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Local probiotic Lactobacillus brevis downregulates LPAR1 and LPAR2 gene expression and reduces invasion of MDA-MB-231 and MCF-7 breast cancer cell lines

Seyedeh Mona Mousavi Esfahani[1], Niloufar Rashidi[2], Shadi Abkhiz[1], Neda Akbari[3], Parastoo Tarighi[1*]

1. Department of Medical Biotechnology, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran
2. Department of Medical Laboratory Sciences, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran
3. Department of microbiology, Faculty of Science, Islamic Azad University, Arak, Iran

ABSTRACT

Introduction: Breast cancer (BC) is the second leading cause of cancer deaths in the world. Studies suggest that the lysophosphatidic acid (LPA) gene is the cause of invasion and metastasis in malignant cancers, including BC. In addition, the PI3K/ PAK1/ ERK cascade in cancer cells helps metastatic BC. It has been observed that LPA can stimulate reactive oxygen species production, which is an important mediator of LPA to stimulate the migration of BC cells and activate the PI3K/ PAK1/ ERK signaling pathway.

Methods: This study aimed to evaluate the Lactobacillus brevis probiotic supernatant's effectiveness in reducing LPA expression in BC cell lines. MCF-7 and MDA-MB-231 cell lines were treated with supernatant of local Lactobacillus brevis for 24 and 48h. mRNA expression levels of LPAR1 and LPAR2 genes were evaluated by qRT-PCR. Furthermore, an invasion assay was performed to assess these cell lines' invasion rate following treatment.

Results: The results indicated a remarkable decline in the survival rate of treated cells. LPAR1 and LPAR2 gene expression declined in MDA-MB-231 and MCF-7 cells. Moreover, the invasion rate of these cells was reduced following treatment.

Conclusion: Considering Lactobacillus brevis supernatant's cytotoxic effects on cancerous cells, this bacteria could be thought of as a promising application for a possible treatment approach with fewer adverse reactions. However, more research is obviously needed. In the future, probiotics could be used in conjunction with currently available therapies.

Keywords:
Lactobacillus brevis
Probiotics
Breast cancer
Invasion
LPA

Introduction

Breast cancer (BC) is among the most common types of cancer. Despite many advances in screening, diagnosis and treatment, it is also the main reason for cancer deaths (Thiagarajan et al., 2018). BC cell metastasis occurs in several organs; however, bone is the main target organ of metastatic BC cells. Lysophosphatidic acid (LPA) is recognized as a major cause of bone metastasis

* Corresponding author: Parastoo Tarighi, tarighi.p@iums.ac.ir
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in this cancer (Boucharaba et al., 2006). LPA levels increase significantly in cancers and its receptors are abnormally expressed in some human cancers (Mills and Moolenaar, 2003). LPA can activate the PI3K/ PAK1/ ERK cascade in cancer cells and help metastatic BC. It has also been observed that LPA can stimulate reactive oxygen species production, which can act as an importtant mediator of LPA to promote the migration of cancer cells and act through PI3K/ PAK1/ ERK signaling (Du et al., 2010). BC cells express approximately three LPAR1, LPAR2 and LPAR3 receptors on their surface with a high LPA affinity (Chen et al., 2007).

Probiotics are “live microorganisms that confer a health advantage to the host when present in adequate quantity,” according to the World Health Organization (Pandey Naik et al., 2015). These microorganisms can inhibit pathogen adherence and invasion of the epithelium by producing short-chain fatty acids, blocking adherence sites and increasing gene expression of mucin 2 and oligomeric mucus gel-forming (Ramakrishna, 2009). In several research studies, probiotics are shown to have anti-cancer properties by enhancing local and systemic antioxidant activity, strengthening the intestinal microbiota, elimination of potential carcinogens, modification of the immune system associated with the gut, as well as antioxidant defenses (Yu and Li, 2016). However, the underlying mechanism of action is still unknown and probiotic efficiency is quite different depending on the strains and the quantities administered (Kang and Im, 2015; Markowiak and Śliżewska, 2017). On the other hand, although cancer risk is intrinsically linked to genetic determinants, the organism’s immunological state, directly linked to probiotic bacteria and commensal bacterial flora found mainly in the digestive system, plays a significant part. Probiotics have received much interest because of their capacity to control cancer cell proliferation and death *in vitro* and *in vivo*. These features’ potential application in innovative therapy could be a viable alternative to more invasive treatments like chemotherapy or radiotherapy (Górska et al., 2019). Lactobacillus and Bifidobacterium are natural intestine bacteria and a type of probiotic that has recently been studied for their anti-cancer properties as well as other health benefits (Kamkar et al., 2020). Lactobacillus metabolizes glucose to produce lactic acid during glycolysis. It contains about 170 facultative, anaerobic, catalase-negative, gram-positive bacteria and non-spore-forming rods. Numerous applications of lactobacillus have been reported in human and animal health, including tumor-inhibiting, anti-proliferative, as well as inhibiting oxidative stress and enhancing cancer cell apoptosis in the ongoing battle against cancer (Marinelli et al., 2017; Yang et al., 2018; Zhang et al., 2018). Among the Lactobacillus species is Lactobacillus brevis, which is a gram-positive, catalase-negative, non-spores, immobile, rod-shaped, or coconut-shaped bacteria. This strain is given special attention due to its qualified presumption of safety status, so it is widely used in the production of fermented foods. Their potential to function as probiotics extend beyond their use in fermentation (Feyereisen et al., 2019).

