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Antiproliferative Effect of *Lactobacillus helveticus* and Low Dose Gamma Radiation on Mammary Carcinogenesis

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IMMUNOTHERAPY plays an important role in cancer prevention and treatment. This study was aimed to investigate the role of milk fermented by *Lactobacillus helveticus* (L. *helveticus*) and low dose of gamma radiation (0.25 Gy) as protector and immunotherapy for breast cancer.

Female rats were divided into 7 groups, control group, fermented milk with L. *helveticus* (FM) Group, whole body gamma irradiated group, injected with 17β-estradiol (E2) group, FM and injected with E2 group, gamma irradiation and E2 group and FM then exposed to gamma radiation and injected with E2.

Results showed that, E2 caused a reduction in the percentage of cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8), an decrease in glutathione (GSH), malonaldehyde (MDA), nitric oxide (NO) levels and glutathione peroxidase (GPx), but increase in catalase (CAT) and superoxide dismutase (SOD) activities as well as an increase in the proliferation marker Ki-67 (Antigen KI-67 also known as Ki-67), with hyperplasia appeared in the histological examinations of mammary tissue epithelium. FM with or without gamma radiation increased CD4 and CD8 count, ameliorated GSH, MDA and NO levels, GPx, CAT and SOD activities and reduced Ki-67 percentage. Histological study showed normal breast tissue. This study demonstrated that FM and gamma radiation (0.25 Gy) induced immunoregulatory and antioxidant capacity in preventing carcinogenic effect of E2 on breast tissue.

Keywords: 17β-estradiol, γ-rays, breast cancer, *L. helveticus*.

Breast cancer is one of the most common cancers in women and many dietary factors are related with it either positively or negatively (Kim *et al*., 2011). Estrogens have been implicated to be complete carcinogens through a mechanism involving oxidative stress in the kidney, liver and breast tissues (De Le-Blanc *et al*., 2005).

Data from epidemiological and experimental studies have also indicated that the ingestion of certain lactic acid bacteria (LAB) strains or their fermented dairy products might alleviate the risk of certain types of cancers and inhibit tumours growth (Liu and Pan, 2010). Probiotic organisms and LAB in fermented milks are beneficial to the immune system and increase the resistance to neoplasia and infections. For these and other reasons, there is a steady increase in the consumption of fermented dairy products containing viable microorganisms (De Le-Blanc *et al*., 2005 and Kato, 2000).

Cellular detoxification, DNA reduction, decreasing the probability of neoplastic transformation, tumour growth delay, antimetastatic effects and sensitization of tumour could be achieved via exposure to low doses of ionizing radiation (Hosoi, 2006 and Redpath and Elmore, 2007). Such exposures may also enhance immune reactions and attenuate harmful effects of higher doses of radiation (Safwat, 2000 and Safwat *et al.*, 2003). These may explain the low incidence of leukaemia and some solid tumours as reported among nuclear workers and in the survivors of the Hiroshima and Nagasaki bombings whose absorbed doses did not exceed 0.25 Gy (Katayama *et al*., 2002 and Matanoski, *et al*., 1990). Previous studies have shown that compounds released during milk fermentation by *L. helveticus* are implicated to have antitumor effect of fermented milk (De Le-Blanc *et al*., 2005). The studies on the beneficial effects of fermented products in the prevention of different types of cancer such as colon cancer showing an inhibition of tumour growth during cyclical yoghurt feeding inhibited promotion and progression of the experimental intestinal tumour (Brady *et al*., 2000 and De Le-Blanc *et al*., 2005). Thus, this study was aimed to evaluate the effects of FM consumption on the immune system, antioxidant responses and a tumour marker of E2 dependent breast hyperplasia.

Materials and Methods

Preparation of fermented milk by L. helveticus

L. helveticus ATCC 15009 was obtained from Microbiological Resources Canter (MIRCEN, Cairo, Egypt). Milk used was sterile non-fat milk by autoclaving at 115 °C for 15 min and yeast extract (0.4%) was added to the milk used to grow *L. helveticus* before autoclaving. Sterile milk was inoculated with *L. helveticus* (2% vol/vol) and incubated statically at 37 °C for 17 h. Fermented

milk had a concentration of $1x10^9$ cfu/ml at the end of the fermentation period (De Le-Blanc *et al*., 2005).

