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Research Article

Exploring the Anticancer Efficacy of a Mixture of Local Probiotics on MDA-MB-231 and MCF-7 Breast Cancer Cell Lines

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Abstract

This study aimed to investigate the cytotoxicity of a probiotic mixture on human breast cancer cell lines. To prepare the mixture, local probiotic bacteria were cultured, and the lyophilized supernatant was applied for downstream experiments. The antioxidant activity, total phenol content (TPC), and fatty acid composition of the bacterial supernatant (BS) were also measured. The possible cytotoxic/anti-proliferative effect of the probiotic mixture was accessed on both breast cancer cell lines at different concentrations using MTT assay. Furthermore, the apoptosis-inducing effects of the same mixture was studied by DAPI staining. The highest level of antioxidant activity and total phenol content (TPC) were detected for the BS at 3200 µg/ml. According to the GC–MS analysis, linoleic acid (37.40 %) and oleic acid (26.93 %) were identified as the major fatty acids of the BS. The MTT assay showed that the BS has anti-proliferative effects on MDA-MB-231 and MCF-7 cells in a time- and dose-dependent manner (IC_{50}: 3200 µg/ml). The apoptosis-inducing effects of the mixture was confirmed in both cell lines through morphological analyses of the cells' nucleoli, and the formation of apoptotic bodies. According to these experiments, cytotoxic effects and apoptosis-inducing potential were confirmed for the BS against two human breast cancer cell lines, including MDA-MB-231, and MCF-7. Hence, it could be considered as a suitable anti-cancer agent.

***Keywords*:** Breast cancer, Probiotics, Cytotoxicity, Apoptosis, Antioxidant activity

Introduction

The International Agency for Research on Cancer (IARC) reported that around 18.1 million new cancer cases and 9.6 million cancer-caused deaths occurred in 2018. Among females, breast cancer is the most commonly diagnosed cancer and is the leading cause of cancer death, followed by colorectal and lung cancers (Bray et al., 2018).

Lactic acid bacteria (LAB) are among the most important types of probiotic bacteria producing acid lactic as the final product during carbohydrates fermentation. Lactobacillus and bifidobacteria are gram-positive lactic acid bacteria, which constitute the bulk of the natural intestinal flora of humans. These microorganisms can fight with cancer by ***i***) metabolizing carcinogens, ***ii***) enhancing immune responses, ***iii***) producing anti-mutation and anti-cancer compounds, antioxidants, and anti-angiogenic factors, and ***iv***) the regulation of apoptosis and cell differentiation processes (Malik et al., 2018). The probiotic properties of lactobacillus and bifidobacteria are varied among different species. In addition, these effects are also probiotic strain-dependent (Irecta-Nájera et al., 2017).

Nowadays, probiotics because of high health potential can be used for prevention and adjuvant therapy of many chronic diseases, including cancer (Sli´zewska et al., 2021).

Different studies have shown that probiotics are effective in preventing, treating, and reducing the progression of several types of cancers such as colorectal, liver, breast, bladder, colon, lung, and cervical cancers (Biffi et al., 1997; Hassan et al., 2016; Kadirareddy et al., 2016; Lee et al., 2015; Malik et al., 2018; Maroof et al., 2012; Modarressi et al., 2014; Nami et al., 2014; Nami et al., 2015; Nouri et al., 2018; Yu and Li, 2016). Chemotherapeutics and synthetic drugs are extensively used as the standard treatment for breast cancer. These drugs are usually accompanied by life-threatening side effects and poor patients outcomes (Di Francia et al., 2013). Probiotics are less harmful and more cost-effective than various pharmacological compounds which are utilized to prevent or treat cancers (Malik et al., 2018).

The previously established cancer prevention

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mechanisms based on the application of probiotics include: ***i***) regulation of gut microbiota, ***ii***) improvement of gut barrier functions, ***iii***) elimination of procarcinogens, ***iv***) protection of the intestinal epithelium, and **v**) improvement of the immune and inflammatory systems. Moreover, other than cancers, probiotics reduce the risk of multiple chronic diseases, such as high serum cholesterol-related diseases, allergic diseases, and HIV (Nazir et al., 2018).

In literature, probiotics have been shown to inhibit the proliferation of several cancer cells types; but, the effect(s) of the mixture of probiotics on human breast cancer cell lines have rarely been investigated. Therefore, the purpose of this study was to investigate these effects as an alternative treatment strategy of breast cancers.

Materials and Methods

Chemicals

The chemicals, including $AlCl_3$, ethanol, ethyl acetate, the Folin-Ciocalteu reagent, $FeCl_3$, $FeSO_4$, gallic acid, glacial acetic acid, hexane, methanol, and TPTZ (tripyeridyl-s-triazine) were obtained from Merck (Germany). The mixture of local probiotic bacteria was purchased from Takgene Zist Company (Iran).