Previous research has shown that Lactobacillus Brevis metabolites can be used as adjuvant therapy and reduce apoptosis in MCF-7 cells (Nasiri et al., 2021). However, no studies have been conducted to assess the cytotoxic effects of Lactobacillus brevis supernatant on MCF-7 and MDA-MB-231 cells, or the LPAR1 and LPAR2 gene expression involved in invasion in cells treated with supernatant.

In addition, tumor recurrence and drug resistance have even been documented after anticancer therapy (Górska et al., 2019). The design of a unique drug delivery system using supplementary medications relying on bacterial and plant extracts may aid in reducing malignant cell resistance and recurrence (Al-Oqail et al., 2019; Jafari-Nasab et al., 2021). Thus, this study is aimed at evaluating the therapeutic effects of Lactobacillus brevis supernatant, including its cell growth inhibition and mRNA expression levels of LPAR1 and LPAR2 genes were evaluated. Furthermore, an invasion assay was performed to assess these cell lines’ invasion rate following treatment.

Material and Methods

Preparation of Lactobacillus brevis supernatant (LBS)

We obtained Lactobacillus brevis from Nasiri et al research (Nasiri et al., 2021). MRS broth is also used to culture in an anaerobic environment at 37°C. The solution was centrifuged in 1000g for 15min at 4°C after 48h (the stationary phase). We took the supernatant and measured it at OD600 with a spectrophotometer. Freeze-drying was then used to lyophilize the supernatant.

