Preventive Effects of Probiotic Bacteria *Lactobacillus plantarum* and Dietary Fiber in Chemically-induced Mammary Carcinogenesis

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Abstract. *Aim: The purpose of the present study was to evaluate the chemopreventive efficacy of a new probiotic bacterial strain, Lactobacillus plantarum LS/07 (PRO), prebiotic oligofructose-enriched inulin (PRE) and PRO-PRE combination in a rat model of breast cancer. Materials and Methods: Mammary carcinogenesis was induced by 7,12-dimethylbenz[a]anthracene (DMBA). Daily oral administration of PRO (at a dose of 8.4×10^8 c.f.u./rat) and PRE (in the diet, 20 g/kg) started two weeks before the first DMBA dose and lasted until the end of the experiment (16 weeks). Results: Administration of PRO, PRE and PRO-PRE combination significantly suppressed the tumor frequency, increased $Cd4^+$ T-cells in tumor tissue and reduced serum tumor necrosis factor-α concentration. In PRO and PRO-PRE groups, the decline of $Cd8^+$ T-cells in blood and their increase in tumor tissue was observed. Conclusion: Long-term administration of Lactobacillus plantarum LS/07 with and without inulin is effective against breast cancer, at least partially, through immunomodulatory mechanisms.*

Breast neoplasia is the leading cause of cancer-related death among women, both in developed and developing countries. New preventive strategies affordable for the general public are required in order to decrease global breast carcinoma incidence in the 21st century. One of the promising ways could be the application of probiotic bacteria. Most

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probiotics in use today belong to the genera *Lactobacillus* and *Bifidobacterium*. Lactobacilli produce lactic acid and comprise a major group of lactic acid bacteria (LAB). *Lactobacillus plantarum* is found in various fermented food products such as vegetables, fish and dairy products [reviewed in (1)]. It is also an indigenous inhabitant of the human gastrointestinal tract.

Consumption of LAB has been shown to improve a wide variety of health disorders, including cancer. Great attention is attributed on preventive effects of LAB on colorectal cancer (2, 3); there are several reports concerning bladder, liver and stomach cancer [reviewed in (4)]. Data demonstrating the antitumor activity of LAB in breast cancer are scarce; direct antiproliferative effect was reported *in vitro*. Fermented milk containing five probiotic species (5), and kefir and yogurt extracts (6) inhibited the growth of MCF-7 breast adenocarcinoma cell line. Liu and Pan reported that *L. plantarum* NTU 102 strain can efficiently inhibit the proliferation of MDA-MB-231 cancer cells *via* G_0/G_1 phase cell-cycle arrest (7). Two case–control studies have suggested that fermented milk products may prevent human breast cancer (8, 9). In several animal studies, a murine syngeneic model using subcutaneous injection of 4T1 tumoral cell line has been used (10-13). In these experiments, milk fermented by *L. helveticus*, as well as kefir (11, 13), was able to delay mammary tumor growth when administered cyclically for 28 days after tumor induction. However, long-term chemopreventive studies are still lacking.

Anti-tumor effects of LAB are primarily related to their immunomodulatory properties. LAB strains have been shown to affect cellular, humoral and non-specific immunity (14-16). Immunomodulatory effects of LAB are strongly strain-specific (17), even the ability of different strains of the same species varies, therefore the efficacy of each strain needs to be carefully evaluated (18).

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon (19). Inulin and oligofructose, the most extensively studied dietary fibers, increase survival and activity of bifidobacteria and lactobacilli (20) and possess anti-carcinogenic activity. Oligofructose-enriched inulin suppressed the formation of colorectal tumors in rats (21), and inhibited the growth of liver tumor (mouse TLT cell line) and mouse mammary carcinoma (EMT6 cell line) (22). The reduced incidence of *N*-methyl-*N*-nitrosourea-induced mammary tumors in rats was also reported (23). Mechanisms involved in these effects are not fully-understood at present; modulation of the intestinal immune system is under consideration (24).

