Anticancer and Immunomodulatory Effects of *Lactobacillus plantarum* LS/07, Inulin and Melatonin in NMU-induced Rat Model of Breast Cancer

MONIKA KASSAYOVÁ[1], NIKITA BOBROV[2], LADISLAV STROJNÝ[3], PETER ORENDÁŠ[1], VLASTA DEMEČKOVÁ[1], RASTISLAV JENDŽELOVSKÝ[1], PETER KUBATKA[4], TERÉZIA KISKOVÁ[1], PETER KRUŽLIAK[5], MARIÁN ADAMKOV[6], ALOJZ BOMBA[3] and PETER FEDOROČKO[1]

[1]Institute of Biology and Ecology, Faculty of Science, P.J. Šafárik University, Košice, Slovak Republic;
Departments of [2]Forensic Medicine and [3]Experimental Medicine,
Faculty of Medicine, P.J. Šafárik University, Košice, Slovak Republic;
Departments of [4]Medical Biology and [6]Histology and Embryology, Jessenius
Faculty of Medicine, Comenius University in Bratislava, Martin, Slovak Republic;
[5]Laboratory of Structural Biology and Proteomics, Central Laboratories, Faculty of Pharmacy,
University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic

Abstract. *Background/Aim: Chemopreventive activity of a new probiotic strain Lactobacillus plantarum LS/07 (PRO) and prebiotic oligofructose-enriched inulin (PRE) in rat mammary carcinogenesis induced by procarcinogen 7,12-dimethylbenz[a]anthracene has been reported before. This study evaluated the anticancer and immunomodulatory efficacy of PRO, PRE, PRO+PRE (PRO/PRE) and combination with melatonin (PRO+PRE+MEL) in a rat model, when breast cancer was induced by a direct-acting carcinogen N-nitroso-N-methylurea (NMU). Materials and Methods: Daily administration of PRO (at a dose of 8.4×10^8 colony-forming units (c.f.u.)/rat), PRE (in the diet, 20 g/kg) and MEL (in tap water, 20 mg/l) started 14 days before the first NMU dose and lasted for 16 weeks. Results: Although tumor growth was not altered, a marked decrease in the ratio of high-/low-grade carcinomas and in tumoral Ki-67 expression was found after PRO+PRE treatment; melatonin augmented these effects. PRO+PRE+MEL combination enhanced $CD4^+$ and $CD8^+$ T-cell tumor infiltration induced by PRO/PRE and increased $CD25^+FoxP3^+$ regulatory T-cells in tumors. Conclusion: In mammary carcinogenesis, Lactobacillus plantarum LS/07 and inulin exert prodifferentiating, antiproliferative and immunomodulatory activities, which are significantly amplified by melatonin co-administration.*

Correspondence to: Monika Kassayová, Institute of Biology and Ecology, Faculty of Science, P.J. Šafárik University, Moyzesova 11, 040 01 Košice, Slovakia. Tel: +421 552341214, Fax: +421 556222124, e-mail: monika.kassayova@upjs.sk and Peter Kružliak, Laboratory of Structural Biology and Proteomics, Central Laboratories, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Palackého tr. 1946/1, 612 42 Brno, Czech Republic. Tel: +420 541561111, e-mail: kruzliakpeter@gmail.com

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Lactobacillus plantarum (*L. plantarum*) belongs to the large group of lactic acid bacteria that can exhibit health-promoting effects on the host. It is one of the most versatile probiotic species dominating in various fermented products common in Western diet, such as sauerkraut, green olives, sourdough, natural wines, beers, as well as cheeses and sausages. It has also been found to be a resident of human gastrointestinal, oral and vaginal mucosa (1).