A stock solution (100mg/ml) of lyophilized bacteria

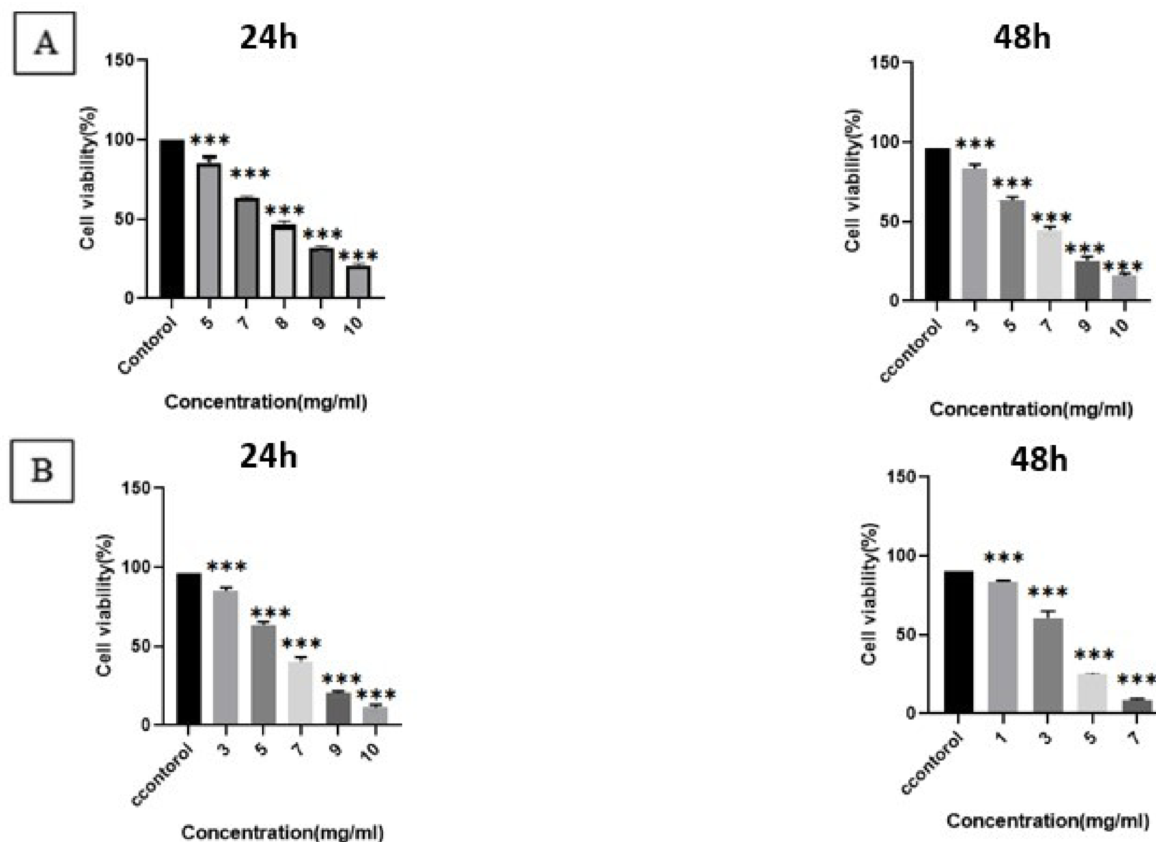

FIGURE 1. Cell viability assay of cells treated with LBS after 24 and 48 h. A). Cytotoxic effects of different concentrations of LBS on MCF-7 cell line. B) LBS cytotoxicity at different concentrations on MDA-MB-231. Results represent the means ± SE of three different experiments. n=3. (*P*<0.05:*, *P*<0.01:**, *P*<0.001: ***).

was prepared in order to select the concentration of the supernatant for inhibiting cancer cells. The 3ml of the culture medium was diluted with 300mg of the lyophilized bacteria to make a sterile solution. This mixture is then sequentially filtered through a 0.22µm filter. Then, concentrations ranging from 10µg/ml to 10mg/ml were made, and each concentration was repeated eight times. After estimating the IC50 (Half-maximal inhibitory concentration) of the supernatant, we chose the combination of the optimum supernatant concentration that had the greatest effect on the lethality of the cells for this purpose.

Cell culture

The MCF-7 and MDA-MB-231 cell lines were provided by the Pasteur Institute of Iran cell bank. The MCF-7 and MDA-MB-231 were cultured in RPMI1640 and DMEM medium, respectively (Biowest, Nuaille, France). The medium was prepared with 10% fetal bovine serum (FBS, Gibco, Billings, MT, United States) and 1% penicillin/ streptomycin (biowest) and incubated at 37°C in a humidified atmosphere containing 5% CO2. After the cells had reached about 70% confluency, they were passaged.

Cell viability assay

The MDA-MB-231 and MCF-7 cells were seeded on a 96-well cell culture plate and incubated for 24h. A supernatant of bacteria (100mg/ml) was prepared as a treatment for cancer cells. Cells were treated with 10µg/ml- 10mg/ml concentrations of LBS for 24 and 48h. In order to assess the cytotoxicity of lyophilized LBS, we used the MTT (3-(4, 5dimethylthiazol-2-yl)2, 5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, Missouri, United States). Briefly, each well was filled with MTT solution (5mg/ml) and the plates were incubated in an incubator for 4h. DMSO (Sigma Aldrich) was then used to dissolve the formazan crystals. The absorbance was measured at 570nm by the Bioteckmicro plate reader (USA) with a 690nm baseline for calibration standard (Figure 1)

RNA extraction, cDNA synthesis and primer design

An RNX solution purchased from the "Sinaclone" company was used to extract RNA from cells treat-