The 7,12-dimethylbenz-*α*-anthracene (DMBA)-induced rat mammary carcinoma model is a well-established tool for efficacy testing of chemopreventive agents. An advantage of this model is that it covers all stages of carcinogenesis – initiation, promotion and progression. DMBA-induced tumors display several similarities to human breast cancer, they are generally hormone-dependent adenocarcinomas of ductal origin (25).

In the present study, the preventive and antitumor effects of a new strain *Lactobacillus plantarum* LS/07, oligofructose-enriched inulin and their combination were analyzed in a long-term preventive-curative study in female rats. Cluster of differentiation 4-positive (Cd4+) and 8-positive (Cd8+) T-cell populations in blood, tumor tissue and in mammary glands, as well as levels of selected cytokines in serum of rats with and without carcinogenesis induction were evaluated in order to identify the possible mechanisms responsible for antitumor effects.

Materials and Methods

Animals. One hundred and twenty female Sprague Dawley rats (AnLab, Czech Republic) aged 30 days were adapted to standard vivarium conditions. The experiment was approved by the State Veterinary and Food Administration of Slovak Republic (accreditation no. 1574/10-221 and 2690/11-221).

Treatment. The rats were fed conventional MP diet (Peter Miško, Snina, Slovakia). The prebiotic (PRE) oligofructose-enriched inulin (Beneo™ Synergy 1; ORAFTI, Tienen, Belgium) was compressed into pellets at a concentration of 20 g/kg and administered *ad libitum*. The probiotic (PRO) *L. plantarum* LS/07, a new strain isolated from human rectal swabs was kindly provided by the Department of Experimental Medicine, Faculty of Medicine, P.J. Šafárik University in Košice Slovak Republic (26). PRO was prepared in MRS broth (Merck, Darmstadt, Germany) at 37°C aerobically to provide 3×10^9 c.f.u./ml and administered orally daily (Mon-Fri) as a volume of 280 µl per rat, corresponding to 8.4×10^8 c.f.u.

Induction of mammary carcinogenesis with DMBA. Mammary carcinogenesis was initiated with three intragastric doses (10 mg/rat each by gavage, on the 45th, 50th and 55th postnatal day) of DMBA dissolved in corn oil (both Sigma, Deisenhofen, Germany).

Experimental design. Treatment with PRO and PRE started two weeks before the first dose of DMBA and lasted until the end of experiment (16 weeks). The animals were divided into eight groups as follows: (i) control group treated with carcinogen only (DMBA); (ii) three carcinogen-treated groups additionally treated with probiotic (DMBA-PRO), prebiotic (DMBA-PRE) and the combination of probiotic and prebiotic (DMBA-PRO-PRE); (iii) three control groups without carcinogenesis induction, treated with probiotic (C-PRO), prebiotic (C-PRE), and the combination of probiotic and prebiotic (C-PRO-PRE); (iv) an intact group of untreated healthy rats (C-INT). Each carcinogen-treated group consisted of 20 animals, the groups not exposed to carcinogen consisted of 10 animals.

All rats were weighed weekly. The rats administered DMBA were palpated once a week to record the presence, number, location and size of each palpable tumor. Food and water intake was monitored.

At the end of the experiment (16th week), the animals were sacrificed by quick decapitation, and blood from each animal was collected. Mammary tumors were excised and tumor size was recorded. The basic parameters of mammary carcinogenesis that were evaluated included tumor incidence, latency period, tumor frequency per group and per animal, and tumor volume (calculated according to the formula $=\pi \times (S_1)^2 \times S_2/12$; $S_1 < S_2$, where S_1 and S_2 are tumor diameters). The tumor samples were fixed in formaldehyde and embedded in paraffin for histopathological analysis and Cd4+ and Cd8+ T-cell immunohistochemistry. All tumors were histopathologically classified by an experienced pathologist. For the rats without carcinogenesis induction, right abdominal mammary glands were removed and processed in the same manner for immunohistochemical determination of Cd4+ and Cd8+.

