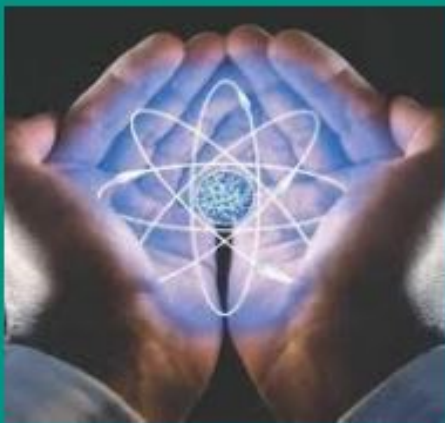


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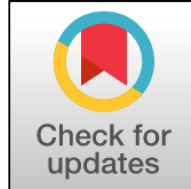
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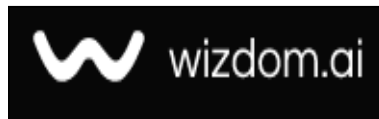
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Lactobacillus, Bifidobacterium and Streptococcus, Probiotic Bacteria Supplements, Induce DNA Repair of Local Breast Cancer Cell Line AMJ13

Lactobacillus, Bifidobacterium dan Streptococcus, Suplemen Bakteri Probiotik, Menginduksi Perbaikan DNA pada Garis Sel Kanker Payudara Lokal AMJ13

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Abstract

General Background: Breast cancer is the most prevalent cancer affecting women, with increasing incidence worldwide. **Specific Background:** Recent research has focused on the role of epigenetic changes in DNA damage, repair mechanisms, and the potential therapeutic effects of probiotics. Probiotics have shown promise in promoting tissue regeneration and DNA repair. **Knowledge Gap:** However, the precise impact of probiotics on DNA repair in cancer cells, specifically breast cancer cells, remains underexplored. **Aims:** This study aimed to evaluate the effects of probiotics on DNA damage repair in AMJ13 Iraqi breast cancer cells and assess the cytotoxic effects of probiotics on these cells. **Results:** Using the comet assay, we found significant increases in DNA damage repair in AMJ13 cells treated with *Lactobacillus plantarum* (T1) and a combination of eight probiotic strains (T2). Exposure to T1 for 48 hours resulted in significant increases in tail DNA ($P \leq 0.001$), head DNA ($P \leq 0.001$), and tail moment ($P < 0.001$), while T2 showed similar significant increases at 72 hours ($P < 0.05$). Image analysis further supported the DNA repair potential of probiotics, as indicated by a small tail curve for treated cells. **Novelty:** This study provides novel insights into the therapeutic potential of probiotics in breast cancer treatment by demonstrating their capacity to enhance DNA repair mechanisms in cancer cells. **Implications:** The findings suggest that probiotic therapy may be a promising adjunct treatment in breast cancer, offering a new avenue for cancer management through the enhancement of DNA repair and reduction of DNA damage.

Highlights:

Probiotics significantly repaired DNA damage in breast cancer cells.

T2 and T2 enhanced DNA repair within 48-72 hours.
Probiotics offer potential as breast cancer adjunct therapy.

Keywords: Breast cancer, probiotics, DNA repair, AMJ13 cells, cytotoxicity

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Introduction

Breast cancer affects female more than any other type of cancer, and its prevalence has significantly increased in recent years [1]. The Iraqi Cancer Registry indicates that breast cancer ranks first among malignancies affecting the Iraqi population and accounts for approximately one-third of cancers affecting women in the country (32%) [2]. Cell lines are a valuable tool for cancer research in general. Their main advantage is that they offer an endless supply of a broadly distributable, self-replicating cell population that is reasonably homogeneous, making comparison studies easier [3]. A novel cell line for breast cancer (AMJ13) has been generated from a patient with breast cancer in Iraq. It has been considered to be exceptional since it is the first for the Iraqi community and could prove beneficial for the study of breast cancer [4]. This cell line will be an invaluable tool for breast cancer research and may help create novel treatments for the condition [4].