TABLE 1: List of primer sequences

Gen	Sequence	Length	TM	GC%	Product Size
LPAR1 Forward	GCGGCAATCTATGTCAACCGC	21	59.94	52.38	87
LPAR1 Reverse	CAACCCAGCAAAGAAGTCTGC	21	60	52.38	87
LPAR2 Forward	GCCTGGTCAAGACTGTTGTCA	21	60.48	52.38	105
LPAR2 Reverse	CATTGCAGGACTCACAGCCTA	21	60.07	52.38	105
GAPDH Forward	GAAGGTGAAGGTCGGAGTCAAC	22	60.87	54.55	71
GAPDH Reverse	CAGAGTTAAAAGCAGCCCTGGT	22	60.82	50	71

ed with LBS for 24 and 48h (Zandi and Ebrahimifard, 2017). Additionally, RNA extraction steps were performed according to the protocol. The RNA concentration is obtained in terms of absorption ratio of 280/260 and 230/260 ng/µl. Finally, the RNA quality and accuracy were evaluated using 1% agarose gels. The separated RNA (500ng) was reverse transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara Bio, Shiga, Japan) (Zhuang et al., 2019). Primers were designed for LPAR1 and LPAR2 genes. The exon-exon junction method was used to increase the specificity of primers. As an internal control, glyceraldehyde 3-phosphate dehydrogenase was used. The primer pairs used in this study are listed in Table 1.

Quantitative RT-PCR reaction

A qRT-PCR was performed using the Takara Bio SYBR Premix Ex Taq II with a light cycler 96 (Roche) detection system. The PCR amplification conditions included 95°, 40 cycles at 95°, and 60° for 30s, 5s and 30s, respectively. In order to figure out the melting temperature of a particular amplicon and primer, a melting curve analysis was carried out. The experiments were repeated three times in duplicate (Toyomane et al., 2022).

Invasion assay

Invasion assay was performed by implanting Matrigel on Transwell insert (Pore size, 8µm, SPL, Korea) under sterile conditions and in the presence of invasive cells. Briefly, 5×10^4 serum-starved cells were dissolved in a fresh medium containing 1% FBS and were added to the Matrigel-coated upper chamber of the insert. A medium containing 20% FBS was put in the lower chamber of the insert to act as a chemoattractant. We used a cotton swab soaked in phosphate buffered saline to remove the remaining cells on the upper chamber after incubation for 48h at 37°C. Invaded cells on the lower level of the insert were fixed with methanol (Merck, Germany) and stained with hematoxylin-eosin (Sigma-Aldrich, USA). We used ImageJ software for counting invaded cells (Dong et al., 2020).

Statistical analysis

Graphpad prism9 was used to conduct the statistical analysis. Results from three independent experiments were expressed as mean±SE, and one-way ANOVA calculated differences. As a threshold for significance, *P* values less than 0.05 were considered.

Results

LBS cytotoxicity on BC cell lines

In order to examine the cytotoxicity effect of lyophilized LBS on MDA-MB-231 and MCF-7 cells, the cells were treated with different concentrations (10µg/ml to 10mg/ml) of LBS, and as indicated in Figure 1, eight samples with varying concentrations were examined.

According to Figure 1A, after 24h of treatment, the cell viability of 10µg/ml concentrations to 4mg/ml reached 95% for the MCF-7 cell line. Therefore, it could not inhibit cell growth. But the cell viability decreased significantly by increasing the concentration between 5 to 10mg/ ml as the 7mg/ml concentration reached to 63%. After 48h of treatment with 5mg/ml supernatants,