Bacterial strains

A mixture of three local probiotic bacteria from Iran (including; *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Lactobacillus casei*) were pre-cultured under sterile conditions on de Man, Rogosa and Sharpe (MRS) broth at 37°C overnight. Afterward, 10% of this stock was inoculated into the main culture medium, and incubated for 24h. Subsequently, the bacterial cultures (concentration: 1.5×10^{10} CFU/ml) were centrifuged at 1100*g* for 15min. Then, the pH was adjusted to 7.2 with NaOH (1N), and lyophilized. The lyophilized cell-free supernatant (CFS) was re-suspended in DMEM medium, filter sterilized, and stored at 4°C for further experiments.

Determination of antioxidant activity

The antioxidant power of the bacterial supernatant was determined by the ferric reducing ability of plasma, the FRAP assay (Benzie and Strain, 1996). Briefly, 50µL of each sample was added to 1450µL of the FRAP solution, and incubated for 30min at 37ºC. The samples' absorbances were read at 593nm, and the antioxidant activity was reported as mmol Fe^{2+}/g of each sample.

Determination of total polyphenol content

The total polyphenol content (TPC) of the bacterial supernatant was estimated in terms of the Folin–Ciocalteu (F-C) method, using gallic acid as the standard (Singleton et al., 1999). Briefly, 200µl of the extract was mixed with 1.3ml of Folin-Ciocalteu solution for 5min. Then, 1.5ml of the sodium carbonate (6%, w/v) was added, and incubated at room temperature for 30min. In addition, the absorbance of the sample was measured at 725nm. The TPC content was reported as gallic acid equivalent (GAE) (mg/l)/g of sample.

Fatty acid (FA) identification and quantification

Free fatty acids (FFAs) were analyzed using the method introduced by Torella et al. (Torella et al., 2013). Briefly, 200µL of the bacterial supernatant was extracted by the addition of 50µL of NaCl (10%, w/v), 50µL glacial acetic acid, and 200µL ethyl acetate, and then the solution vortexed for 20s. The mixture was centrifuged at 16000g for 10min, and then ethyl esters were generated by mixing 50µL of the organic phase with 450µL of a 30:1 mixture of EtOH and HCl (37%, v/v); they were incubated for 1h at 55°C. Then the mixture was cooled to the room temperature, and dH_2O (250µL) and hexane (250 µL) were added to this mixture. The mixture was vortexed for 10s, and 75µL was taken from the hexane layer for the GC-MS analysis. Extracts were run on the Agilent 5977A MSD GC-MS system (Agilent Technologies, USA) using the HP-5MS column (length: 30m; diameter: either 0.25 or 0.50mm; film: 0.25µm); the temperature was increased from 75 to 290°C (10°C/minute). Different compounds were determined via GC retention times compared to normal alkanes and verified by mass spectra. To calculate the retention indices (RI), n-Alkanes (C8-C40) were used as reference points (Babushok et al., 2011). The fatty acids were identified by computer matching with the Wiley Registry of Mass Spectral Data, 7th Edition (WILEY7 MS library), and confirmed by comparing their retention indices.

Cell culture

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin-Streptomycin, and Trypsin-EDTA were purchased from Gibco (UK). Cell culture vessels were obtained from the Greiner Bio-One and Falcon. DAPI (4', 6-diamidino-2-phenylindole) was purchased from Roche (Germany).

The breast carcinoma cell lines MDA-MB-231 (ATCC HTB-26) and MCF-7 (ATCC HTB-22) were seeded in DMEM medium containing 10% FBS, and incubated in a 5% CO_2 incubator at 37°C.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to study the cytotoxic effects of the bacterial supernatant on MDA-MB-231 and MCF-7 cells (Rezaei et al., 2012). Briefly, 5000 cells/well were seeded in 96-well plates. After 24h the cells were exposed to different concentrations of the bacterial supernatant (100, 200, 400, 800, 1600, and 3200µg/ml) for 24, 48, and 72 h. At each time point, the MTT reagent (4 mg/ml in PBS) was added to the wells, samples were incubated at 37°C for 4h, and absorbances were determined using a microplate reader (Stat Fax 2100 Microplate ELISA Reader, Awareness Technologies) at 570nm. The IC_{50} value was defined as the concentration leading to a 50% inhibition of cell growth in comparison to the control cells.

