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## Cellular Cytotoxicity and Epigenetic Alteration in RP1 and RASSF1A Genes as Response for Anticancer Capabilities of Some Probiotic Bacteria in Breast Cancer

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### Abstract

This study aimed to assess the anti-proliferative capabilities of the three probiotic strains on breast cancer (MCF7) and test their anticancer capabilities on RP1 and RASSF1A Genes. Three probiotics bacterial strains: *Lactobacillus casei* ss. *casei* (LC 1093), *Lactobacillus delbreuckii* ss. *bulgaricus* LD (1102) and *Bifidobacterium bifidum* (BB 1334) were tested for their anti-proliferative capabilities on cell lines via trypan blue test and MTT assay. Their anti-methylation activities were tested using methylation-specific PCR (MSP). Results revealed that *Lactobacillus casei* strain achieved the highest percentage of cancer cell death. The effects of these strains on the methylation status of RASSF1A and RB1 promotor regions in breast cancer cells were tested. The unmethylation-specific primers of both genes were able to generate a defined band. The methylation patterns were reshaped when compared to the untreated MCF7 cell line showing the epigenetic delaying mechanism of the probiotic cell free filtrate by interfering the methylation mechanism of breast cancer on two tested genes.

**Key word:** probiotics, breast cancer, MTT, RB1, RASSF1 genes

### Introduction

Cancer is a group of disorders characterized by abnormal cell proliferation that can infiltrate or spread to other regions of the mammalian body [1]. It is one among the world's top causes of death [2].

Breast cancer (BC) is the most frequent malignancy among women globally, with risk factors including hereditary genetics, hormone replacement therapy,

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lifestyle, and eating habits [3-5]. Cancer risk is influenced by hereditary factors as well as the body's immune system. The development of cancer can be explained by a number of molecular processes. The development of this disease is linked to epigenetic changes such as DNA methylation and histone and non-coding RNA modification [6,7]. DNA methylation is a technique that modulates gene expression levels without changing the sequence of tumor suppressor genes' promoter regions [8]. As a result, gene expression is completely suppressed. The influence of methylation of CpG islands in a tumor suppressor gene in cancer was initially discovered in the instance of retinoblastoma (RB1), which is hyper methylated in 10% to 20% of examined cases. RB1 is a nucleoprotein that plays a main role in cell cycle regulation.

RAS-association domain family (RASSF), a family of ten members (RASSF1-10) involved in cell cycle regulation, apoptosis, and microtubule stability, was another tumor suppressor shown to be linked to cancer. RASSFs expression has been demonstrated to be lost in a number of human malignancies, and this loss has been linked to methylation of the gene's CpG-island promoter region [9].

Because of their failure to target cancer cells and toxicity owing to non-specific effects on normal cells, all traditional cancer therapies, including various operations, hormone therapies, immune therapies, and so on, demonstrate a lack of efficacy in terms of long-term prognosis [10]. Radiation therapy (also known as X-ray therapy or irradiation) is a type of treatment that can be used to kill cancer cells and decrease tumors. It can be given superficially through external beam radiotherapy (EBRT) or internally through brachytherapy. Although the effects of radiation treatment are localized and limited to the area being treated, it frequently causes substantial side effects, particularly in children aged 0–14, and has an impact on the young patients' lifestyle [11, 12].

Chemotherapeutic medicines, like any other drug, have side effects. These chemotherapeutic medicines' long-term safety and stability attack cancer cells while also harming healthy cells and causing treatment resistance. Furthermore, these cytotoxic medications are linked to life-threatening adverse effects, which are frequently worse than the cancer's malignancy [13, 14]. As a result, scientists are researching cancer clinical care with minimum side effects and high efficacy. With

high consumption of fruits and vegetables, a good diet may be one of the most important measures for reducing cancer risk [15].

Incorporating microorganisms, also known as probiotics, into people's daily diet has proven to be an effective method for boosting their immune systems and lowering their risk of cancer during the last two decades. These probiotics (health-promoting bacteria) have been progressively added in a variable foods, pharmaceuticals, and other items, particularly fermented milk products, since they are thought to provide effective protection against pathogenic microorganisms and cancers in humans [10].