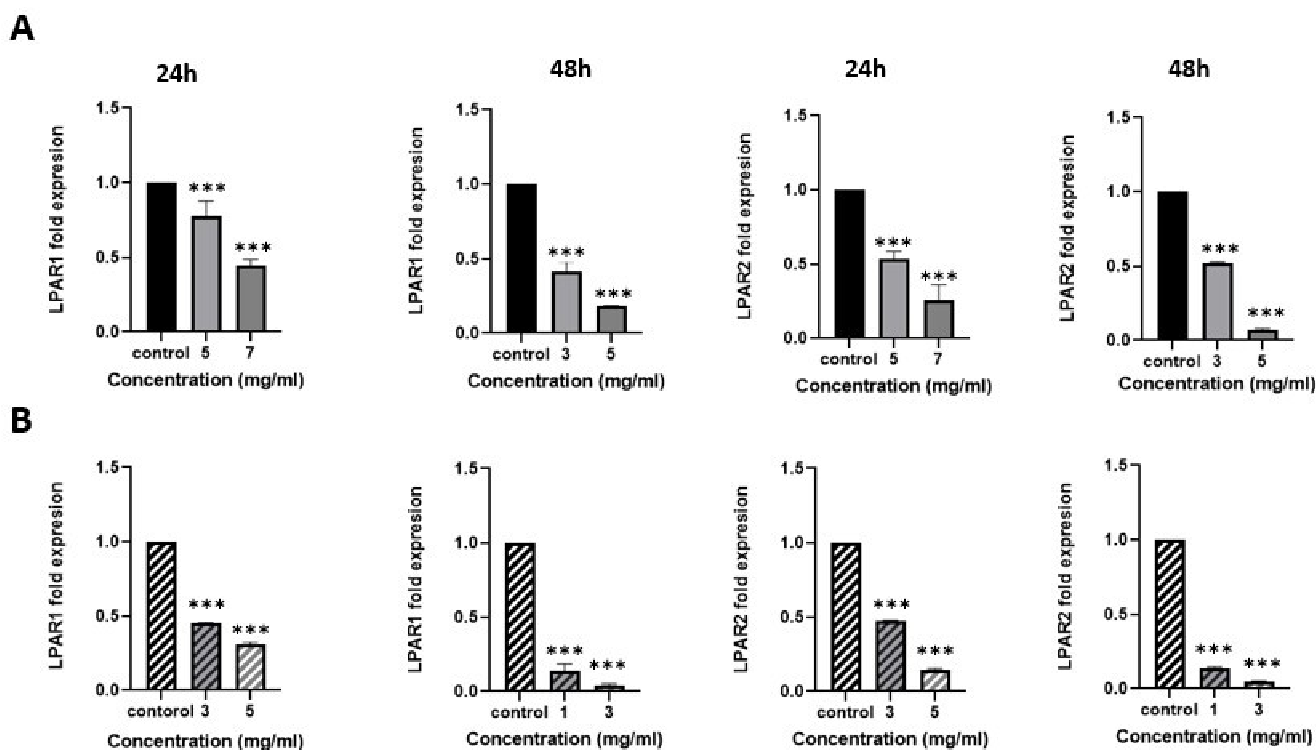

FIGURE 2. Quantitative RT-PCR gene expression of LPAR1 and LPAR2 in (A) MCF-7 and (B) MDA-MB-231. Gene expression was normalized with GAPDH as internal control. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

the cell growth decreased to 63%. (IC50=5mg/ml)

As shown in Figure 1B, after 24h of treatment of MDA-MB-231, cell viability at the concentrations of 10µg/ml to 2mg/ml reached 95%, so it could not inhibit cell growth. But the cell viability decreased significantly by increasing the concentration between 3 to 10mg/ml as of 5mg/ml concentration reached 61%. After 48h of treatment with 3mg/ml supernatants, the cell viability decreased to 61% (IC50=3mg/ml). According to the concentrations mentioned above, MDA-MB-231 cells were shown to be more sensitive than MCF-7 cells. *P*-Value was less than 0.0001.

The effects of LBS on the mRNA expression of LPAR1 and LPAR2 genes of BC cell lines

mRNA levels of LAPR1 and LAPR2 were evaluated by qRT-PCR, and the results showed transcription of these genes was reduced significantly in LBS treated cells. As shown in Figure 2, two samples of significant concentrations were selected.

In MCF-7 after 24h treatment with concentrations of 5 and 7mg/ml of supernatant, 23% and 56% reduction in LPAR1, 47% and 74% reduction in LPAR2 expression were observed compared to the control group (Figure 2A). Also, treatment with concentrations of 3 and 5mg/ml of supernatant for 48h decreased LPAR1 expression by 59% and 82%, while LPAR2 expression was substantially reduced by 48% and 93%.

As shown in Figure 2B, in MDA-MB-231 after 24h of treatment with concentrations of 3 and 5mg/ml of supernatant, 55% and 69% reduction in LPAR1 mRNA expression, and 53% and 86% reduction in LPAR2 expression were observed. After 48h treatment with concentrations of 1 and 3mg/ml of supernatant, 69% and 96% reduction in LPAR1 expression and 86% and 95% reduction in LPAR2 expression were observed. *P*-Value was less than 0.0001.

Effects of LBS on invasion of BC cells

As shown in Figure 3, treated cells exhibited a considerable decrease in invasion compared to control after 48h, hence three samples of significant concentrations were collected. In order to reach this reduction in MCF 7 and MDA-MBA-231, 5mg/ml and 3mg/ml LBS were applied, respectively. *P*-Value was less than 0.0001.