Animals and treatments

Female adult Wister albino rats weighing 100± 10g were housed in the animal house of NCRRT, Egypt. Animals were cared in accordance with the standards outlined in Guide for the Care and Use of Laboratory Animals (DHHS publication 85-23) and received food and water *ad libitum*. Animals were divided into 7 groups (n=8). Control, rats were received 1 ml of 0.9% saline by gavages, FM group, rats received FM 1 ml/ kg body wt/ day by gavages, radiated group, rats were exposed to whole body of 0.25 Gy of γ-rays, E2 group, rats were inter peritoneum (i.p.) injected once with E2 (50 mg/ kg body wt), Sigma Chemical Co. (St. Louis, MO, USA), E2+ FM group, rats were received FM 1ml/ kg body wt/ day, for 15 days then injected with E2 (50 mg/kg body weight) and continued receiving FM, Group 6 (E2+radiation): rats were exposed once to gamma radiation (0.25 Gy) and injected with E2 (50 mg/kg body wt) and E2+ radiation+ FM group, rats were received FM for 15 days and exposed once to γ-rays (0.25 Gy) then injected once with E2 (50 mg/kg body wt) and continued receiving FM for 28 days.

Irradiation

Whole-body gamma irradiation was performed at the NCRRT, using ^{137}Cs Gamma Cell-40 biological irradiator. Animals were exposed to a single dose of 0.25Gy γ-rays at a dose rate of 0.46 Gy/ min.

Antioxidant assays

Antioxidant parameters were measured in the liver homogenate (10 % in 0.9 % saline). Tissue content of glutathione (GSH) was measured calorimetrically according to the method described by Beutler *et al*. (1963). The activity of glutathione peroxidase (GPx) was measured calorimetrically according to the method described by Gross *et al*. (1967). The activity of superoxide dismutase (SOD) was measured according to the method described by Minami and Yoshikawa (1979) and catalase (CAT) activity was measured calorimetrically according to the method described by Sinha (1972). Lipid peroxide concentration was determined by measuring the Malonaldialdahyde (MDA) end product content described by Yoshioka *et al*. (1979). Nitric oxide (NO) was determined according to Miranda *et al*. (2001).

Determination of CD4, CD8 and Ki-67

FACS calibar flow cytometer in Mansoura Children Hospital was used (Becton Dickinson, sunnyvale, CA, USA) equipped with a compact air cooked low power 15m watt Argon ion laser beam (488nm) was used to determine percentage of cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8). The average number of evaluated nuclei per specimen was 20.000 and the number of nuclei scanned was 120 per second. CD4and CD8 histograms were obtained by a mathematical analysis according to Dean and Jett (1974).

Determination of cell proliferation marker (Ki-67) in breast tissue was determined using Anti-Ki-67 antigen conjugate (Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone Ki-67) according to methodology described by Tribukati (1984).

Evaluation of apoptosis and cell cycle analysis

Flow cytometric analysis was performed for cell cycle analysis and evaluation of apoptosis via DNA stained with propidium iodide. As the DNA content is duplicated prior to cell division, mathematical models can estimate the percentage of cells in different phases of the cell cycle (G0/1, S, G2/M).

Data analysis was conducted using DNA analysis program MODFIT (verity software house, Inc. Topsham, ME 04086 USA), version: 2.0.

Histopathological study

Sections of mammary glands were stained with haematoxylin and eosin (H&E) and examined by light microscope (Banchroft *et al*., 1996).

Statistical analysis

The values representing mean± standard deviation (S.D.), values were considered statistically significant if the *P*-value was less than or equal 0.05. Comparisons among the different groups were carried out by ANOVA tests using SPSS (statistical package for social sciences, 1999; ver.10.0).