Probiotics have recently been suggested as an alternate course of treatment for cancer prevention. Particularly, probiotic lactic bacteria have been considered to be significant microorganisms with anti-carcinogenic properties [5]. Probiotics have been shown to effectively support cancer case management, according to mounting research [6-8]. For instance, by lowering intestinal absorption of pro-carcinogenic aflatoxins, probiotics comprising *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* dramatically reduced risk of liver cancer [9]. Studies utilizing *Lactobacillus* spp. on several breast cancer cell lines and on xenograft models of breast cancer have been conducted, and they have demonstrated the anti-cancer benefits of probiotics [10]. Probiotic strains such as *Bifidobacterium animalis*, *Lactobacillus acidophilus*, *Bifidobacterium infantis*, *Lactobacillus paracasei*, and *Bifidum* have been shown to inhibit the growth of cancer cells in MCF7 cells, which makes them effective as natural cancer treatment agents. *Lactobacillus* and *Streptococcus* are the main bacteria in the breast microbiota of healthy individuals [11, 12]. They control the formation of malignancies by stimulating the activation of natural killer (NK) cells. Moreover, *Streptococcus thermophilus* produces antioxidants with anticancer properties by reducing DNA damage and reducing reactive oxygen species (ROS) [13, 14]. According to studies, probiotics, as functional nutrients, may be able to prevent breast cancer in animal and cell models. Probiotics have the ability to alter the immune system and gut microbiota, which may help prevent or treat breast cancer. However, more clinical trials and research are needed to verify the in vitro and in vivo results as well as to gain insight into the metabolic, immunological, and molecular pathways connected to probiotics in breast cancer [10]. The current study aims to evaluate the cytotoxic effects of *Lactobacillus planetarium* and a combination of eight probiotic strains on breast cancer cell lines under in vitro conditions. These strains include *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Lactobacillus plantarum*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium bifidum*. Additionally, measurements of the alterations in AMJ13 cells' DNA following exposure to probiotic bacteria were examined, which could provide support for the use of probiotic bacteria in breast cancer treatment. The comet assay was used to measure genetic alterations, while the cell viability assay was used to measure cytotoxicity.

Methods

Preparation of probiotic bacteria

Two probiotic supplements were selected at random from the pharmacy. One supplement contained one strain of *Lactobacillus planetarium* 299v, which was used as treatment 1 (T1). The other supplement contained eight different strains of probiotic bacteria, including *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Lactobacillus plantarum*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium bifidum*, and was used as treatment 2 (T2). 0.5 gm of powder of each groups was added into De Man-Rogosa-Sharp (MRS) broth (Oxoid Ltd., Basingstoke, Hampshire, UK), then the broth was incubated for 24 hrs in 37°C and 5% CO₂. Microorganisms were streaked on MRS agar media to verify their purity. Subsequently, broth media was inoculated with a single colony, which was then cultured under ideal conditions for 24 hrs.

The biological density of probiotics (colony forming factors, cfu/ml)

In basically, the ratio of tissue culture macrophages per milliliter determined the probiotic bacteria ratio used in this investigation which was 10:1, according to [15-17].

Cell culture

The study utilized the Iraqi human breast cancer cell line AMJ13 which was provided by the Iraqi Centre for Cancer and Medical Genetic Research (ICCMGR) at AL-Mustansiriyah University (Baghdad, Iraq). The cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco), supplemented with 10% FBS, and incubated at 37 °C in a 5% CO₂. Cells were passaged twice a week. Experiments were conducted within ten passages of cell recovery, and for the experimental methods, cells were seeded at a density of 2×10^5 cells per milliliter. The media was removed, the cells were passaged through using 1-3 ml of Trypsin-EDTA, and they were then incubated in the cell culture incubator for two to three minutes when the cells reached 80-90% confluence. Trypsin was inhibited by adding medium supplemented with 10% FBS. The pellet was re-suspended in culture medium and aliquoted 1:4 in

tissue culture flasks 75 cm². The cells were centrifuged for five minutes at 1300 rpm, after which the supernatant was discarded. The cell count was performed using a hemocytometer.

