the murine mammary carcinoma 4T1: Endogenous IFN-γ affects tumor phenotype, growth and metastasis. Exp Mol Pathol 85:174–188. https://doi.org/10.1016/j.yexmp.2008.05.002
38. LeBlanc AJG, Matar C, Valdez JC, LeBlanc J, Perdigon G (2002) Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. J Dairy Sci 85(11):2733–2742. https://doi.org/10.3168/jds.S0022-0302(02)74360-9
39. Biffi A, Coradini D, Larsen R, Riva L, Fronzo GD (1997) Antiproliferative effect of fermented milk on the growth of a human breast cancer cell line. Nutr Cancer 28(1):93–99. https://doi.org/10.1080/01635589709514558
40. Wang CL, Ng TB, Yuan F, Liu ZK, Liu F (2007) Induction of apoptosis in human leukemia K562 cells by cyclic lipopeptide from *Bacillus subtilis* natto T-2. Peptides 28:1344–1350. https://doi.org/10.1016/j.peptides.2007.06.014
41. Cao X, Wang A-H, Wang C-L, Mao D-Z, Lu M-F, Cui Y-Q, Jiao R-Z (2009) Surfactin induces apoptosis in human breast cancer MCF-7 cells through a ROS/JNK-Mediated Mithocondrial/Caspase pathway. Chem Biol Interact 183:357–362. https://doi.org/10.1016/j.cbi.2009.11.027
42. Maroof H, Hassan ZM, Mobarez AM, Mohamadabadi MA (2012) *Lactobacillus acidophilus* could modulate the immune response against breast cancer in murine model. J Clin Immunol 32:1353–1359. https://doi.org/10.1007/s10875-012-9708-x
43. Yazdi MH, Mahdavi M, Kheradmand E, Shahverdi AR (2012) The preventive oral supplementation of a selenium nanoparticle-enriched probiotic increases the immune response and lifespan of 4T1 breast cancer bearing mice. Arzneimittelforschung 62:525–531. https://doi.org/10.1055/s-0032-1323700
44. Aragon F, Carino S, Perdigon G, de LeBlanc ADM (2014) The administration of milk fermented by the probiotic *Lactobacillus casei* CRL 431 exerts an immunomodulatory effect against a breast tumour in a mouse model. Immunobiology 219(6):457–464. https://doi.org/10.1016/j.imbio.2014.02.005