Results

To evaluate the effect of FM and gamma radiation on antioxidant parameters, GSH level, GPx, CAT and SOD activities were assayed. The results showed that E2 injection significantly decreased GSH and CAT

activity and significantly increased GPx and SOD activities compared to the control. Oral intake of FM and low dose of gamma radiation significantly ameliorated GSH, GPx, CAT and SOD activities, while a marked ameliorative effect in antioxidant parameters was the result of combined treatment of FM and gamma radiation in E2 treated rats compared to E2 group (Table 1).

To determine the effect of FM and gamma radiation on oxidative stress parameters in E2 treated group, lipid peroxidation as MDA and NO levels were measured. MDA and NO levels were significantly increased by E2 and ameliorated by FM intake and combined treatment of FM and gamma radiation when compared to E2 treated group (Table 2).

TABLE 1. Effect of fermented milk by *L. helveticus* and gamma radiation on GSH, GPx, CAT and SOD activities in the liver.

Group	GSH (mg/ g)	GPx (µmol oxidized GSH /min/ g)	CAT (µM H_2O_2/ g)	SOD (µg/ g)
Control	30.6± 1.4	148± 2.1	101± 2.8	9.3± 0.5
FM	32.9± 1.2[a]	146± 2.5	98.8± 3.0	9.2± 0.5
Rad	32.7± 1.6[a]	147± 2.0	98.6± 4.4	9.1± 0.5
E2	26.3± 1.4[a]	171± 2.6[a]	86.6± 2.9[a]	12.2± 0.4[a]
E2+ FM	28.5± 1.8[ab]	156 ± 2.2[ab]	91.6± 2.7[ab]	11.4± 0.4[ab]
E2+ Rad	28.9± 1.3[b]	155± 1.2[ab]	92.7± 1.8[ab]	10.9± 0.2[ab]
E2+ Rad+ FM	29.8± 0.4[b]	150± 1.7[b]	94.2± 2.7[ab]	10.1± 0.3[ab]

Values were represented as means± S.D.

Rad= gamma irradiation.

a: Significant compared to control ($P \leq 0.05$).

b :Significant compared to E2 ($P \leq 0.05$).

TABLE 2. Effect of FM and gamma radiation on lipid peroxidation (MDA) and nitric oxide (NO) levels in the liver.

Group	MDA (µM/ g)	NO (nM/ g)
Control	85.8± 2.8	6.3± 0.4
FM	84.6± 4.2	5.4± 0.4[a]
Rad	89.2± 4.0	5.5± 0.4[a]
E2	160.7± 4.1[a]	14.6± 0.4[a]
E2+ FM	107.6± 4.1[ab]	7.5± 0.4[ab]
E2+ Rad	139.0± 3.8[ab]	9.6± 0.4[ab]
E2+ Rad +FM	94.0 ± 3.8[ab]	7.8± 0.4[ab]

Legends as in Table 1.

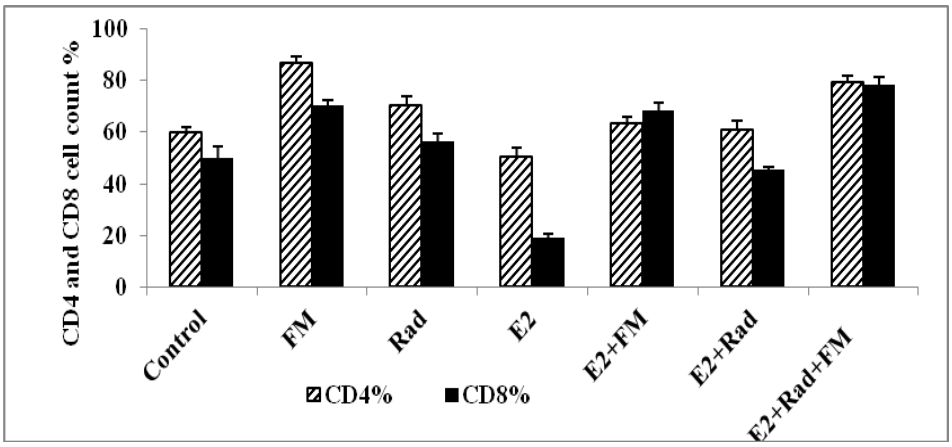

Fig. 1. Immuno-modulatory effect of FM and low dose of gamma radiation on CD4 and CD8 cell count percentage in the blood.