Viability assay and dose response

In order to determine the cytotoxic effect, AMJ13 cell line was seeded at 1×10^4 cells/well. the crystal violat assay (CV) of cell viability was carried out on 96-well plates for 24 h, 48 h and 72 h [18]. A confluent monolayer was established after 24 hours, and probiotic supplements T1 and T2 were added at doses of 1×10^5 cfu/ml. The medium was removed after 24 hours, 48 hours, and 72 hours, and each well was given 50 μ l of CV dye solution with a concentration of 2 mg/ml (Bio-World, USA). After leaving it sit for half an hour, 50 microliters of methanol were added to dissolve the remaining dye, dried overnight after being cleaned with tab water. Using a Flourometer (BMG LABTECH, Germany) at a wavelength of 492 nm, the optical density (OD) was measured.

Comet Assay

According to [19] Singh et al. (1988), the comet assay test has been conducted, with modifications according to the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), AL-Mustansiriyah University (Baghdad, Iraq) [20]. First, cells were suspended in phosphate buffer saline (PBS) with 0.75% low melting point agarose (USBiological, USA). The samples were cast onto microscope slides and then covered with 0.5% normal melting agarose (USBiological, USA). Subsequently, the cells were lysed for an hour at 4°C in a lysis solution containing 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10 mM Tris. After lysis, DNA was allowed to unwind for 40 minutes in an electrophoretic solution (pH>13, 300 mM NaOH, 1 mM EDTA). Electrophoresis was carried out for 30 minutes at 4°C with an electric field strength of 0.73 V/cm (30mA). After neutralizing the slides with nutriliation buffer (0.4 M Tris, pH 7.5), 100 μ l of ethidium bromides (2 ug/ml) (Sigma Chemicals, USA) were added for staining, and cover slips were placed on top. The slides were examined using a 200x magnification fluorescent microscope (Micros MCX 500, Austria). It was connected to a computer-based image processing system via a CCD camera (Infinity Capturer, Micros, Austria). With the use of CASP software (casp_1.2.3b1.exe), images were analyzed. The endogenous DNA damage was calculated using the mean comet tail DNA of an AMJ13 cell, and each sample comprised of fifty randomly selected photographs. The following dimensions were measured and recorded. The olive tail moment is the result of multiplying the length of the tail by the proportion of total DNA in the tail. The Tail Moment measurement takes into account both the comet tail length, which represents the smallest detectable size of moving DNA, and the intensity of DNA in the tail, which represents the quantity of relaxed or fragmented bits. The distance that DNA migrates from the nuclear core center is known as the tail length, and it is used to determine the degree of DNA damage.

Statistics

For the comet test and cytotoxicity experiment, samples were run in triplicate. Independent replicates were also conducted in triplicate for publication. Using the f-test, the significance between samples was determined, with $p < 0.05$ being deemed significant. The head% DNA tail length, tail% DNA, olive tail moment, and tail moment measures were displayed as mean $\pm$ SD.

Result and Discussion

Result

Cell viability tests showed a significant time-dependent anti-proliferative effect when AMJ13 cells were exposed to live probiotic bacteria T1 or T2. Based on the cell viability test, Figures 1, 2, and 3 show the cytotoxicity of the probiotic on the Iraqi breast cancer cell line. Iraqi breast cancer cells did not significantly respond to any of the two treatments (T1 or T2) when they were exposed to 24 hours $p < 0.5$ for both treatments. While, cytotoxic effect increased in exposure time 48 hours $p < 0.05$ for T2. However, as shown in Figure 3, exposure AMJ13 cells to 72 hours induced cell death $p < 0.05$ for both T1 and T2.