Determination of Cd4+ and Cd8+ T-cells in blood by flow cytometry. Blood (100 µl) was collected in heparin-treated tubes, diluted with equal amount of phosphate buffered saline (PBS), mixed with 1 ml of red blood cell lysis buffer (RBCLB), then incubated for 5 min at room temperature (RT); it was then pelleted ($1000 \times g$ for 5 min at RT), lysed again in 1 ml of RBCLB for 5 min at RT and pelleted again ($400 \times g/5$ min at RT). It was then mixed with 1.3 ml of PBS, washed by centrifugation ($250 \times g$ for 5 min at 4°C) and finally resuspended in 0.5 ml of RPMI-1640 supplemented with 10% of fetal calf serum (both GIBCO Invitrogen, Carlsbad, CA, USA). An aliquot (100 µl) was pelleted ($1000 \times g/5$ min/4°C), resuspended in 20 µl of monoclonal antibodies pre-mixed in PBS (anti-rat Cd4-Phycoerythrin (PE)-Cy7 (0.7 µl/sample) and anti-rat Cd8a-Fluorescein isothiocyanate (FITC) (0.3 µl/sample) (BD Biosciences PharMingen, Erembodegem, Belgium), incubated for 20 min/4°C, diluted with 200 µl of PBS and analyzed by FACS Calibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Debris was eliminated by forward scatter and side scatter (FSC×SSC) gating and the proportions of lymphocyte subpopulations expressed as a percentage of total lymphocytes.

Detection of Cd4+ and Cd8+ T-cells by immunohistochemistry. Four-micrometer tumor tissue sections on slides were deparaffinized, rehydrated by ethanol series, then washed in 10 mM PBS. Nonspecific protein blocking was achieved by 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA). The sections were incubated at 37°C with primary antibody (Cd4 DOMAIN 1, clone W3/25 or Cd8 ALPHA, clone OX-8; both AbD Serotec, Düsseldorf, Germany) diluted at 5 µg/ml in PBS. Secondary antibody of biotinylated anti-mouse IgG (2 µg/ml in PBS; Vector

Table I. *Effect of Lactobacillus plantarum and oligofructose-enriched inulin on tumor growth parameters in 7,12-dimethylbenz/a/anthracene-induced mammary carcinogenesis in female rats. Data are expressed as the mean±S.E.M.*

	DMBA (n=19)	DMBA-PRO (n=20)	DMBA-PRE (n=20)	DMBA-PRO-PRE (n=18)
Incidence (%)	100	95 (−5%)	90 (−10%)	83 (−17%)
Latency (days)	58.21±2.84	63.37±2.11 (9%)	64.56±3.23 (11%)	59.06±2.71 (1%)
Frequency per group	5.16±0.69	2.75±0.40 (−47%)**	2.75±0.40 (−47%)**	2.61±0.38 (−49%)**
Frequency per animal	5.16±0.69	2.89±0.39 (−44%)**	3.06±0.37 (−41%)*	3.13±0.31 (−39%)*
Cumulative tumor volume (cm^3)	94.67	87.62 (−8%)	84.15 (−11%)	59.45 (−37%)

Values in brackets are the percentage deviation from the control DMBA-treated group. n, Number of animals per group; significance *versus* DMBA treatment alone (*$p<0.05$; **$p<0.01$).

Laboratories) was used. Both Vectastain® Elite ABC Kit and DAB Substrate Kit (Vector Laboratories) were used for visualization according to the manufacturer's instructions. Representative areas of malignant tumors (three *in situ* and four invasive tumors from each group of animals) were examined using a light microscope with a digital camera. At least 10 different vision fields per section containing tumorous parenchyma and stroma were evaluated. Digital images were morphometrically analysed by Ellipse v.2.0.7.1 software (ViDiTo, Košice, Slovakia).

Concentration of interleukin 10 (Il10) and tumor necrosis factor-α (Tnfα) in serum. Capture enzyme-linked immunosorbent assay (ELISA) was utilized for determination of serum Il10 and Tnfα concentrations. Commercial kits (Quantikine® ELISA; R&D Systems, Minneapolis, MN, USA) were used according to the manufacturer's instructions.