The microbial composition of the human body (microbiota) has gotten a lot of interest in recent decades in a various fields, including cancer biology. The human host and bacteria have a dynamic and complex connection. Bacteria and their metabolites have the ability to influence several signaling pathways, produce DNA double-strand breaks, and induce apoptosis [3].

Lactic Acid Bacteria (LAB) has been a source of endless health advantages to the human population and an efficient chemo preventive food element against various cancer types as a prospective member of the probiotic family. Though the major goal of LAB formulations is to balance the microbial population of the intestine, consumption of LAB with food or as supplements was proven to reduce blood cholesterol levels and suppress harmful bacteria in the intestine [16]. *L. acidophilus* is a member of the intestinal microflora and is one of the most commonly used non-pathogenic strains for a variety of health advantages [17].

Until recently, probiotic bacteria were thought to prevent cancer by attaching to monoamines generated during meat processing and inactivating carcinogen-producing enzymes (e.g. -glucuronidase). Another study looked at the influence of *Lactobacillus* free cell supernatants on the growth of a human colon cancer cell line and found that the higher the supernatant concentration, the worse the cell viability was [16].

As a result, the current research focused on determining the anti-proliferative effects of various probiotic bacteria against breast cancer, as well as their hypermethylation inhibitory activities of the RB1 and RASSF1 genes.

## Materials and methods

### 1. Probiotic strains

Four probiotic bacterial strains were used in the study; *Lactobacillus casei* ss. *casei* (LC 1093), (isolated from cheese), *Lactobacillus delbreuckii* ss. *bulgaricus* (Isolated from yoghurt), LD 1102) and *Bifidobacterium bifidum* (isolated from the intestine of adult) (BB 1334) which is isolated from human. These four bacterial strains were purchased from Cairo MIRCEN, Faculty of agriculture, Ain shams university, Cairo, Egypt.

#### 1.1 Culture Supernatants Preparation

Probiotic strains were cultured in 250 ml of the Erlenmeyer flask containing 100 ml of de Man Rogosa Sharpe (MRS) broth media (pH = 6.5, Oxoid, Germany) with a single colony of tested isolate. The inoculated flasks were incubated at 37°C for 48 h in CO<sub>2</sub> incubator. The turbidity of each suspensions was adjusted to match 0.1ml of culture were aseptically withdrawn to measure optical density (O.D.) at 600 nm and the cell count was determined using plate count technique (1ml contained  $3 \times 10^5$  CFU/ml for [18].

All probiotic cultures were maintained in MRS medium (Oxoid) and stored at 4°C. For long-term preservation, the cultures were stored in vial tubes containing 1ml aliquots plus 20% glycerol at -20°C [19]

To obtain cell-free extract (supernatant), bacterial cultures were centrifuged at 5000 RPM for 15 minutes at 4°C and filtered through a 0.45 and 0.2 µm membrane filter (Finetech, Taiwan) to exclude the culture debris [11].

### 2. Cell culture

#### 2.1 Cancer cell lines

Human breast cancer (MCF7) cell line was purchased from Holding Company for Biological Products & Vaccines (VASCERA), S.A.E., Cairo, Egypt

Culturing of cancer cell line was carried out according to the protocol of Invitrogen, USA. Chilled vials of MCF-7 cell line was placed in water bath at 37 °C till the entire chilled block was thawed. The pre-warmed culture media were prepared from PRPMI 1640 (Oxoid) supplement with 10% fetal bovine serum (FBS) [20].

Few drops of pre-warmed media were added in the vial, aspirated gently and then transferred to T-75 cell culture flask preloaded with 50ml complete culture media. The flask was incubated with 5% CO<sub>2</sub> at 37°C for 12 hours. The suspended cells were centrifuged for 10 minutes at 3000. Supernatants were discarded and the pellets were washed with PBS (phosphate buffered saline) (pH 7.2). The pellets were then re-suspended again in a new flask containing fresh media for 24 hours [20].