Microscopic analysis of apoptotic cells

The apoptosis-inducing effect of the bacterial supernatant was confirmed by DAPI (4′,6-diamidino-2-phenylindole) staining (Rezaei et al., 2011). Briefly, the cells (3×10^5 cell/well) were seeded in 6-well plates, and then treated by the IC_{50} concentration of the bacterial supernatant for 24, 48, and 72h. The methanol-fixed cells were stained with 0.02% DAPI solution. Also, the plates were observed, and pictures taken using the Cytation5 Cell Imaging Multi-Mode Reader (BioTeK instruments).

Statistical analyses

The results were expressed as mean of three independent experiments±standard deviation (STDEV). Moreover, the obtained data were statistically compared by IBM SPSS Statistics22.0 using one-way analysis of variance (ANOVA). The $P<0.05$ was considered as statistically significant, and correlations among pairs of variables were determined using the Excel software.

Results

The BS content analysis

The antioxidant potential of the bacterial supernatant was calculated using a standard curve ($y=0.00289X+0.03340$, $R^2=0.99$) of the ferrous sulfate ($FeSO_4$) solution. As it was demonstrated by the results, the antioxidant activity increases along with the increase in the concentration. Also, the highest antioxidant activity (123.5 mmol Fe^{2+}/g) of the samples was measured at the concentration of 3200 µg/ml of the bacterial supernatant (Table 1).

The total phenolic content of the extract was calculated using a standard curve ($y=0.00221X + 0.04578$, $R^2=0.93$) of gallic acid. As shown in Table 1, total polyphenol content is increases in accordance with the increasing concentrations. The highest amount of 30.9 mg GAE/g was observed at 3200 µg/ml of the bacterial supernatant.

As described in the Table 2, in the bacterial supernatant, linoleic acid has the highest percentage (37%) among other investigated fatty acids, which is followed by oleic acid (27%). It is while, palmitic acid (3.42%) showed the lowest percentage.

Table 1. Antioxidant activity and TPC of the bacterial supernatant.

Bacterial supernatant concentrations (µg/ml)	TPC (mg GAE/g of sample)	Antioxidant activity (mmol Fe^{2+}/g of sample)
100	3.78±0.001	28.201±0.03
200	5±0.02	32. 83±0.05
400	7.24±0.024	45.15±0.062
800	8.91±0.035	50.77±0.051
1600	16±0.051	63.05±0.01
3200	20±0.021	100.52±0.1

Values are expressed as mean±standard deviation.
Abbreviations: TPC, total phenol content; GAE, galic acid equation.

Table 2. Fatty acid composition of the bacterial supernatant.

C number: double band number	FA	RI	Fatty acid (%)
14:0	Tetradecanoic acid	1558	6.11
16:0	Palmitic acid	1882	3.42
18:2	Linoleic acid	1974	37.40
18:1	Oleic acid	1440	26.93
20:0	Eicosanoic acid	2856	13.42
22:0	Docosanoic acid	1520	12.03

RI indices on HP-5MS column, experimentally determined using the homologue series of n-alkanes.
Abbreviations: C, carbon; FA, fatty acid; RI, linear retention.

Cytotoxic effects of the probiotic mixture

The effects of the bacterial supernatant on the viabilities of two human breast cancer cell lines MDA-MB-231 (Figure 1) and MCF-7 (Figure 2) were assessed using MTT assay at similar concentrations for 3 consecutive days. The bacterial supernatant showed a significant growth inhibitory effect in a dose- and time-dependent manner on both cell lines. The IC_{50} value on both cell lines was determined as 3200µg/ml in 72h following the exposure. The IC_{50} concentration was then used for performing further studies.