Anticancer activities of different *L. plantarum* strains have been observed in various types of tumors. *In vitro* cytotoxic effects have been reported on human cervical, gastric, colon, breast (2), as well as melanoma (3) cancer cell lines. Genetically modified *L. plantarum* WCFS1 expressing cancer testis antigen has been shown to evoke antigen-specific immunity, including T-cell responses in mice, thus indicating the potential of its use in cancer immunotherapy (4). Antitumor activity of several *L. plantarum* strains against colon cancer has been demonstrated *in vitro* (5, 6), as well as *in vivo* (7, 8). However, epidemiologic and clinical evidence are still lacking. Immunomodulatory properties and anticarcinogenic capacity may vary substantially among the strains (9, 10), therefore, efficacy of each strain needs to be precisely assessed.

L. plantarum LS/07, the new probiotic strain isolated from the gut of a healthy man, has been shown to exert a preventive effect on rat dimethylhydrazine-induced colon carcinogenesis when administered alone or in combination with prebiotic (oligofructose-enriched inulin) and plant bioactive compounds (chestnut extract and/or flaxseed oil) (11, 12). Authors reported the reduced number of coliform bacteria and the decreased activity of bacterial carcinogenic enzymes, which indicates the antimutagenic actions of this strain. Moreover, immunomodulatory and antiinflammatory properties have been recorded. Co-administration of *L. plantarum* LS/07 and bioactive compounds increase tumor necrosis factor α (TNFα) serum level (11) and decrease TNFα concentration in jejunal mucosa (13). When co-administered with prebiotic, *L. plantarum* LS/07 induces inhibition of proinflammatory (interleukin (IL)-6 and TNFα) cytokine production, suppression of NFκB activity and stimulation of antiinflammatory IL-10 cytokine synthesis in mucosal cells (14). Reduced serum concentration of TNFα has been observed in mammary tumor-bearing rats, as well (15). However, precise mechanisms of the immune response induced by *L. plantarum* LS/07 applied alone or in combination with other natural agents remain unclear.

Prebiotics are non-digestible food constituents that can promote the growth and activity of probiotic bacteria. Inulin and oligofructose, usually classified as dietary fibers, possess immunomodulatory (16) and anticancer properties (17). Oligofructose-enriched inulin enhanced the efficacy of *L. plantarum* LS/07 in mammary carcinogenesis in our previous study (15).

Melatonin is a naturally-occurring substance, a hormone that is primarily secreted by the pineal gland. Melatonin displays pleiotropic physiological functions in vertebrates (18), is involved mainly in the regulation of circadian rhythms. As a dietary supplement, melatonin is available in many countries over the counter and often used to treat insomnia or circadian rhythm disorders, such as jet-lag syndrome. Antineoplastic activities of melatonin have been proven in a variety of cancer types, especially in hormone-dependent tumors, such as breast cancer. Melatonin is known to counteract the proliferative effect of estrogens at the level of their gonadal synthesis, local production in extragonadal tissues and intracellular receptor activation as well (19). Numerous *in vitro* studies have demonstrated capability of melatonin to inhibit tumor cell proliferation, promote apoptosis, stimulate cell differentiation and suppress cancer cell invasion (20-22). Potent antioxidative, antiinflammatory and antiangiogenic properties have also been reported (23-25). Melatonin's immunomodulatory actions comprise stimulation of both innate and adaptive immunity (26, 27). Activation of lymphocytes, monocytes/macrophages and natural killer cells may be one of the key mechanisms in preventing tumor development by this hormone (28). Melatonin administration has been shown to stimulate cytokine production by cluster of differentiation 4-positive ($CD4^+$) T-lymphocytes (29). In addition, melatonin synthesis in lymphocytes has been proven (30) to have a substantial impact on local immune responses within tumor microenvironment. Due to melatonin's ability to improve efficacy of various drugs, it would be a very useful therapeutic tool for combination therapy (31). Nevertheless, its role in anticancer and immunoregulatory activities, when co-administered with probiotics and prebiotics, has not been established yet.