### 3. MTT Assay

Cytotoxicity of probiotic cell free culture filtrate was measured using MTT assay [21] by adding serial dilutions of culture filtrate 50%, 25%, 12.5%, and 6% in the growth medium. Untreated cells were used as control. The plates were incubated for 24 hours at 37°C for 24 hours in 5% CO<sub>2</sub> [22]. After incubation, 100µl of cells cultured and 10 µl of MTT solution to each well. The cells were then incubated for 3 hours in CO<sub>2</sub> incubator, 50 µl of the media was then aspirated and 150 µl Dimethyl Sulfoxide (DMSO) was added to each well. Plates were transferred to a shaker water bath at 37°C and 250 RPM for 30 minutes to dilute the crystals from formazan. Specimens were read by ELISA reader (biotek elx-800 -USA) at 545 nm and reference filter 630nm [23].

The cytotoxic effect of probiotics was expressed as the relative viability (% control). To calculate the percentages of cell viability, the following equation was applied:

$$\text{Relative viability} = \frac{\text{Sample absorbance} - \text{blank absorbance}}{\text{Control absorbance} - \text{blank absorbance}} \times 100 \quad (1)$$

## 4. Epigenetic study

### 4.1 DNA Extraction

Genomic DNA of human breast cancer MCF7 treated with the supernatant of probiotics bacteria, was extracted using the Quick-DNA Plus (Zymo research, USA) following the instructions of the manufacturer [24].

### 4.2 Genomic DNA Bisulfite Treatment and Methylation-Specific PCR (MSP)

The genomic DNA (gDNA) was treated with sodium bisulfite which changes unmethylated cytosines to uracil, while methylated cytosines remain unchanged. Bisulfite-induced modification of gDNA was performed using EZ DNA

Methylation<sup>TM</sup> kit (Zymo research, USA) according to the manufacturer's instruction manual [25].

The status of promoter methylation of the tumor suppressor genes; RASSF1A and RB1 genes, was determined by chemical cure with sodium bisulfite followed by MSP analysis. The bisulfite-treated DNA was diluted 1:4 with nuclease-free water. Primer sequences of RASSF1 and RB genes represented in

. All primer pairs were dissolved in TE (Promega, USA) to stock concentration (100  $\mu$ M), then the work concentration (10  $\mu$ M) of each forward and reverse primer were prepared in nuclease free water (Promega, USA). The amplicons were confirmed with the expected fragment size [24].

The methylation-specific PCR (MSP) reactions were achieved in PCR tube (Axgen, USA) using Promega GoTaq green master mix. MSP was performed in a 25  $\mu$ l total volume containing: 12.5  $\mu$ l 2x PCR master mix (Promega, USA), 1  $\mu$ l 10  $\mu$ mol methylated/unmethylated forward primer, 1  $\mu$ l 10  $\mu$ mol methylated/unmethylated reverse primer, 2  $\mu$ l 1 bisulfite converted DNA, and 8.5  $\mu$ l nuclease-free water. The thermal cycling conditions used for the two genes were proceeded with 5 min at 95  $^{\circ}$ C as the primary denaturation, followed by 39 cycles of each 95  $^{\circ}$ C for 30 seconds, 58  $^{\circ}$ C (for RASSF1 primers)/ 63  $^{\circ}$ C (for RB1 primers) for 30 seconds, and 72  $^{\circ}$ C for 45 seconds, and a final extension at 72  $^{\circ}$ C for 5 min. Finally, the PCR amplicons were electrophoresed on a 2% agarose gel stained with Ethidium bromide dye.

**Table 1: List of primers used in MSP**

Gene	Primer	Primer sequence (5' to 3')	bp
	Un methylated Forward "UF"	AGGTTAATTTTGTGTGGGG	18 0
	Un methylated Reverse "UR"	AAAACAAAAACAAATACCAAC ATCC	
<b>RASSF1</b>			
	Methylated Forward "MF"	GAGGTTAATTTTGCGCGG	16 0
	Methylated Reverse "MR"	AACGAAAACGAATACCAACG	
	Un methylated Forward "UF"	GGGAGTTTTGTGGATGTGAT	16 3
	Un methylated Reverse "UR"	ACATCAAAACACACCCCA	
<b>RB</b>			
	Methylated Forward "MF"	GGGAGTTTCGCGGACGTGAC	16 3
	Methylated Reverse "MR"	ACGTCGAAACACGCCCCG	