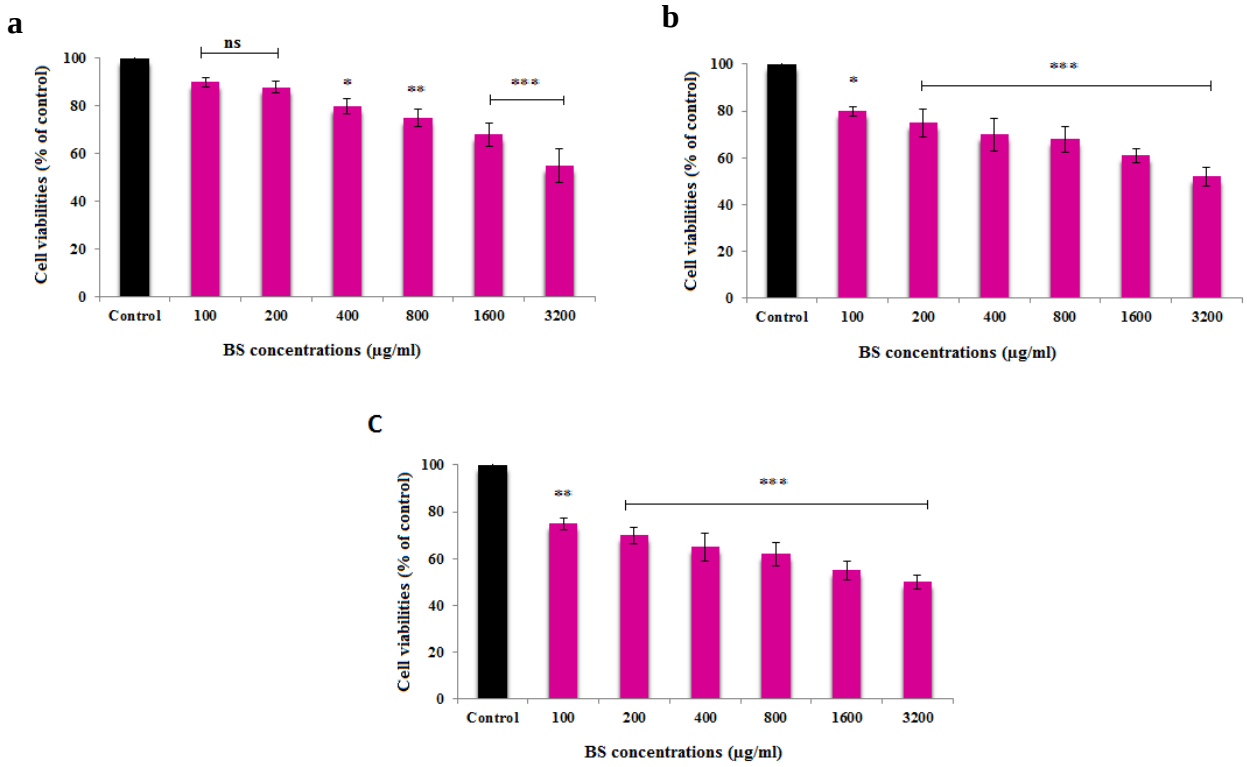

Figure 1. The cytotoxic effects of the bacterial supernatant on MDA-MB-231 cells as it was obtained by MTT assay. The MDA-MB-231 cells were exposed to different concentrations of the bacterial supernatant (100, 200, 400, 800, 1600, and 3200µg/ml) for 24 (a), 48 (b), and 72 h (c). The $p<0.05$ was considered as statistically significant in comparison to the control cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$, and "ns" showed no significant difference was observed between treated and untreated groups).