Breast cancer is the most frequently diagnosed cancer worldwide accounting for 25% of all cancer cases. Despite significant advances in diagnosis and treatment, it remains the leading cause of cancer death among women (32). Therefore, a lot of attention is devoted to finding innovative preventive strategies. Because of no toxicity and easy accessibility in healthy populations, probiotic bacteria, along with prebiotics and other immunomodulating agents, could be a good choice (33). The investigation of novel chemopreventives needs appropriate and validated animal models of carcinogenesis. 7,12-dimethylbenz/a/anthracene (DMBA) and N-nitroso-N-methylurea (NMU) are the two most widely used chemical inductors of mammary carcinogenesis in rodents (34). DMBA is a polycyclic aromatic hydrocarbon (PAH), a procarcinogen, which is usually administered orally or intragastrically, thus attacking the DNA after its passage through the gastrointestinal tract and subsequent metabolic activation in the target tissue, *i.e.* in mammary gland. On the other hand, NMU is a direct carcinogen administered intraperitoneally that does not require activation and directly induces point mutations on DNA in mammary tissue. Mammary tumors developed in both models are known to be hormone-dependent adenocarcinomas exhibiting many similarities with human breast cancer (35). Considering the promising antitumor and immunoenhancing effects of *L. plantarum* LS/07 and inulin in DMBA-induced mammary carcinogenesis in rats (15), the aim of this study was to evaluate efficacy of these agents in the NMU model. The probiotic and prebiotic under study were administered alone or in combination with melatonin that is considered to be a physiological anticancer substance (36) with potent immunomodulatory activities.

Materials and Methods

Animals. Ninety female rats of Sprague-Dawley strain (Velaz, Prague, Czech Republic) aged 30-35 days and weighing 90-119 g were used in the experiment. The animals were adapted to standard vivarium conditions with temperature of 22±2˚C, relative humidity of 55±10% and artificial 12:12 h light:dark regimen. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic (Accreditation No. Ro-1574/10-221 and Ro-2690/11-221).

Treatment. During the experiment, the rats were fed the 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*. Beneo™ Synergy 1 is a commercially available fiber food ingredient composed of long linear fructan chains (95%) and shorter oligofructose chains (5%), both obtained from chicory roots. The probiotic (PRO) *L. plantarum* LS/07 was kindly provided by the Institute of Experimental Medicine, Faculty of Medicine, P. J. Šafárik University in Košice (12). PRO was prepared in MRS broth (Merck, Darmstadt, Germany) at 37˚C aerobically to provide 3×10^9 colony-forming units (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. Melatonin ((MEL) Sigma, Deisenhofen, Germany) was administered in drinking water at a concentration of 20 mg/l *ad libitum*.

Induction of mammary carcinogenesis with NMU. Mammary carcinogenesis was initiated with two intraperitoneal doses (50 mg/kg of body weight (b.w.) each) of N-methyl-N-nitrosourea (Sigma, Deisenhofen, Germany) dissolved in isotonic saline solution. The first dose was injected on 45th postnatal day and the second one on the 53rd postnatal day on average.

Experimental design. Administration of PRO, PRE and MEL started two weeks before the first dose of NMU and lasted until the end of experiment (16 weeks). The animals were randomly divided into five experimental groups: (i) CONT (control group with NMU only); (ii) PRO (NMU + probiotic); (iii) PRE (NMU + prebiotic; (iv) PRO+PRE (NMU + combination of probiotic and prebiotic; (v) PRO+PRE+MEL (NMU + combination of probiotic, prebiotic and melatonin). Each group consisted of 18 animals.

All rats were weighed and palpated weekly for detection of the presence, number, location and size of each palpable tumor. Food and water intake was monitored during weeks 8 and 15 of the experiment. Based on average daily water consumption by rats drinking melatonin, the daily dose of melatonin per rat was 310.2 μg (1.2 mg/kg b.w./day).