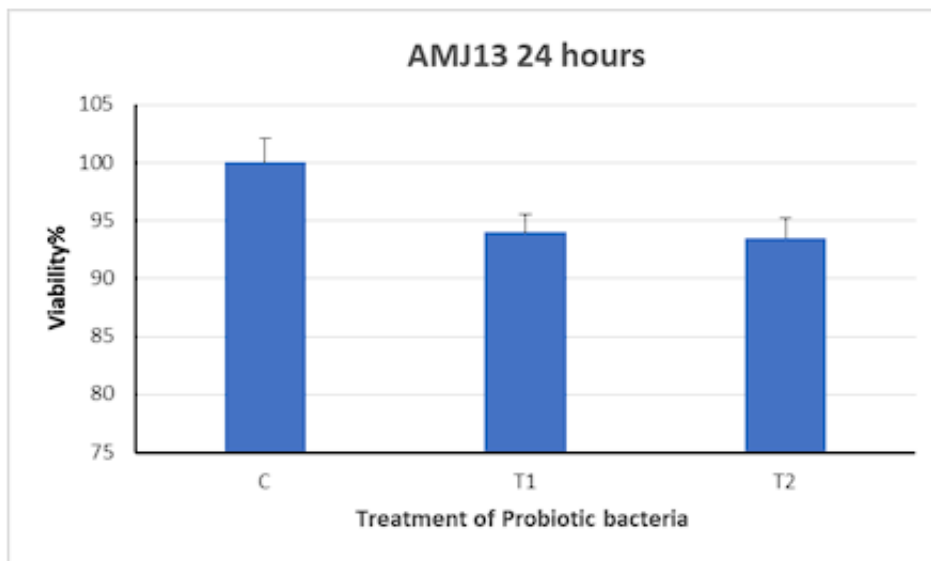


Figure 1. Cytotoxic activity of (T1) and (T2) on AMJ13 cell line which had incubated for 24 h.

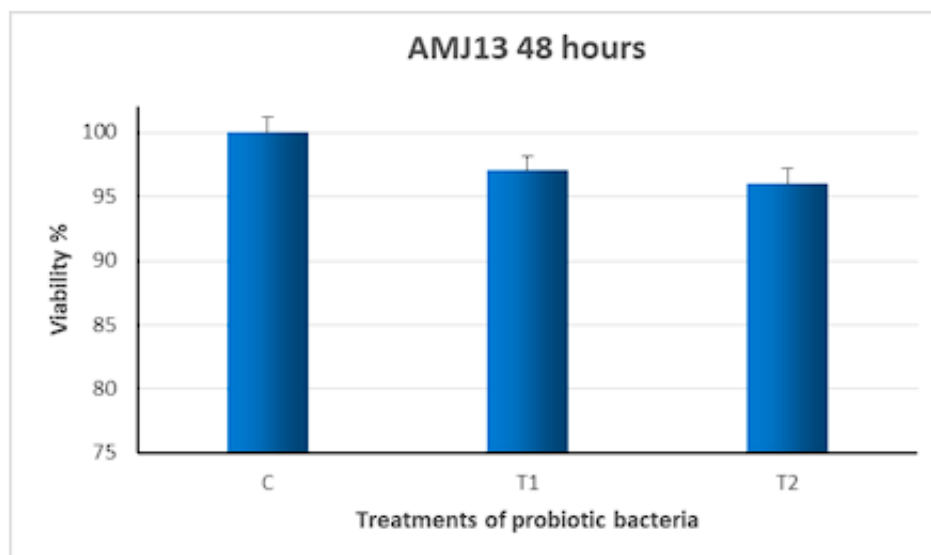


Figure 2. Cytotoxic activity of (T1) and (T2) on AMJ13 cell line which had incubated for 48 h.