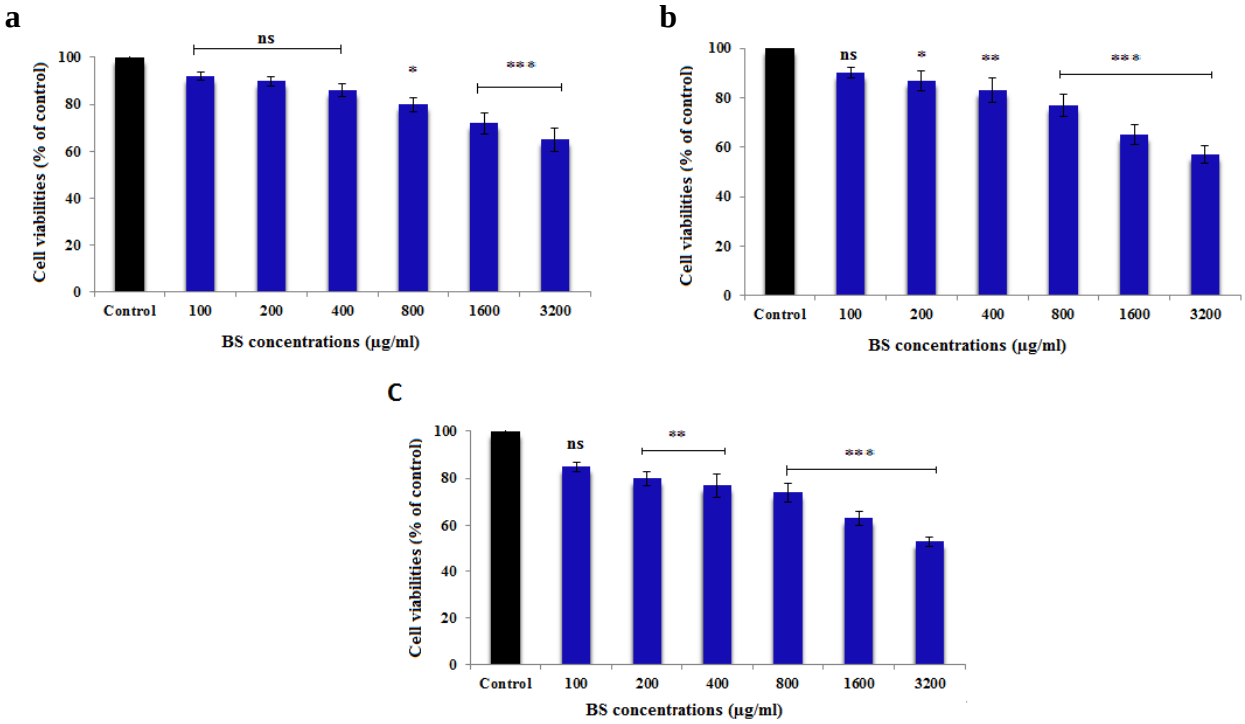

Figure 2. The cytotoxic effects of the bacterial supernatant on MCF-7 cells as it was obtained by MTT assay. The MCF-7 cells were exposed to different concentrations of the bacterial supernatant (100, 200, 400, 800, 1600, and 3200 µg /ml) for 24 (a), 48 (b), and 72 h (c). The $p<0.05$ was considered as statistically significant in comparison to the control cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$, and "ns" showed no significant difference was observed between treated and untreated groups).

Correlation among total phenolic content and antioxidant capacity of BS with the cells' viability

The correlations between total phenolic contents and cell viabilities are shown in Figure 3a. The correlation coefficients of linear regression (R^2) were calculated as 0.90 and 0.82 for MCF-7 and MDA-MB-231 cells, respectively. There is a positive and high correlation between TPC values and the viabilities of MCF-7 cells.

The correlations between the antioxidant capacities and cell viabilities are shown in Figure 3b. The R^2 were calculated as 0.89 and 0.94 for MCF-7 and MDA-MB-231 cells, respectively. There is a positive and high correlation between antioxidant capacities and MDA-MB-231 cells viability.

The correlation between the TPC and the antioxidant capacity (FRAP) of the bacterial supernatant is presented in Figure 3c (R^2=0.97). Hence, there is a positive and high correlation between TPC values and antioxidant capacities of the bacterial supernatant.

In addition, significant correlations were found between antioxidant capacities and cell viabilities of MDA-MB-231 (R^2=0.94, Figure 3b); and between MCF-7 cells viabilities and TPCs (R^2=0.90, Figure 3a). Moreover, the lowest correlation was found between the antioxidant capacities and MCF-7 cell viabilities (R^2=0.89, Figure 3b; R^2=0.82, Figure 3a) with the TPC and MDA-MB-231 cell viabilities, respectively. Data are shown in Table 3.