## 5. Statistical analysis

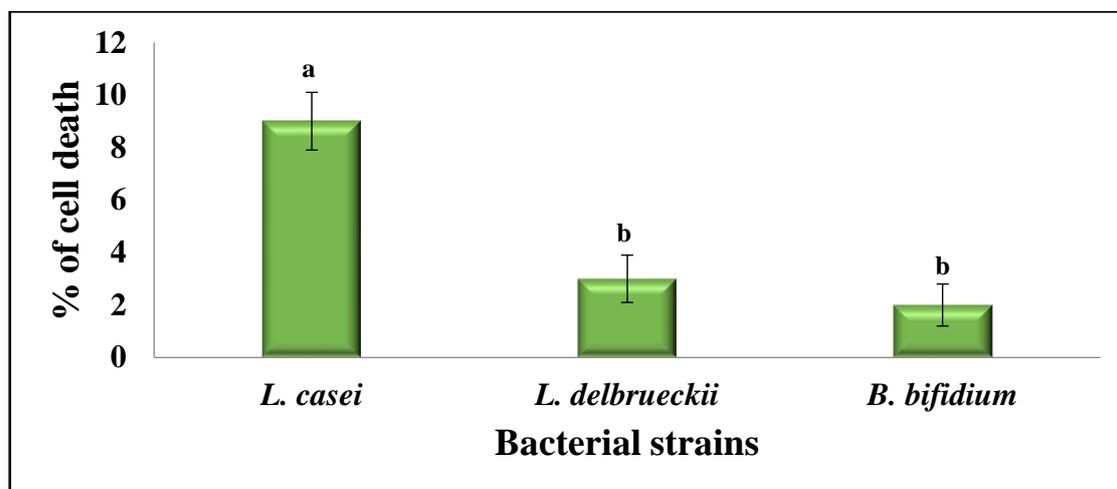
Results of all experiments were statistically analyzed by one-way Analysis of variance (ANOVA) for multiple comparisons among groups using Costat computer program V6.303 (2004). Significant differences ( $P < 0.05$ ) between the means were determined by the least significant Difference (LSD) test [26].

## Results and discussion

Three strains of probiotic bacteria (*Lactobacillus casei* ss. *casei* (LC 1093), *Lactobacillus delbreuckii* ss. *bulgaricus* LD (1102) and *Bifidobacterium bifidium* (BB 1334)) were tested and compared for their potentiality in anti-proliferation on Breast cancer cell line. Strains were grown in MRS medium, incubated at CO<sub>2</sub> incubator and after cultivation, cultures were centrifuged at 5000 RPM for 15 minutes at 4°C then the supernatant was used to treat the cancer cell lines. Cell viability was examined by MTT assay.

### 1. MTT anti-proliferative assay

This experiment was completed using MTT assay to determine the potential killing effect of probiotic strains upon cancer cell lines, as according to [27], whatever the limitations of MTT; it is the first widely accepted method of measuring cell viability. For breast cancer, results indicated that percentage of dead cells produced by the treatment with supernatants of *B. bifidium*, *L. delbrueckii* and *L. casei* were 2, 3 and 9%, respectively (Figure 1).



**Figure 1: Percent of breast cancer dead cells by MTT assay as a function of treatment with probiotic strains supernatants.**

In the current study, *L. casei* demonstrated a higher significant cytotoxic activity than the other probiotic bacteria. In former studies *Lactobacillus casei* spp. was found to have anti-proliferative activity against a breast cancer cell line (MCF7), as well as if it used as cell free suspension. The secretion of soluble compounds during growth was thought to be responsible for these valuable effects [28]. Another *in vitro* activity of *L. casei* was observed in a study performed by [29], at which *L. casei* was mixed

with *Lactobacillus acidophilus*, and *Lactococcus lactis* in Kefir grains water 50 mg/mL and examined against breast cancer cell line (4T1) using MTT assay. They found that The LC50 values after 48 h and 72 h were 12.5 and 8.33 mg/mL, respectively.