Legends as in Table 1.

Estradiol in E2 group significantly decreased percentage of CD4 and CD8 cells count which was then ameliorated by FM intake as compared to E2 group. FM as well as low dose of gamma radiation induced CD4 and CD8 cell count percentage compared to control (Fig.1)

To study the effects of FM and gamma radiation on cell cycle of tumour tissue induced by E2, mammary tissue cells were analyzed by flow cytometer. The results show that E2 markedly increased the cell count percentage in S and M phases but decreased in G0 phase when compared to that of the control. However the counts were ameliorated in G0, S and M phases with oral intake of FM or gamma radiation and combined treatment of FM and gamma radiation (Table 3).

TABLE 3. Effect of fermented milk by L. *helveticus* and low dose gamma radiation on cell cycle in mammary gland of E2 injected group.

Group	Go%	S %	M %
Control	82.7± 0.8	5.8± 0.9	1.9± 0.9
FM	84.7± 0.8[a]	9.6± 0.7[a]	3.8± 0.8[a]
Rad	82.4± 0.6	7.5± 0.6[a]	1.2± 0.5
E2	52.4± 0.6[a]	19.5± 0.6[a]	8.8± 0.8[a]
E2+FM	79.4± 0.6[ab]	10.3± 0.5[ab]	5.5± 0.6[ab]
E2+Rad	67.8± 0.8[ab]	8.8± 0.9[ab]	4.3± 0.6[ab]
E2+Rad+FM	84.6± 0.7[ab]	8.7± 0.8[ab]	3.6± 0.5[ab]

Legends as in Table 1.

Estradiol induced proliferation marker Ki-67 percentage and apoptosis, FM significantly reduced Ki-67 marker but with an increase in apoptotic thus causing reduction in growth index when compared to the E2 group, while combined treatment of FM with low dose of gamma radiation markedly caused reduction in Ki-67 marker with the same growth index as that of FM alone (Fig.2).

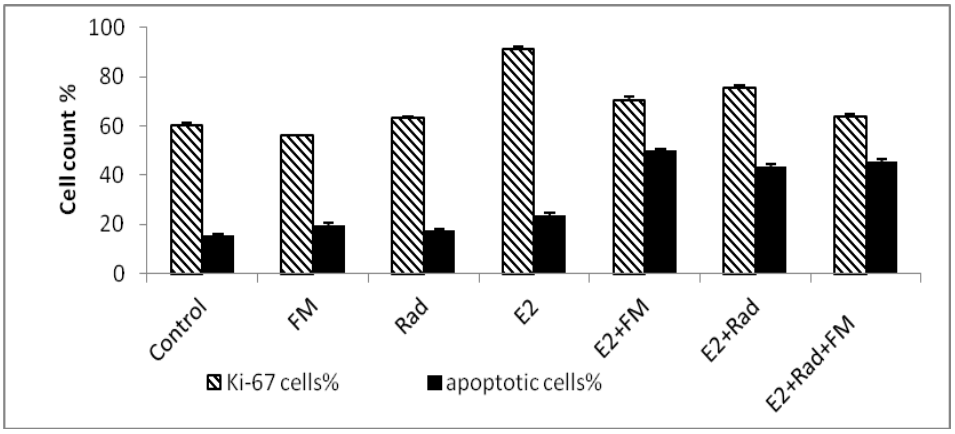

Fig. 2. Effect of fermented milk by L. *helveticus* and low dose gamma radiation on Ki-67 and apoptotic marker.

Legends as in Table 1.