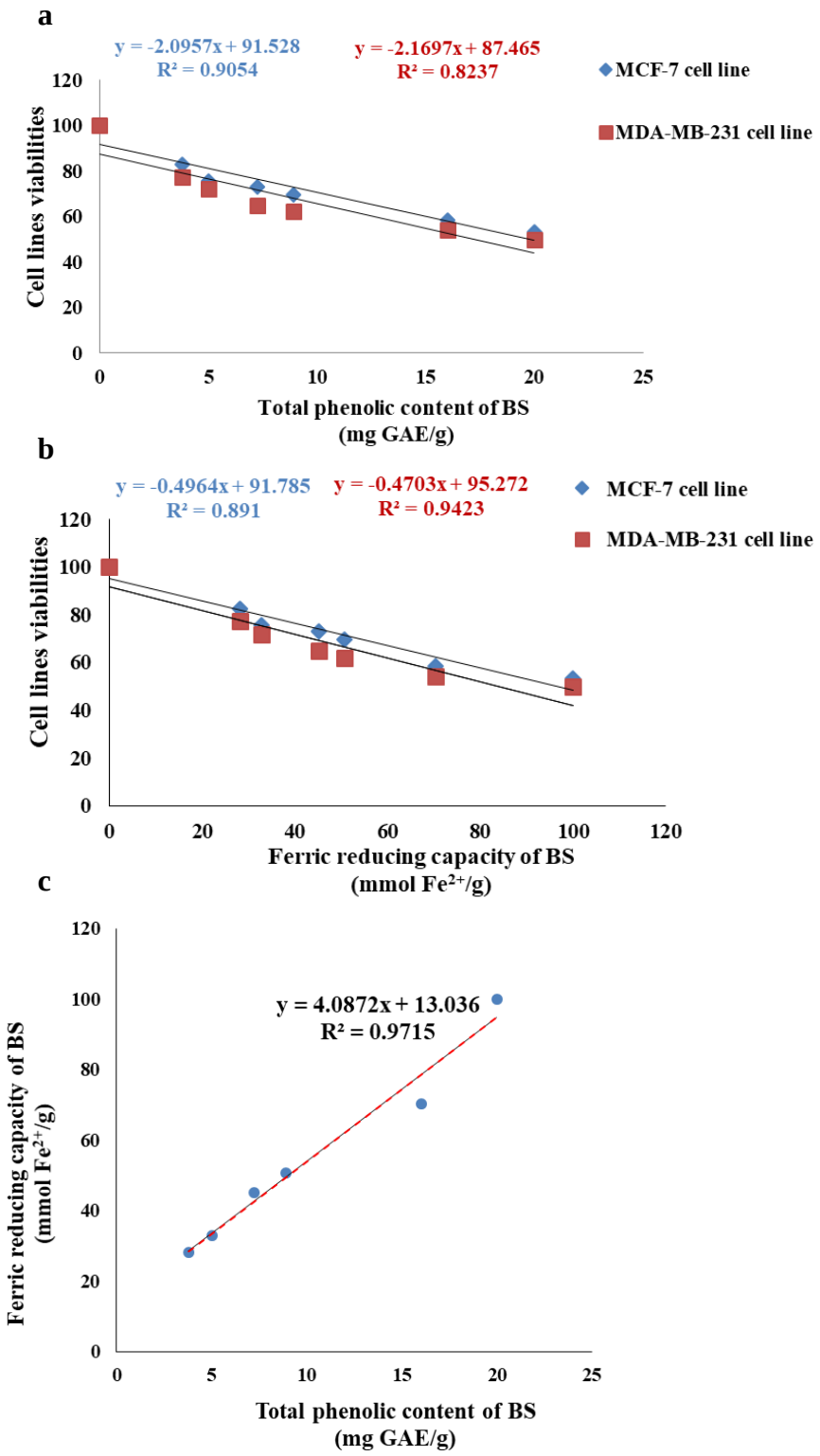

Figure 3. Linear regression among FRAP, TPC and cell lines viabilities. a) Total phenolic contents (mg gallic acid/g DW) and cell viabilities (%); b) Antioxidant capacity as it was evidenced by the FRAP assay (momol Fe^{2+}/g DW) and cell viabilities (%); c) total phenolic contents and antioxidant capacities.

Table 3. Correlation coefficient matrix between data.

	[probiotic]	TPC	FRAP	MDA-MB-231	MCF-7
[probiotic]	1				
TPC	0.948682	1			
FRAP	0.931299	0.983209	1		
MDA-MB-231	-0.76253	-0.90759	-0.9439	1	
MCF-7	-0.83134	-0.95154	-0.9707	0.989193	1

Abbreviations: [probiotic], probiotic concentration; TPC, total phenolic content; FRAP, ferric reducing ability of plasma.

Apoptosis-inducing effects of the bacterial supernatant

The apoptotic type of the cell death was confirmed through morphological analyses. The nuclear morphological changes of MDA-MB-231 cells are clearly shown in Figure 4. In BS-treated cells, nuclear morphological changes, such as nuclear condensation and fragmentation were observed 48h post treatment. As shown in Figure 5, morphological changes of the MCF-7 cells' nucleoli, including condensation in the peripheral zone of the nucleus and DNA fragmentation were observed following 24h. Dead cells were detected upon increasing the exposure time following the treatment with the bacterial supernatant.