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, as well as tumor volume (calculated according to the formula $V=\pi\times(S_1)^2\times S_2/12$; $S_1<S_2$, where S1 and S2 are tumor diameters). The fresh tissue samples of selected tumors (five of each experimental group) were used for preparation of single-cell suspension and subsequent determination of regulatory T-cells by flow cytometry. The tumor samples were fixed in formaldehyde and embedded in paraffin for histopathological analysis, as well as Ki-67, caspase-3, $CD4^+$ and $CD8^+$ T-cell immunohistochemistry.

Histopathological classification of tumors. All tumors were histopathologically classified according to the criteria for the classification of rat mammary tumors (37) by an experienced pathologist. An additional parameter, grade of invasive carcinomas, was determined. Tumor samples were divided into low-grade (LG) and high-grade (HG) carcinomas. The criteria for categorization (solidization, cell atypia, mitotic activity index and necrosis) were chosen according to the standard diagnostic method of classification. HG carcinomas were considered to be tumors with ≥2 positive criteria; LG carcinomas were tumors with ≤1 positive criterion.

Determination of Ki-67 and caspase-3 in tumor cells by immunohistochemistry. The detection of selected proteins was carried out by indirect immunohistochemical method on whole paraffin sections, utilizing commercially available rat-specific antibodies (Ki67, Dako, Glostrup, Denmark; caspase-3, Bioss, Woburn, MA, USA). After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 30 minutes. Sections were pretreated in a microwave generator for 15 min in 10 mM citrate buffer (pH 6.0) and incubated with the primary antibody in PBS containing 1 % BSA, for 60 min at room temperature (RT). The primary antibodies were visualized by a secondary staining system (EnVision, Dual Link System-HRP; Dako North America, Carpinteria, CA, USA) using diaminobenzidine tetrahydrochloride (DAB). Immunohistochemically detected antigen expression was evaluated by morphometric method. Expression of caspase-3 was analyzed in the cytoplasm of tumor cells, while Ki-67 was detected within the nuclei. Expression of proteins was quantified as the average percentage of antigen-positive area in standard fields (0.5655 mm^2) of tumor hot-spot areas. Morphometric analysis of the digital images was performed using QuickPHOTO MICRO software, version 3.0 (Promicra, Prague, Czech Republic).

Determination of $CD4^+$ and $CD8^+$ T-cells in blood by flow cytometry. Blood (25 μl) was collected in heparin-treated tubes and incubated with monoclonal antibodies 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) for 20 min at RT in the dark. Subsequently, samples were mixed with 500 μl of BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA), incubated for 10 min at RT in the dark, washed with phosphate buffered saline (PBS), centrifuged (300 × *g* for 5 min at RT), diluted with 200 μl of PBS and analyzed by BD FACSCalibur flow cytometer (BD Biosciences). Debris was eliminated by forward scatter and side scatter (FSC×SSC) gating and the proportions of lymphocyte subpopulations were expressed as percentages of total lymphocytes. The results were analyzed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Detection of $CD4^+$ and $CD8^+$ T-cells by immunohistochemistry. Four-micrometers tumor tissue sections on slides were deparaffinized, dehydrated by ethanol series and, then, washed in 10 mM PBS. Non-specific 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 from AbD Serotec, Düsseldorf, Germany) diluted at 5 μg/ml in PBS. Secondary antibody of biotinylated anti-mouse IgG (2 μg/ml in PBS; Vector 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 were examined using a light microscope with a digital camera. At least 10 different vision fields per section containing tumorous parenchyma and stroma were

Table I. *Effect of Lactobacillus plantarum, oligofructose-enriched inulin and melatonin on tumor growth parameters in N-nitroso-N-methylurea-induced mammary carcinogenesis in female rats.*