Statistical analysis. Tumor incidence was evaluated by Mann–Whitney *U*-test, and other parameters by one-way analysis of variance or Kruskal–Wallis test, respectively. Significance levels are indicated in the legend of each figure.

Results

Effects of PRO, PRE and their combination on rat mammary carcinogenesis are summarized in Table I. The PRO-PRE combination slightly lowered tumor incidence (by 17%) and cumulative tumor volume (by 37%). PRO and PRE administered alone led to a prolonged latency period at approximately six days. Application of PRO, PRE and PRO-PRE resulted in a significant decline in tumor frequency per group (by 47%, 47% and 49%, respectively) as well as the frequency per animal (by 44%, 41% and 39%, respectively), compared to the DMBA-treated group.

Histopathological examination revealed the highest ratio of invasive carcinomas and *in situ* carcinomas (71:26) in the group treated with DMBA-alone (Figure 1). In all DMBA-treated groups, we recorded a decrease in the number of invasive carcinomas (40, 21, 20) in the DMBA-PRO, DMBA-PRE and DMBA-PRO-PRE groups, respectively,
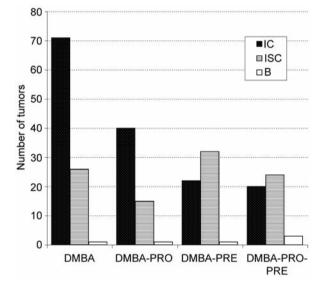

Figure 1. *Histopathological analysis of mammary tumors. IC: Invasive carcinomas; ISC: in situ carcinomas; B: benign lesions.*

when compared to the group treated with DMBA alone, whereas the number of *in situ* carcinomas did not differ among groups. The highest number of benign lesions (n=3) was detected in the DMBA-PRO-PRE group.

In blood, the percentage of Cd4+ T-lymphocytes did not vary among groups. The decline of Cd8+ T-cell count was detected in DMBA-treated groups supplemented with PRO alone and PRO-PRE combination, as compared to the group treated with DMBA alone. In rats without DMBA, no significant changes in Cd8+ T-cells were registered (Figure 2).

In tumor tissue, a significant increase in Cd4+ T-lymphocytes was observed in all DMBA-treated groups; the PRO-PRE combination was more effective than PRO or PRE administered alone. Cd8+ T-cells increased in DMBA-PRO
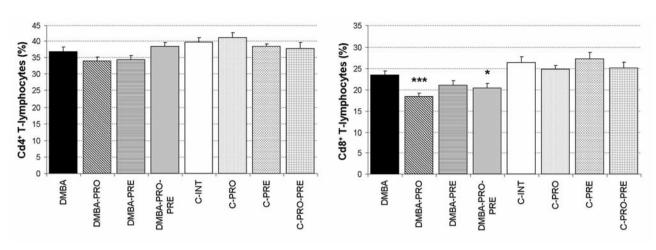
Figure 2. *Effect of Lactobacillus plantarum and oligofructose-enriched inulin on percentage of Cd4+ and Cd8+ T-lymphocytes in blood. Data are expressed as the mean±S.E.M. 7,12-Dimethylbenz/a/anthracene (DMBA)-treated groups were evaluated independently from control (C) groups. PRO: Probiotic bacterial strain L. plantarum LS/07; PRE: prebiotic oligofructose-enriched inulin. Significance versus DMBA alone: *p<0.05; ***p<0.001.*
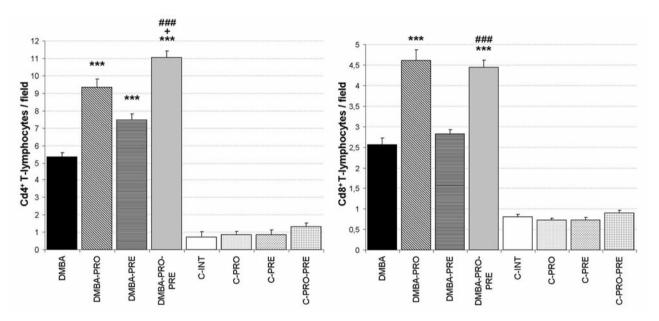