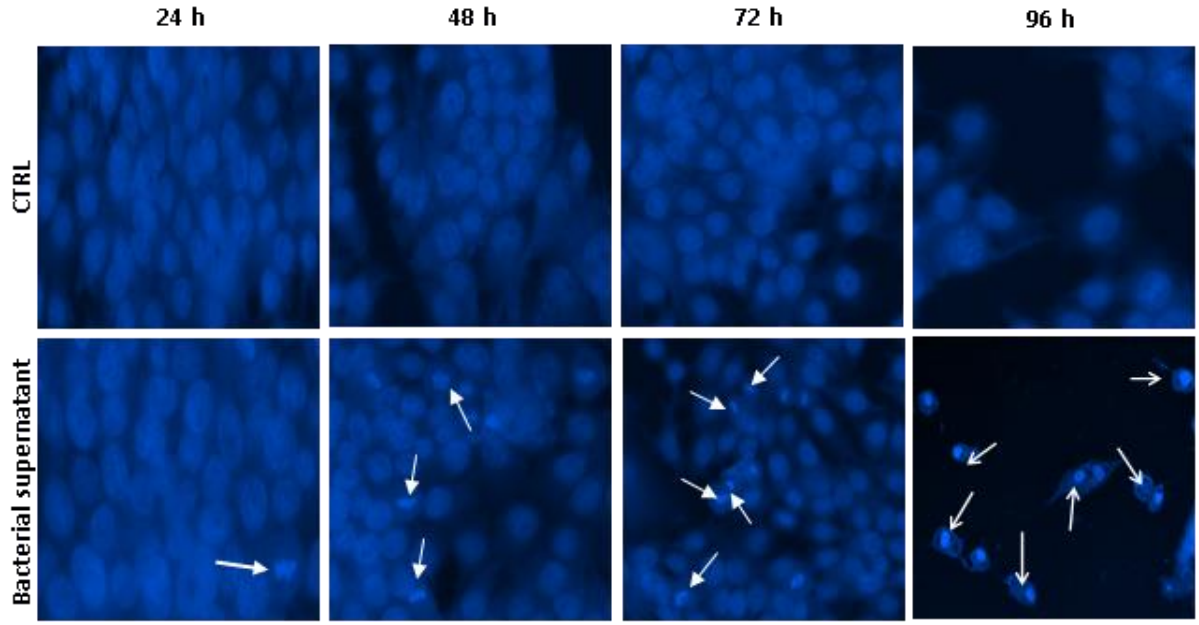

Figure 4. Effects of bacterial supernatant on nuclear morphology of MDA-MB-231 cells. MDA-MB-231 cells were cultured and exposed to the bacterial supernatant (3200 µg/ml) or left untreated (CTRL). DAPI staining was performed at different time points (24-96h) to detect apoptosis-inducing effects of the BS. The images were taken using the Cytation5 Cell Imaging Multi-Mode Reader. White arrows indicate apoptotic cells with condensed nucleoli (10X).

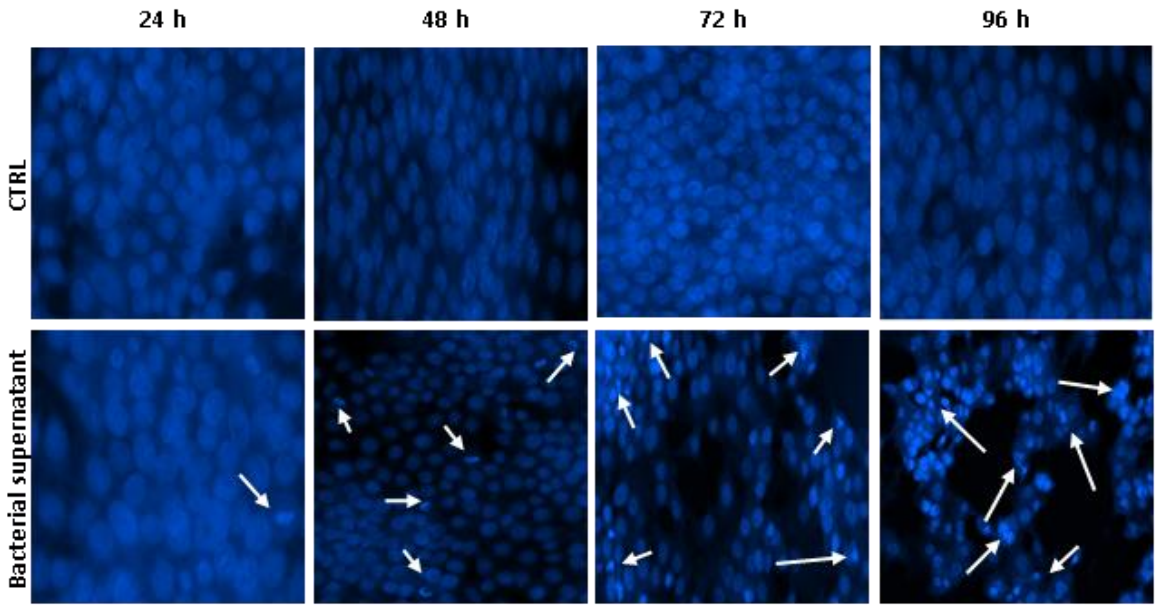

Figure 5. Effects of bacterial supernatant on nuclear morphology of MCF-7 cells. MCF-7 cells were cultured and exposed to the bacterial supernatant (3200 µg/ml), and DMEM (CTRL) for 24-96 h. The images were taken using the Cytation5 Cell Imaging Multi-Mode Reader. The white arrows indicate apoptotic cells (10 X).

Discussion

Probiotics and their metabolites can regulate key cellular processes, including proliferation, differentiation, apoptosis, and angiogenesis (Motevaseli et al., 2017). In some cases, synbiotics and a combination of two or more probiotics are more efficient than a single probiotic compound (Yu and Li, 2016). It is believed that, many commercially available probiotics are safe and have some beneficial health effects for the host. Also, probiotics could act against breast cancer progression, as it was evidenced by several *in vitro* cell-based studies, pre-clinical experiments, and large-scale clinical trials (Malik et al., 2018).