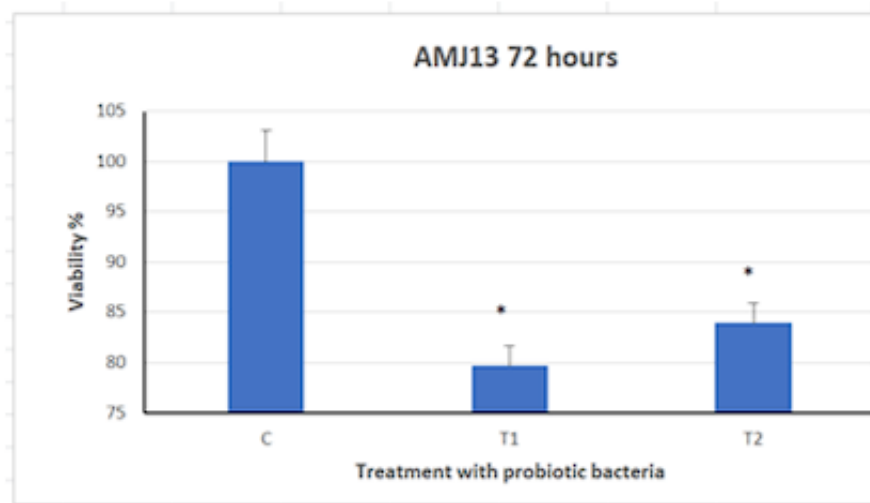


Figure 3. Cytotoxic activity of (T1) and (T2) on AMJ13 cell line which had incubated for 72 hours. * referred to significant cytotoxic effect of probiotic on AMJ13 cells.

The AMJ13 cells in this study were exposed to 105 cfu/ml of live probiotic bacterial strains, either *Lactobacillus planetarium* 299v (T1) or a mixture of strains, including *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Lactobacillus plantarum*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium bifidum* (T2). The DNA changes that occurred in the AMJ13 cells were examined using the comet assay. With the use of CASP software (casp_1.2.3b1.exe), images were examined. In a comet experiment, the amount of damage can be measured by calculating out the percentage of DNA in the tail.

As shown in Table 1, there were no significant differences found in L head, L comet between control and 48 hours treated cells by T1. While AMJ13 cells exposed to T1 for 48 hours showed a significant increase in L Tail ($P \leq 0.005$), Head DNA ($P \leq 0.001$), Tail DNA ($P \leq 0.001$), TM ($P < 0.001$) and OTM ($P < 0.001$) compare with control. Also, exposure of AMJ13 cells to T1 for 72 hours demonstrated significant increase in L Head ($P < 0.05$), L Tail ($P < 0.05$), Head DNA ($P < 0.0001$), Tail DNA ($P < 0.0001$), and TM ($P < 0.001$) compare with control. Additionally, the analysis of the curve of images showed that T1 may play a role in the treatment of breast cancer, as showing green small tail and red large head in treated cells compare with control Figure 4.

Groups Parameters	Control	T1 48 h	P value	T1 72 h	P value
LHead Mean $\pm$ SD	151.3 $\pm$ 55.8	154 $\pm$ 6.1	NS	184.2 $\pm$ 31.8	$P < 0.05^*$
LTail Mean $\pm$ SD	47.6 $\pm$ 60.2	7.7 $\pm$ 4.9	$p < 0.05^*$	15.1 $\pm$ 14.2	$P < 0.05^*$
L comet Mean $\pm$ SD	198.9 $\pm$ 60.2	161.7 $\pm$ 5.00	NS	199.3 $\pm$ 35.1	$P < 0.5$
Head DNA Mean $\pm$ SD	79.00 $\pm$ 22.3	97.9 $\pm$ 2.2	$P < 0.001^{**}$	98.5 $\pm$ 2.6	$P < 0.0001^{***}$
Tail DNA Mean $\pm$ SD	219.9 $\pm$ 11.1	2.03 $\pm$ 2.1	$P < 0.001^{**}$	1.5 $\pm$ 2.6	$P < 0.0001^{***}$
TM Mean $\pm$ SD	10.7 $\pm$ 7.6	11.2 $\pm$ 1.6	$P < 0.001^*$	0.7 $\pm$ 2.7	$P < 0.001^{**}$
OTM Mean $\pm$ SD	11.02 $\pm$ 7.1	10.5 $\pm$ 1.7	$P < 0.001^{**}$	1.3 $\pm$ 2.5	$P < 0.5$

Table 1. Comparison of T1 effect on AMJ13 cells depending on exposure time. * a significant, **more significant, ***most significant.