Discussion

Probiotic bacteria have recently received much atten-

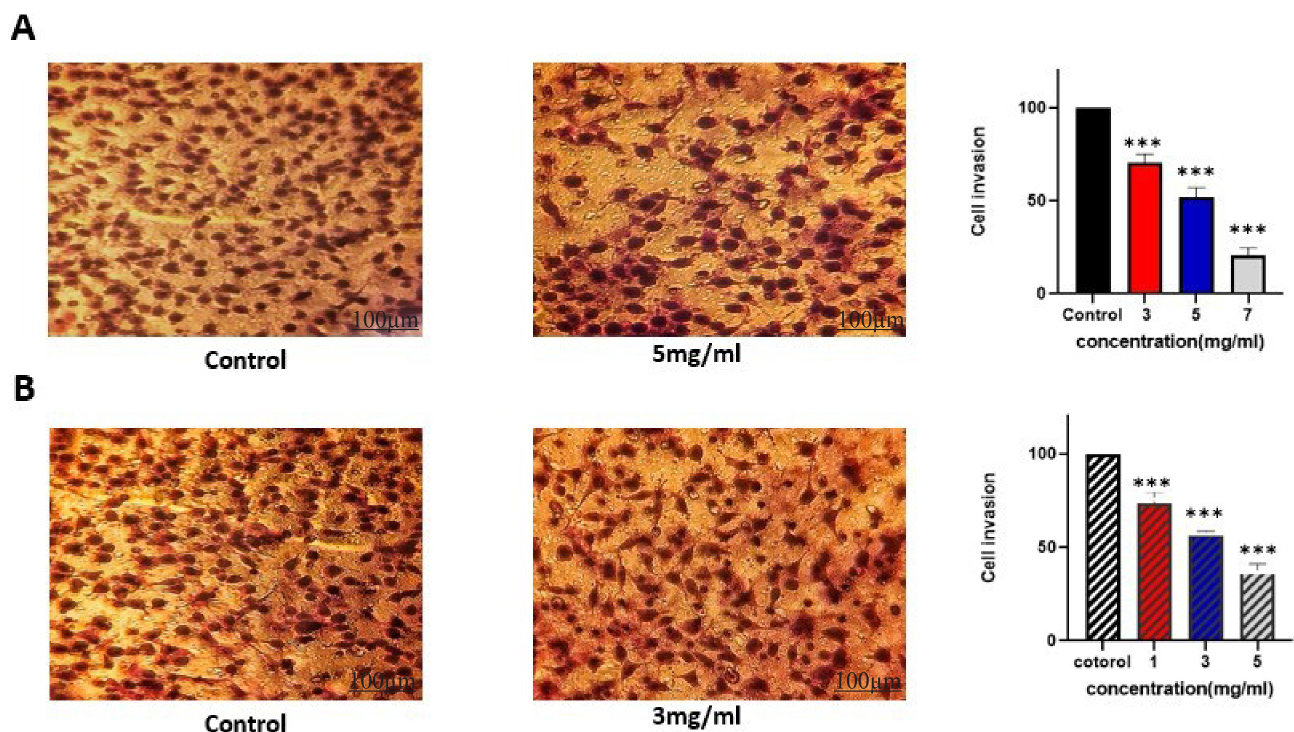

FIGURE 3. Lactobacillus brevis supernatant's effect on invasion of BC cell lines. As compared to a control, cell invasion of A) MCF-7 and B) MDA-MB-231 decreased significantly after 48h. (Original magnification × 40; scale bar = 100μm). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

tion due to their anti-cancer properties and emerging as future biotherapeutics with demonstrated efficacy and multifunctional roles for preventing and treating disease (Dimitrovski et al., 2014; Abedin-Do et al., 2015). Additionally, probiotics may act at various steps of signaling pathways, including (i) COX-2 inhibition, (ii) caspase-3 activation and polarization of mitochondrial membrane potential, (iii) autophagic cell death activation, (iv) inflammasome inactivation, (v) down-regulation of NF-B and mitogen-activated protein kinases signaling, and (vi) intestinal metabolites secreted (Daniluk, 2012; Song et al., 2015). As one of the probiotic bacteria, Lactobacillus has shown antitumor activity. Some epidemiological evidence indicates that fermented milk products by Lactobacillus casei CRL 431 effectively prevented tumor development in a murine BC model (Aragón et al., 2015). In a study published by Pan et al. (2009) the killed Lactobacillus bacterium inhibited breast and intestinal cancer cell survival. It has been found that dead bacteria and their supernatants have anti-cancer properties. Another study by Nasiri et al. (2021) showed that LBS could be a useful adjuvant treatment and prevention option for breast cancer. One limitation of LBS is that it takes a very long time, typically around a week, so the microbiological contamination in the supply chain cannot be managed in time. However, it could be incorporated as a factor in therapeutic procedures due to their affordability and high efficacy (Chen et al., 2019; Coulup and Georg, 2019; Shamsadin-Azad et al., 2019; Karimi-Maleh et al., 2020).