Several significant studies have illustrated that probiotics have pro-apoptotic or anti-proliferative effects on various human cancer cell lines. The induction of apoptosis is the best mode of cell proliferation inhibition or suppression. Apoptosis is an evolutionarily conserved pathway of cell death which is responsible for the programmed elimination of the cells during homeostasis and normal eukaryotic development (Singh et al., 2019). Apoptosis of eukaryotic cells is associated with specific morphological changes in their cell membranes and nuclear DNA. These morphological changes could be observed during microscopic studies (Elmore, 2007). The anti-carcinogenic effects of different probiotics are attributed to a combination of events and mechanisms. Their mechanism of function is similar to the tumor suppressor protein p53, which induces cell apoptosis and high levels of DNA damage (Yu and Li, 2016).

It was shown in a previous publication that the proteinaceous postbiotic metabolites (PPM) of *L. plantarum* I-UL4 are cytotoxic against MFC7 cells in a dose- and time-dependent manner (IC_{50}: 10.83µg/µl, 72h). In addition, they demonstrated that 48h following the treatment apoptosis is detectable in 80.87% of the cells (Tan, 2015). Also, treatment of human cancerous cell lines (HeLa, MCF-7, AGS, HT-29, Caco-2) with the *Enterococcus lactis* IW5 supernatant at concentrations less than 50µg/ml confirmed its cytotoxic effects in all investigated cell lines, and indicated its apoptosis-inducing capacity in HeLa cells (Nami et al., 2015). Lee and coworkers reported that *L. lactis* KC24 isolated from kimchi exerted antimicrobial, anti-inflammatory, antioxidant, and anti-cancer effects. Its anti-cancer effect were shown against AGS, HT-29, LoVo, MCF-7, and SK-MES-1 cells (>50% cytotoxicity) (Lee et al., 2015).

The cell free supernatant (CFS) of *B. bifidum* has the ability to inhibit the growth of SW742, Caco-2 and HT-29 colon cancer cells (Bahmani et al., 2019).

Kadirareddy et al. reported the inhibition of MDA-

MB-231 proliferation by *Lactobacillus plantarum*-conjugated linoleic acid (LP-CLA) from *Lactobacillus plantarum.* Cell detachment, rounding of cells, and oligo-nucleosomal fragmentation of DNA were reported as the main morphological changes observed following the treatment (Kadirareddy et al., 2016).

Metabolites of *E. durans* 39C showed anti-cancer characteristics against MCF-7, HeLa, HT29, and AGS cell lines mainly via induction of apoptosis (Haghshenas et al., 2014). The supernatants of *L. rhamnosus* strain GG (LRS) and *L. crispatus* strain SJ-3C-US (LCS) showed growth inhibitory effects on the HeLa cells (Nouri et al., 2018). Also, *L. acidophilus* and *L. crispatus* supernatants indicated antitumor activity (15%, v/v) on the MDA-MB-231 cells (Modarressi et al., 2014). Several probiotic strains, including *B. animalis*, *L. acidophilus*, *Bifidobacterium infantis*, *Lactobacillus paracasei*, *and Bifidobacterium bifidum* have been shown to reduce the MCF7 cancer cell's growth (Kim et al., 2007).

Probiotics produce compounds, such as polysaccharides and fatty acids, which are powerful in inhibiting the proliferation of cancerous cells. Short chain fatty acids (SCFA), by increasing the amount of butyrate, can affect the regulation of the balance amongst proliferation, differentiation, and apoptosis (Bougnoux et al., 2010). Probiotics can also stimulate the antioxidant system of the host cells, and efficiently elevate the activity of antioxidant enzymes (Wang et al., 2017).

According to the results, the antioxidant capacity of the BS is increased in a dose-dependent manner, and a positive and high correlation observed between its antioxidant capacity and the viability of MDA-MB-231 cells. The correlation between antioxidant activity and the viability of MCF-7 cells was positive and a little less than MDA-MB-231. Based on the results, linolenic acid is the most common fatty acid of the BS which may have growth inhibitory effects against both cell lines. The present findings suggest that the mixture of local probiotic bacteria, including *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Lactobacillus casei* not only has the antioxidant capacity, but also exert cytotoxic effects on MCF-7 and MDA-MB-231 cell lines, partially through the induction of apoptosis. The exact effector molecules and mechanisms which are responsible for the probiotic mixture properties should be further explored. In addition, as a natural bioactive compound, its suitability for clinical applications needs further evaluations and complementary studies.

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