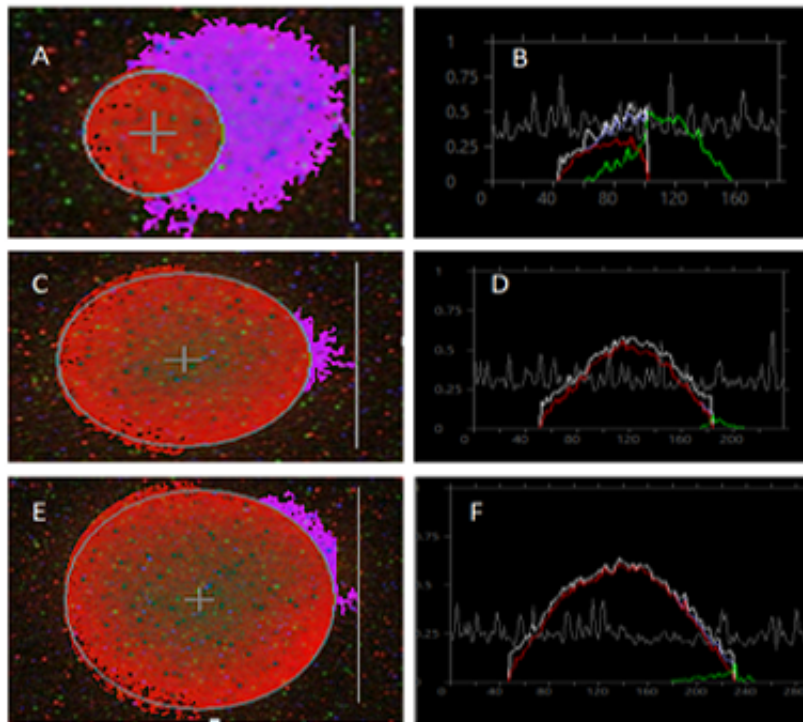


Figure 4. The image generated by the analysis software of the T1 treatment provides an illustration of the head-tail connection of the DNA distribution in the comet. The CASP program's image analysis outputs A, C, and E showed the AMJ13 control, AMJ13 cells treated with T1 48 hours of exposure, and AMJ13 cells treated with T1 72 hours of exposure, respectively. The picture analysis displayed curves are B, D, and F. When comparing D and F to control B, the green small tail and red large head are seen.

The results in Table 2 showed that the L comet, Head DNA, Tail DNA, TM and OTM did not significantly alter between control and 48 hours treated cells by T2. In contrast to the control, AMJ13 cells treated with T2 for 72 hours demonstrated a significant increase ($P < 0.05$) for all measurement parameters. Furthermore, the analysis of the image curve indicated that T2 might be involved in the treatment of breast cancer, as demonstrated by Figure 5, which shows a green small tail and a red large head in contrast to the control, which exhibited a green tail equivalent to a red head.

Groups	Control	T2 48 h	P value	T2 72 h	P value
Parameters					
LHead Mean $\pm$ SD	150.4 $\pm$ 56.1	86.2 $\pm$ 23.8	$P < 0.05^*$	144.2 $\pm$ 39.01	$P < 0.05^*$
LTail Mean $\pm$ SD	46.4 $\pm$ 59.5	14.6 $\pm$ 16.05	$P < 0.05^*$	12.9 $\pm$ 17.5	$P < 0.05^*$
L comet Mean $\pm$ SD	199.0 $\pm$ 59.5	100.8 $\pm$ 34.04	$P < 0.5$	157.1 $\pm$ 50.8	$P < 0.05^*$
Head DNA Mean $\pm$ SD	80.00 $\pm$ 21.7	87.4 $\pm$ 15.8	$P < 0.5$	95.3 $\pm$ 13.05	$P < 0.05^*$
Tail DNA Mean $\pm$ SD	220.1 $\pm$ 10.8	12.6 $\pm$ 15.8	$P < 0.5$	5.3 $\pm$ 12.9	$P < 0.05^*$
TM Mean $\pm$ SD	11.2 $\pm$ 8.1	4.5 $\pm$ 6.01	$P < 0.5$	2.3 $\pm$ 5.8	$P < 0.05^*$
OTM Mean $\pm$ SD	10.05 $\pm$ 7.5	3.5 $\pm$ 5.4	$P < 0.5$	2.6 $\pm$ 4.7	$P < 0.05^*$