	CONT	PRO	PRE	PRO+PRE	PRO+PRE+MEL
Number of all animals/ tumor-bearing animals	18/17	16/16	17/16	17/16	17/15
Incidence (%)	94	100 (+6%)	94 (0%)	94 (0%)	88 (–6%)
Latency (days)	73.88±4.46	71.00±2.96 (–4%)	70.56±4.19 (–5%)	66.13±4.61 (–11%)	72.27±4.35 (–2%)
Frequency per group	5.61±0.83	3.38±0.66 (–40%)	4.06±0.84 (–28%)	3.82±0.58 (–32%)	4.47±0.92 (–20%)
Frequency per animal	5.94±0.81	3.38±0.66 (–43%)	4.31±0.85 (–27%)	4.06±0.57 (–32%)	5.07±0.94 (–15%)
Cumulative tumor volume (cm^3)	62.08	73.61 (+19%)	80.37 (+29%)	54.73 (–12%)	62.99 (+1%)

Data are expressed as the mean±S.E.M. Values in parentheses are the percentage deviation from the control NMU-treated group.

evaluated. Digital images were morphometrically analyzed by Ellipse v.2.0.7.1 software (ViDiTo, Košice, Slovakia).

Determination of rat regulatory T-cells in tumors by flow cytometry. Single-cell suspensions from fresh tumor tissue were prepared according to the protocol of Bayne and Vonderheide (38). Briefly, tumor samples were minced on ice into small fragments and incubated at 37°C for 30–45 min in an enzymatic cocktail (collagenase I (2.5 mg/ml), collagenase II (1.5 mg/ml), collagenase IV (1 mg/ml) and hyaluronidase IV (0.25 mg/ml) all from Worthington (Lakewood, NJ, USA) in RPMI 1640 medium (Sigma-Aldrich). Dissociated cells were passed through a 70 μm cell strainer twice and washed three times in RPMI-1640 medium supplemented with 10% fetal calf serum (PAA, Pasching, Austria), 2 mM L-glutamine (1:100 dilution of a 200 mM stock (Gibco, Paisley, Scotland, UK)) and 83 μg/ml gentamicin (1:600 dilution of a 50 mg/ml stock (Sigma-Aldrich). Single tumor cells were counted to monitor recovery, adjusted to desired cell concentration and used immediately for flow cytometry.

For detection of rat regulatory T-cells, Rat Regulatory T-Cell Multi-Color Flow Cytometry Kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions. Samples were analyzed by a flow cytometer BD FACSCAria II SORP (BD Biosciences). An acquisition gate was established based on FSC-A×SSC-A to exclude fragments of dead cells or debris. Data were evaluated and proportions of CD25+FoxP3+ population were expressed as percentages of total intact cells. The results were analyzed using FlowJo software (TreeStar Inc.).

Concentration of transforming growth factor β1 (TGFβ1) and interleukin 6 (IL-6) in serum. Enzyme-linked immunosorbent assay (ELISA) was utilized for determination of serum TGFβ1 and IL-6 concentrations. Commercial kits for TGFβ1 (Platinum ELISA kit; eBioscience Bender Medsystems, Vienna, Austria) and IL-6 (ELISA kit; Invitrogen, Camarillo, CA, USA) were used according to the manufacturer's instructions.

Statistical analysis. Tumor incidence and HG/LG ratio were evaluated by the Mann-Whitney *U*-test. Other parameters were evaluated 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, MEL and their combinations on rat mammary carcinogenesis are summarized in Table I. No significant differences were found among the groups when assessed in terms of tumor incidence, latency or volume. Administration of PRO, PRE, PRO+PRE and PRO+PRE+MEL led to moderate decrease in tumor frequency (per group, as well as per animal) in the range of 15% (in PRO+PRE+MEL group) to 43% (PRO group); nevertheless, statistically significant differences were not recorded.