The present study included two other probiotic bacteria *B. bifidum* and *L. delbrueckii* that showed significant lower activities against MCF7 cell line than *L. casei*. Cell free suspension of *L. delbrueckii* was found to have good activity against breast adenocarcinoma cell (caused 13.59% cell mortality on using 100 µg HKC/mL on the MDA-MB-231 cell) [30].

Probiotic metabolites are known to contain Lipopolysaccharide, exopolysaccharide (EPS), and polysaccharide molecules, which can suppress tumor formation through many ways. The probiotic *L. delbrueckii* fermentation (LBF) solution suppresses the proliferation of human colon cancer SW620 cells by inducing apoptosis [31]. Cell wall components of *Lactobacillus acidophilus* and *Lactobacillus casei* also have anticancer properties. The EPS of *L. acidophilus* 20,079 exerts a direct cytotoxic effect on malignant cells via apoptotic pathways [13].

In vivo and in vitro, researchers observed a significant effect of several probiotic bacteria against various malignant cells. By passing through numerous critical molecular pathways, metabolic components of probiotic bacteria and yeasts promoted various forms of cell death. EPSs produced by probiotics *Kluyveromyces marxianus* (*K. marxianus*) and *Pichia kudriavzevii* (*P. kudriavzevii*) inhibit numerous colon cancer cell lines, making them viable therapeutic weapons against some cancers like colorectal cancer (CRC) [32].

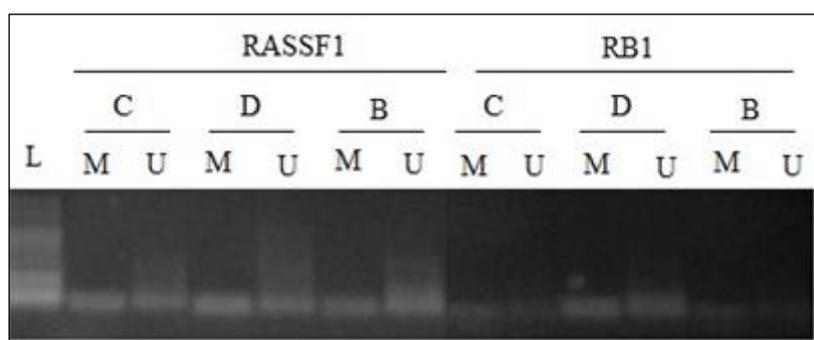
*Bifidobacterium breve* lw01 EPS was also discovered to increase immune response while retaining anticancer and anti-inflammation properties. Rhamnose, galactose, glucose, arabinose, and mannose are all established by EPS. By regulating apoptosis and cell cycle arrest, EPS had anticancer properties against a head and neck squamous cell carcinoma cell line. The EPS of *B. breve* lw01 was discovered to be used in genetic and metabolic engineering assessments, as well as in the application of functional food and medicinal sectors [33].

According to Zhang *et al.*, *Lactobacillus acidophilus* and *Lactobacillus casei* produce substances that suppress the proliferation of the MCF7 breast cancer cell line.

Similarly, through generating soluble polysaccharides, *L. acidophilus* 606 inhibits the proliferation of human pancreatic carcinoma cell lines. *Lactobacillus*, both live and heat-inactivated, had anticancer properties through its extracellular metabolites and cytoplasmic fractions. In vitro, *Lactobacillus sp.* derived compounds from yoghurt fermentation limit the development of human tongue squamous carcinoma cells and induce apoptosis [13, 34].

## 2. Detection of DNA Methylation via MSP-PCR

Human breast cancer cell line (MCF7) after probiotics supernatant treatments were subjected to MSP-PCR to investigate promoter methylation status of RASSF-1 and RB1 genes in these cells. Each treated cell line sample experienced MSP-PCR test using both unmethylated (U) and methylated (M) primers (**Error! Reference source not found.**).