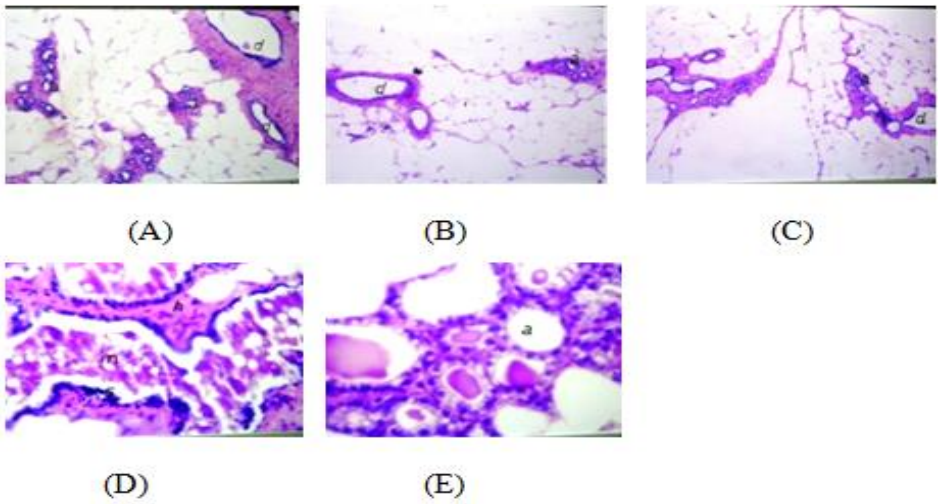

Fig. 3. Histological study of mammary gland tissue of female rats: (A) control group (40x), (B) FM group (40x), (C) gamma radiated group (40x), (D) E2 group (160x), FM+ radiated group+ E2 (160x). a: acini and d: lactiferous ducts.

Histopathological study by light microscope showed that healthy mammary gland of female rat was distinguished with acini and lactiferous ducts. Mammary gland of FM treated and gamma irradiated rats showed no alteration with normal histological structure of the acini and lactiferous ducts were observed. E2 caused dysplastic and anaplastic alterations associated with necrosis, also there was hyperplasia in the lining epithelium of the lactiferous duct. Rats supplemented with FM before and after E2 injection with low radiation exposure showed active acini and duct system and well differentiated lining epithelium (Fig.3).

Discussion

Spontaneous tumours frequently express antigens that can be recognized by the immune system. Specific CD4 with CD8 cells can reject tumours (Klein *et al.,* 2003). Numerous research studies have focused on probiotics anticancer property exerting resistance to neoplasia and infections (Kato, 2000). Data from epidemiological and experimental studies have also indicated that the ingestion of certain LAB strains or its fermented dairy products might alleviate the risk of certain types of cancers and inhibit the growth of tumours (Liu and Pan, 2010).

Absorption of low doses of ionizing radiation may stimulate cellular detoxification and repair mechanisms leading to reduction of the DNA damage even below the spontaneous level and decreasing the probability of neoplastic transformation (Redpath and Elmore, 2007). Such low dose exposures may also enhance immune reactions of the organism and attenuate harmful effects of higher doses of radiation (Safwat, 2000 and Safwat *et al.*, 2003). These mechanisms may explain that nuclear workers and in the survivors of the Hiroshima and Nagasaki bombings whose absorbed doses did not exceed 0.25Gy, were reported to have low incidence of leukaemia and some solid tumours when compared to the respective control groups (Katayama *et al.*, 2002 and Matanoski *et al.*, 1990).

In the present study, E2 caused significant changes in the measured antioxidant markers as reduction in GSH level and CAT activity was observed with an increase in GPx and SOD activities accompanied with a marked increase in oxidative stress markers MDA and NO levels. The induced changes in antioxidant parameters in the E2 group might be the result of a mechanism

through which a controlled increase in reactive oxygen species (ROS) may act to potentiate growth at the expense of increasing sensitivity to DNA damage (Davies, 1999) and inducing oxidative stress resulted in elevated MDA and NO levels (Karabi *et al*., 2012). The decrease in the level of GSH following E2 injection might be due to oestrogen metabolites conjugate forming GSH conjugates in human breast tissue (Rogan *et al*., 2003). A decrease in peroxide metabolism was reported to be the result of a change in antioxidant enzyme activities or antioxidant substrates, total reduced and oxidized glutathione levels and the activities of CAT, SOD and GPx (Mense *et al*., 2009 and Mobley and Brueggemeier, 2004). Also oestrogen receptor-mediated mechanism was reported to be responsible for changes in all of the enzymes studied. According to Nishio and Watanabe (1997), significant increase in SOD and GPx activities and decrease in total GSH level and CAT activity is consistent with the observed decrease in cellular peroxide metabolism**.**