Table 2. Comparison of T2 effect on Iraqi breast cancer cell line AMJ13 cells depending on exposure time. * a significant

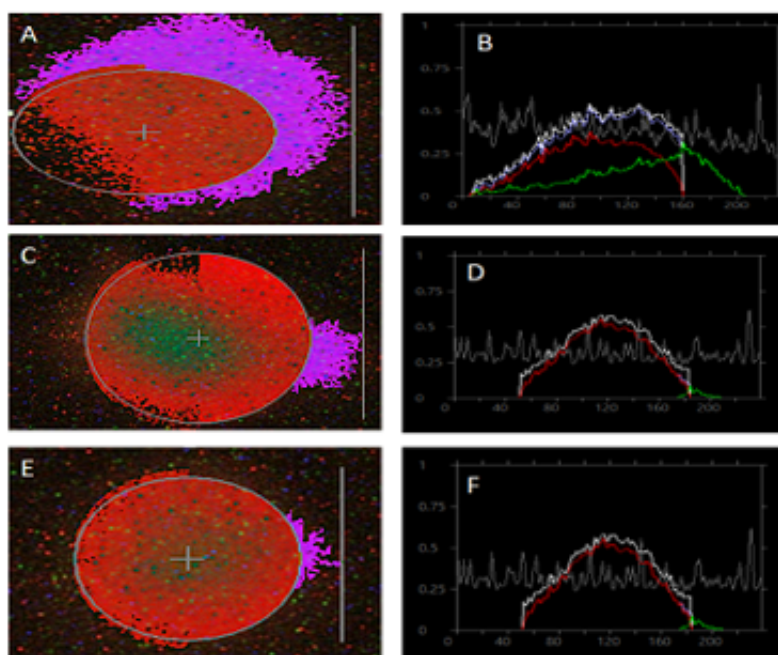


Figure 5. A demonstration of the head-tail relationship of the DNA distribution in the comet is provided by the T2 treatment's image analysis software. The image analysis outputs A, C, and E of the CASP program displayed control AMJ13 cells, AMJ13 cells exposed for 48 hours, and AMJ13 cells exposed for 72 hours, respectively. In B, D, and F, the analysis image curve is shown. The red big head and green small tail are seen when D and F are compared to control B.