Figure 3. *Effect of Lactobacillus plantarum and oligofructose-enriched inulin on Cd4+ and Cd8+ T-lymphocytes in tumor tissue (7,12-Dimethylbenz/a/anthracene DMBA-treated groups) and in mammary gland (C) groups. Data are expressed as the mean±S.E.M. DMBA-treated groups were evaluated independently from C groups. Significance versus DMBA-alone: ***p<0.001; versus DMBA-PRO: +p<0.05; versus DMBA-PRE: ###p<0.001.*

and DMBA-PRO-PRE groups compared to animals treated with DMBA-alone. Neither Cd4+ nor Cd8+ T-cell count changed in mammary gland of rats without DMBA administration (Figure 3).

The serum Il10 levels were increased non-significantly in DMBA-PRO (176±34.2 pg/ml) and DMBA-PRO-PRE (202±30.9 pg/ml) groups compared to the DMBA-alone group (159±25.1 pg/ml). In rats not exposed to DMBA, administration of PRO, PRE and PRO-PRE combination

resulted in a non-significant increase in serum Il10 concentration (absolute concentration range=184-212 pg/ml) in comparison with intact controls (122±23.2 pg/ml).

The highest serum concentration of Tnfα was observed in the group treated with DMBA alone. Administration of PRO, PRE and PRO-PRE combination resulted in significant decrease of Tnfα levels in animals exposed to DMBA. In rats without DMBA application, no significant changes in Tnfα levels were recorded (Figure 4).

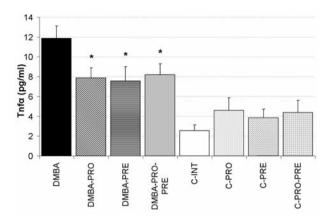Figure 4. *Effect of Lactobacillus plantarum and oligofructose-enriched inulin on serum tumor necrosis factor-α (Tnfα) concentration. Data are expressed as the mean±S.E.M. 7,12-Dimethylbenz/a/anthracene (DMBA)-treated groups were evaluated independently from control (C) groups. Significance versus DMBA-alone: *p<0.05.*

Discussion

To our knowledge, this study is the first report demonstrating preventive and curative effects of a new probiotic strain, *L. plantarum* LS/07, and prebiotic inulin in mammary carcinogenesis. Administration of PRO, PRE and their combination led to the decline of tumor frequency, which is considered the most sensitive parameter in antitumor evaluation of various agents in rat mammary tumorigenesis (27). Anti-carcinogenic properties of LABs in chemically induced mammary tumorigenesis are not clear and might be strain-specific. Anti-neoplastic efficacy of fermented milk products and LAB (bifidobacteria and *L. acidophilus*) was evaluated in mouse DMBA-induced mammary carcinogenesis. Neither the initiation nor the promotion phase was affected by probiotics (28). In contrast, dietary supplementation of *B. longum* suppressed 2-amino-3-ethylimidazol[4-5-f]quinoline-induced mammary tumor growth in a rat curative study (29). PRE application (15% oligofructose in the diet) reduced the incidence and the total number of tumors in *N*-nitroso-*N*-methylurea-induced rat mammary carcinogenesis (23). In our experiment, PRE administration slightly enhanced the antitumor activity of PRO, which was seen as maximal reduction of tumor incidence and cumulative tumor volume, the lowest proportion of invasive tumors and the highest number of benign tumors in DMBA-PRO-PRE group.