It is well documented, that antioxidants play an important role in ameliorating the damaging effects of oxidative stress on cells. According to our findings, oral administration of FM before and after E2 injection accompanied with low dose of radiation normalized antioxidant enzymes of GPx, CAT and SOD activities and GSH level and also normalized stress markers of MDA and NO levels. This could be attributed to potent antioxidant activity and free radical scavenging capability of FM due to antioxidant property of LAB. Therefore the LAB strains are able to decrease the risk of ROS accumulation through food ingestion and also to degrade the superoxide anion and hydrogen peroxide (Liu and Pan, 2010). The results support the role of FM in scavenging free radicals and its antioxidant properties. *Lactobacillus* species have been reported to play a significant role in the production of bioactive peptides in fermented dairy products by showing a significant increase in radical scavenging activity. *Lactobacillus* may be used to combat oxidative stress by involving in cholesterol metabolism through several anti-oxidative mechanisms: CAT, glutathione-system-related compounds and SOD, attenuating proliferation caused by ROS (Ramesh *et al*., 2012) and reducing oxidative stress parameters such as NO (Fernanda *et al*., 2012).

Exposure to low dose of gamma radiation stimulates cellular metabolic activities such as antioxidant activities; a phenomenon termed as radiation

hormesis which can cause an increase in GSH level in the spleen 4 hours after irradiation in mice **(**Liu, 1996) and might be effective for the prevention of various ROS-related diseases and exerts a protective effect upon cells and whole animals (Otsuka *et al*., 2006).

In the present study, E2 markedly inhibited the production of T cells represented by reduced CD4 and CD8 counts which were then ameliorated by oral administration of FM or exposure to gamma radiation and markedly normalized by the combined treatment of FM and gamma radiation.

It was reported that E2 caused a reduction in CD4 and CD8 T cells number in rats (Kim *et al*., 2013). Oral administration of fermented milk induced the CD4 and CD8 percentage (De Le-Blanc *et al*., 2005), while exposures to low doses of ionizing radiation enhanced CD4 and CD8 production in the thymus and CD8 T cells in the spleen (Ina and Sakai, 2004 & 2005a & 2005b) hence increased the natural killing activity post-irradiation with reduction in the percentage of B cells in blood lymphocytes concomitant with an increase in helper T cell population (Kojima, 2006).

In this study, analysis of cell cycle by flow cytometry showed that the E2 markedly increased the cell population at S and M phases which were inhibited by the FM treatment as cell cycle was arrested at S and M. Exposure to gamma radiation caused cell arrested at S phase. These effects were markedly ameliorated by the combined treatment of FM and gamma- radiation. The mechanism of cancer cell growth inhibition by LAB could be related to modulation of apoptotic signalling regulated proteins (Liu and Pan, 2010).

Acquired resistance to the programmed cell death mechanism, apoptosis, is an important hallmark of cancer (Lowe *et al*., 2004). Antiproliferative effects of LAB in this study, was exerted via the induction of apoptosis, necrosis and growth inhibitory effects, which was also reported by Kim *et al*., (2003). Fermented milk by *L. helveticus* has an antiproliferative effect on breast cancer cells. However, the precise mechanism by which LAB exerts its anticancer effects remains unknown (Liu and Pan, 2010). Combined treatment of FM with exposures to low level of gamma rays may suppress further the development and progression of tumours which can be associated with stimulation of anti-neoplastic functions of the immune system by irradiation (Nowosielska *et al*., 2012).

Ki-67 marker is frequently measured as a marker of proliferative activity and a possible dynamic marker of tumour treatment efficacy. In the present study Ki-67 was evaluated as prognostic factor and monitoring marker during the treatment. We have demonstrated that E2 induced preneoplastic changes by elevated Ki-67 in the breast in addition to histological analysis as preneoplastic and neoplastic changes, which were ameliorated by FM or low dose of gamma radiation or combined treatment of both.