Discussion

Breast cancer is still a significant worldwide health concern. Its increasing occurrence is linked to a number of unfavorable patient outcomes, including delayed diagnosis, the complexity of its subtypes, and rising medication resistance. Epigenetic abnormalities play a major role in the development of treatment resistance and the establishment of cancer stem cell characteristics in breast cancer. These include changes to the expression of non-coding RNAs, histone modifications, and DNA methylation [21]. A wide range of complex and diverse subgroups, each with an individually distinct collection of biological traits and clinical pathways, define the disease. The incidence rate will increase to about 3 million new cases and 1 million deaths year by 2040, which has greatly increased the need for novel and efficient prognostic, treatment, and detection methods [22]. The AMJ13 cell line, which was obtained from an Iraqi patient who had poorly differentiated infiltrative ductal carcinoma identified histologically, is the first breast cancer cell line from the Arabian and Middle Eastern regions [4]. The AMJ13 cell karyotype analysis revealed aberrant chromosomal structural and numerical alterations, suggesting a complex karyotype caused on by chromosomal translocation, breaks, and rearrangements. These intricate anomalies reveal chromosomes with an aberrant, poorly defined structure, which refer to as "marker chromosomes" [4]. When AMJ13 cells were exposed to live probiotic bacteria either T1 or T2, there was a strong time-dependent anti-proliferative effect, as demonstrated by cell viability assays in the current study. Increased exposure time of AMJ13 cells to 72 hours induced cell death $p < 0.05$ for both T1 and T2. While, AMJ13 cells were shown no significant respond to any of the two treatments T1 or T2, when they were exposed to 24 hours ($p < 0.5$) for both treatments compare with control. Based on cell viability studies, the administration of live *Lactobacillus casei* (and its bacterial components) to human (HT29) and murine (CT26) colon cancer cell lines resulted in a strong anti-proliferative impact that was concentration- and time-dependent. Particularly, it was found that colon cancer cells co-incubated with 10(9) CFU/mL *L. casei* for 24 hours had a markedly reduced viability (78% for HT29 and 52% for CT26 cells). Additionally, annexin V and propidium iodide staining demonstrated that live *L. casei* produced apoptotic cell death in both cell lines [23]. Two gene families within the BCL2 family are responsible for driving the genes involved in the mitochondrial apoptosis pathway. Anti-apoptotic genes involve the BCL2 gene and apoptotic genes include BAX gene. Apoptotic proteins are truly activated when a stimulation like probiotics cause a cell to enter apoptosis. This results in the release of cytochrome c from the mitochondria, which then activates caspase-9, caspase-3, and ultimately fragmented DNA, which kills the cell [24, 25]. The cytotoxic and anti-cancer characteristics of bacteria that produce lactic acid, such as *Lactobacillus* and *Bifidobacterium*, have been the focus of numerous studies [26-28]. Probiotics may be essential in avoiding oxidative stress-induced DNA damage. *Lactobacilli* and *Bifidobacteria* in this context have reportedly been demonstrated to increase antioxidant enzyme activity or alter signaling, preventing oxidative stress [29]. It has been demonstrated that probiotic supernatants cytotoxicity

affect breast cancer cells. This suggests that the bacteria would be a good candidate for an innovative medicinal strategy with fewer side effects, although further study is absolutely required [30].

The comet test was used in the current investigation to assess probiotic-induced genetic changes in breast cancer cells. The recent comet study's findings demonstrated that comets in the control group had large tails and small heads, which represented large DNA fragment damage. The analysis of the curve of images showed that T1 and T2 may play a role in the treatment of breast cancer, as showing green small tail and red large head in treated cells compare with control. Furthermore, probiotics may help in the repair of DNA damage by stimulating cell proliferation, activating DNA repair enzymes, and promoting tissue regeneration and repair [31, 32]. After bile damage, the addition of lactobacillus reduced NF κ B-associated inflammation in esophageal cells and accelerated the repair of bile-induced DNA damage by attracting pH2AX/RAD51. According to this study, lactobacilli exhibit anti-inflammatory and anti-genotoxic qualities, which makes them highly promising for preventing Barrett's esophagus and esophageal cancer in individuals with gastroesophageal reflux disease (GERD) [33]. Studies have shown that the DNA double strand breaks are repaired via homologous recombination (HR) or canonical non-homologous end joining [34, 35]. Double strand break repair by canonical non-homologous end joining takes place throughout the cell cycle, but the HR pathway only functions during the S and G2 phases because it necessitates extensive DNA-end processing and a homologous DNA sequence from the sister chromatids to serve as a template for DNA-synthesis-dependent repair. It is therefore thought to be extremely accurate [36, 37]. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a dietary mutagen, was employed in the study to cause DNA damage in mice. Lactobacillus rhamnosus IMC501 suspensions were given orally to the mice for ten days prior to the administration of food mutagen. The decrease in tail length was explained by the comet assay, which was used to measure the extent of DNA damage in colon and liver cells. It also revealed that Lactobacillus rhamnosus IMC501, when taken as a dietary supplement, can function as an antimutagen food component [38]. In future decades, probiotics and gut bacteria will most likely be important in the prevention and treatment of cancer [39].

Conclusion

The short tail curve in the image analysis indicates that probiotics were effective in repairing DNA damage, whereas the head curve was substantial. Thus, the therapeutic potential of probiotics in the treatment of breast cancer is reflected in these results.

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