Within the tumor microenvironment, T-lymphocytes recognize the tumor antigens as principal targets for antitumor defence. Cd8+ Cytotoxic T-cells are directly capable of killing tumor cells, and breast cancer tumors with higher levels of infiltrating Cd8+ cells have been associated with better patient survival (30). Cd4+ helper lymphocytes are a heterogenous class of T-cells and their helper function for Cd8+ T-cell-mediated response is well documented particularly in initial stages of tumor progression (31). In our study, the effect of long-term administration of PRO and PRE on the Cd4+ and Cd8+ T-cell count was evaluated in two compartments – in the systemic circulation and in the tumor tissue. In blood, no changes in Cd4+ T-cells were seen. Administration of PRO alone or in combination with PRE induced the decline of blood Cd8+ T-cell number in rats exposed to the carcinogen, favouring Cd4+ T-cell balance. On the contrary, Lee et al. demonstrated that administering *L. casei* and *B. longum* to teratocarcinoma-bearing animals for four weeks enhanced the number of Cd8+ T-cells in blood (32). No changes were observed in Cd4+ T-cells, resulting in a decreased Cd4+/Cd8+ ratio. Differences in tumor type or LAB strain used in both experiments could be related to the different response of circulating Cd8+ lymphocytes. In rats at the end of our experiment, advanced cancer might also have played a role in Cd8+ suppression.

Stimulation of local immune response by increasing in tumor-infiltrating Cd4+ and Cd8+ T-cell number is probably the crucial mechanism of antitumor activity of PRO used in the present study. Our findings are consistent with the report of Rachid *et al.*, where increase in the tumor Cd4+ and Cd8+ population was seen 26 days post-inoculation of breast cancer cell line and mice were cyclically treated with *L. helveticus*-fermented milk (10). Several animal studies have demonstrated that *L. plantarum* can stimulate local immunity *via* interaction with Peyer's patches of the small intestine, thereby activating cellular migration of B- and T-lymphocytes to the distant mucosal sites such as respiratory, urogenital, salivary and mammary glands (14, 15). In our experiment, the most pronounced tumor infiltration by Cd4+ and Cd8+ cells was recorded in DMBA-exposed animals treated with PRO and PRO-PRE combination, respectively. As prebiotics are able to increase the survival and activity of lactobacilli, the enhancing effect of inulin was anticipated, but it was seen in Cd4+ local immune response only. On the other hand, most of the Cd4+ T-cells in the chemically induced rat mammary tumors were identified as regulatory T-cells that may be involved in the suppression of antitumor immune responses (36), therefore the significance of the Cd4+ rise in tumor tissue needs to be assessed in future experiments. In healthy rats without DMBA exposure, no significant changes in Cd4+ and Cd8+ T-cells were observed, neither in circulation nor in mammary gland.

Il10 is a cytokine with anti-inflammatory properties which can inhibit Cd4+ proliferation as well as enhance Cd8+ proliferation (33). Cyclical administration of *L. helveticus* to mammary cancer-bearing mice led to increase in serum Il10 concentration (13). In our study, however, no significant changes in serum Il10 level were observed either in tumor-bearing rats or in healthy animals.

Tnfα signaling is critically required for effective tumor immune surveillance and function of tumor-specific T-cells (34). However, high circulating levels of Tnfα can contribute to tumor progression (35, 37). In our study, increased serum Tnfα levels were observed in the group treated with DMBA, 14 weeks after carcinogen administration. Similarly, in mice inoculated with mammary tumor cells, serum Tnfα levels increased simultaneously with tumor volume during 28 days (10, 13). PRO application resulted in the decline of serum Tnfα concentration in tumor-bearing mice (13), which is in agreement with the decrease in Tnfα after PRO and PRE treatment in our experiment.

In conclusion, our study has shown that long-term oral consumption of new probiotic *L. plantarum* LS/07 strain and prebiotic oligofructose-enriched inulin is effective in prevention and treatment of experimentally-induced breast cancer. Immunomodulatory capacity of both agents has been demonstrated on the level of the systemic immune response, local response in the mammary tumor tissue and Tnfα production. Further investigations are required to confirm using them in protection against breast cancer, mainly in human studies.

Acknowledgements

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