**Figure 2:** Effect of probiotics, *Lactobacillus casei* (C), *Lactobacillus delbrueckii* (D) and *Bifidobacterium bifidum* (B) on promoter methylation status of RASSF-1 and RB1 genes in human breast cancer cell line. The MSP-PCR products were migrated with 100 bp DNA ladder.

Our results indicated that, probiotic secretion of *L. casei*, *L. delbrueckii* and *B. bifidum*, had significant effect on the methylation status of RASSF1A and RB1promotor region in breast cancer cells (Figure2).

It has been found that gene hypermethylation is a common and early alteration in many tumor types, including breast [35], hence it is considered as a hopeful target for diagnosis and cancer treatment.

As mentioned before, Lactic acid bacteria, including *Bifidobacteria*, were revealed to exert chemo preventative effects on breast cancer. Secreted peptides from *B. bifidum* and *L. acidophilus*, have cytotoxicity effect on MCF-7 cell [36]. The

results suggested that a relationship exists between the ability of probiotic secretions to induce apoptosis and decreasing the hypermethylation level in breast cancer cells.

Our finding revealed that, there was a difference in optical density of MT-PCR bands of RASSF1A and RB1 methylation status with the probiotic extract treatment. Meanwhile, different strains of probiotic bacteria had different effects on the viability of breast cancer cells. Same finding was observed by [13].

The probiotic secretion in current research had lower effect on the methylation status of RB promotor region in breast cancer cells in *vitro*. Since the loss or inactivation of the RB gene is infrequent in mammary carcinomas, and the reduced RB expression in these cells is probably due to a cellular regulatory mechanisms. [21]

RAS genes were the first altered genes found in human cancer, and these mutations were able to initiate carcinogenesis, according to previous studies. Moreover, despite the ability of oncogenic RAS to alter mammary cancer cell lines in *vitro*, the prevalence of RAS mutations in clinics does not indicate a key function of RAS proteins in breast tumor genesis [37]. This corresponded to what we discovered.

Hence the main action of probiotic of the used probiotic strains in the present study may be due to its action against the down regulation of RASSF1 gene.

In a study performed by [38], they studied the RASSF1A gene hypermethylation in tissue and serum together in patients with breast cancer. They observed that loss of RASSF1A protein expression was associated with clinic pathological features of bad prognosis in breast cancer patients.

## Conclusion

In the light of our findings that showed the good abilities of the three used probiotic strains in cell cytotoxicity and hypermethylation prevention in case of RB1 and RASSF1 genes, they achieved a promising strategy in Brest cancer treatment using their metabolites.

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## الملخص العربي

السمية الخلوية والتغيير فوق الجيني في جينات **RP1** و **RASSF1A** كاستجابة للقدرات المضادة للسرطان لبعض بكتيريا البروبيوتيك في سرطان الثدي

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## الملخص

هدفت هذه الدراسة إلى تقييم القدرات المضادة للتكاثر لسلاسل البروبيوتيك الثلاثة على سرطان الثدي (MCF7) واختبار قدراتها المضادة للسرطان على جينات (RP1 و RASSF1A). تم اختبار القدرات المضادة للتكاثر لثلاث سلالات بكتيرية من البروبيوتيك؛ (*Lactobacillus casei ss. casei* (LC 1093) (LD) ، *Bifidobacterium bifidum* (BB 1334) و *Lactobacillus delbreuckii ss. bulgaricus* (1102)). أظهرت النتائج أن سلالة *Lactobacillus casei* حققت أعلى نسبة من موت الخلايا السرطانية. و ظهر تأثيرا مختلفا لهذه السلالات على الحالة المثيلة للمناطق المحفزة للجينات RASSF1A و RB1 في خلايا سرطان الثدي التي

تم اختبارها. حيث كانت البادئات الخاصة بالمثيل المضاد لكلا الجينين قادرة على توليد نطاق محدد و تم إ عادة تشكيل الأنماط المثيلة عند مقارنتها بخط خلية MCF7 غير المعالج مما يعكس قدرة المكونات الحيوية للملخص الخالي من الخلايا للبروبيوتك كمعطل لميكانيكية المثيلة لسرطان الثدي في الجينات تحت الدراسة.