Apoptosis has been consistently reported to be positively correlated with Ki-67 (Lipponen, 1999). In the present study, tumour growth index was markedly reduced by FM, gamma radiation and combined treatment of FM and gamma radiation. The cell turnover or growth index is an index based on the ki-67 apoptosis ratio developed to approximate the contribution that these two factors may have on tumour growth, thus it can be used as an early marker of response to treatment in primary therapy of breast cancer (Bundred *et al.*, 2002 and Cleator *et al*., 2002). Low dose total body irradiation showed antitumor effects which could be explained by immune enhancement, induction of apoptosis, and intrinsic hypersensitivity to low dose of radiation and it is effective as chemotherapy (Hosoi, 2006).

In conclusion our results suggest that *L. helveticus* can be used as adjuncts in fermentation of food which is the potential candidate for the prevention of breast cancer when combined with low doses of gamma radiation leading to inhibition of tumour growth.

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التأثير المثبط للاكتوباسيلس هيلفيتكس مع جرعة منخفضة من أشعة جاما للإنقسام الخلوى لسرطان الثدى

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الغذاء المختمر ببكتريا البروبايوتك له تأثيرات مضادة للأورام. تهدف هذه الدراسة الى بحث الدور الوقائى و العلاجى المحتمل للبن المختمر بلاكتوباسيلس هيلفيتيكس و الجرعة المنخفضة من أشعة جاما (٠,٢٥جراى) على خلايا ورم الثدى المحدث بواسطة ١٧ بيتا استراديول. تم تقسيم اناث الجرذان الى ٧ مجموعات: المجموعة الأولى: المجموعة الضابطة و المجموعة الثانية: المجموعة تم تعريضها لأشعة جاما ٠,٢٥(جراى) و المجموعة الثالثة: المجموعة المعاملة باستراديول.

المجموعة الرابعة: المجموعة المعاملة باللبن المختمر و المجموعة الخامسة: المجموعة المعاملة باللبن المختمر ثم باستراديول و المجموعة السادسة: المجموعة تم تعريضها لأشعة جاما و الاستراديول معا و المجموعة السابعة: المجموعة المعاملة باللبن المختمر و أشعة جاما ثم باستراديول.

و أوضحت النتائج أن الاستراديول له تأثير مثبط على مستوى الجهاز المناعى مما أدى الى نقص فى عدد خلايا T بنوعيها CD4, CD8 و على عوامل مضادات الأكسدة: الجلوتاثيون و نشاط انزيم جلوتاثيون بيرأوكسيداس و مع زيادة نشاط كلا من كاتاليز و سوبر أوكسيد ديسميوتاز و زيادة فى النسبة المئوية لعدد خلايا Ki-67 و مستوي المالون داي آلدهيد و النيتريك أكسيد مع ظهور خلايا سرطانية فى التحليل الهستولوجى لأنسجة الثدى. أظهرت الفئران المعاملة باللبن المختمر أو التى تم تعريضها لأشعة جاما أو المعاملة بكلا من اللبن المختمر و أشعة جاما معا زيادة فى عدد خلايا كلا من CD4 وCD8 و رفع مستوى الجلوتاثيون و نشاط انزيمات جلوتاثيون بيرأوكسيداس و كاتاليز و سوبر أوكسيد ديسميوتاز و انخفاض فى عدد خلايا Ki-67 و مستوي المالون داي آلدهيد وو النيتريك أكسيد مع عدم تأثر خلايا نسيج الثدى بالاستراديول بالمقارنة بالمجموعة الضابطة.

من هذه الدراسة يمكن استخلاص ان اللبن المختمر ببكتريا لاكتوباسلس هيلفيتيكس مع جرعة منخفضة من أشعة جاما مجتمعين لهم قدرة عالية على تنظيم الاستجابة المناعية و منع التأثير المسرطن للإستراديول على أنسجة الثدى و استعادة التركيب التشريحى الطبيعى للأنسجة بالمقارنة بالمعاملات الاخرى.