Based on the evidence mentioned above and supporting the benefits of probiotics in cancer treatment, we aimed to evaluate the LBS's effectiveness in reducing LPA expression in BC cell lines. LPA is a ubiquitous phospholipid that binds with certain cell surface receptors in a G-protein-dependent way, as well as significant component of serum found in eukaryotic tissues (Plastira et al., 2020). LPA promotes cell proliferation, migration and survival, all of which contribute to the initiation or development of malignant disease. The LPA receptor couples to multiple signaling pathways, including those triggered by the small GTPases RAS, RHO and RAC. RAS controls cell growth, while RHO/RAC signaling is involved in cell migration and invasion. There is growing evidence that an autotaxin (ATX/lysoPLD) produces LPA extracellularly from lysophosphatidylcholine. LPA receptors and ATX/lysoPLD are both overexpressed in a variety of malignancies, and suppress tumor metastasis with inhibitory drugs that inhibit LPA receptors and/or ATX/lysoPLD, might be useful (Choi et al., 2010;

Geraldo et al., 2021). Probiotics can act as an inhibitory agent for LPA and its receptor.

Thus, this study examined the effectiveness of probiotics in suppressing LPA gene expression in MDA-MB-231 and MCF-7 BC cells. According to the findings, the antitumor activity of LBS on these cells has been proved for the first time. Besides, the effect of the supernatant on LPA expression, cell viability and invasion rate was investigated, and Lactobacillus supernatant could reduce the proliferation and invasion of MCF-7 and MDA-MB-231 cancerous cells after 24 and 48h of treatment. Despite the lack of clarity regarding how probiotics work, their general contribution to health relies on boosting the immune system, inducing apoptosis and suppressing carcinogenic metabolites produced by pathogens. We also presented our findings from analyzing the expression of LPAR1 and LPAR2 after treatment with LBS. After 24h of treatment with 7mg/ml of supernatant, LPAR1 and LPAR2 expression in MCF-7 cells significantly decreased to 56% and 74%, respectively. Furthermore, LPAR1 and LPAR2 expression have been shown to be significantly reduced to 55% and 53% in MDA-MB-231 cells after 24h of treatment with 3mg/ml of supernatant. As a result of our Real time-PCR, we can conclude reducing LPA expression is both time and concentration-dependent, and our treatment is effective at reducing LPA expression. On the other hand, numerous studies demonstrate probiotics' ability to reduce invasion; for example, two probiotics (L. acidophilus and L. casei) supernatants and cell extracts have reduced cell invasion potential (Motevaseli et al., 2017). Previously, in a study conducted by Escamilla et al. (2012) the Lactobacillus rhamnosus GG combination significantly reduced the expression of MMP-9 and ZO-1, which are the most significant proteins in invasion. This is consistent with our findings, which invasion assay shows that the treatment of MCF-7 and MDA-MB-231 with supernatant decreased invasion after 48h compared to the control.

Besides that, probiotics have different effects depending on the species, so their effects cannot be generalized (Mogna and Mogna, 2012). Consequently, it is necessary to examine the effects of different species on different cell lines. Although our findings suggest that probiotics may be useful in the treatment of breast cancer due to their anti-cancer properties, adding probiotics to cancer treatment and prevention may be possible through further *in vivo* studies.

Conclusion

According to the reported results, the LBS could significantly reduce the proliferation and cell growth in MCF-7 and MDA-MB-231cell lines after 24 and 48h of treatment. Real-time PCR results demonstrated LBS's efficacy in decreasing LPAR1 and LPAR2 gene expression as well as decreasing the invasion of cancerous cells in MCF-7 and MDA-MB-231 cell lines. So this bacteria could be thought of as a promising application for a possible treatment approach with fewer adverse reactions. However, more research is obviously needed. In the future, probiotics could be used in conjunction with currently available therapies.

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Conflict of interest

The authors declare no conflict of interest.

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