Histopathological examination revealed the lowest number of invasive carcinomas (IC) and the highest number of *in situ* carcinomas (ISC), as well as benign tumors (BT) in the PRO+PRE+MEL group (18:56:3) among all experimental groups. In contrast, in the control group, the highest proportion of IC, lower number of ISC and no BT were detected (60:39:0). The IC:ISC:BT ratio in other groups was as follows: PRO=30:24:0; PRE=43:26:0; PRO+PRE=21:43:1. As a result of combination treatment with PRO+PRE and PRO+PRE+MEL, a marked decrease in the ratio of poorly differentiated (HG) and well-differentiated (LG) mammary tumors was found, which demonstrates an apparent shift to the induction of tumor cell differentiation, particularly after PRO+PRE+MEL administration (Figure 1). The differences were significant in comparison with controls, as well as PRE group.

Immunohistochemical analysis of rat mammary tumor cells showed a marked decrease in Ki-67 expression after PRO+PRE and PRO+PRE+MEL treatment as compared to control, PRO, as well as PRE group (Figure 1). Compared to controls, a significant positive correlation between histological grade and Ki67 expression in PRO+PRE+MEL group ($r=0.35$; $p=0.029$) was observed. The changes in the expression of caspase3 between controls and treated groups were not recorded; the percentage of caspase-3-positive tumor cells ranged from 19.01±4.68 in PRE group to 23.06±1.74 in control group.

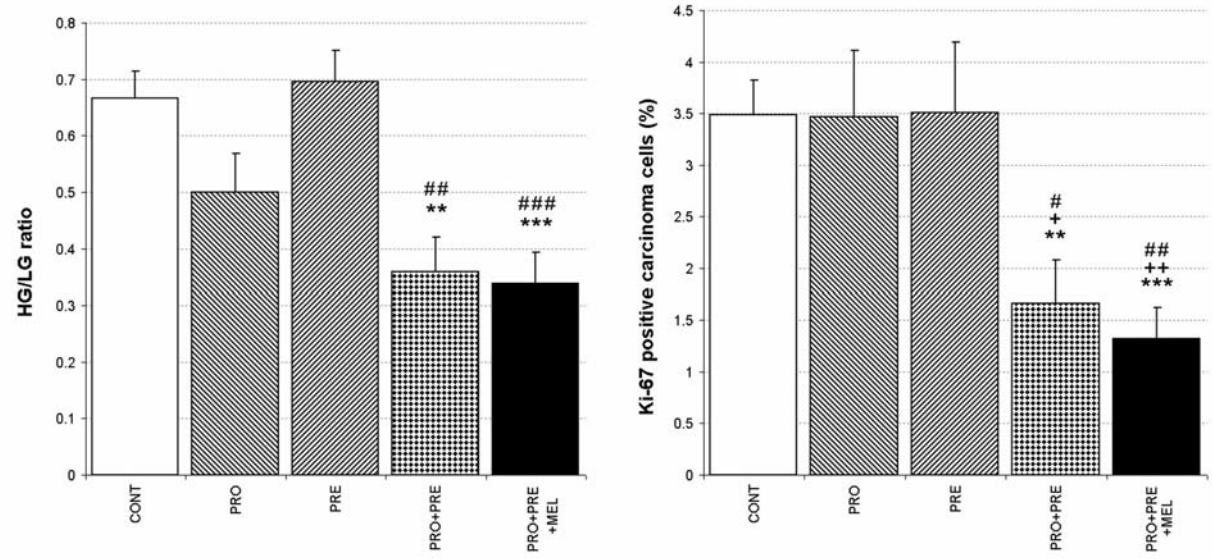

Figure 1. *Effect of Lactobacillus plantarum LS/07, oligofructose-enriched inulin and melatonin on high-grade/ low-grade (HG/LG) tumor ratio and percentage of Ki-67 positive carcinoma cells. Data for Ki-67 are expressed as mean±S.E.M. Significance versus CONT (**p<0.01; ***p<0.001) versus PRO ($^{+}p<0.05$; $^{++}p<0.01$) versus PRE ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$).*

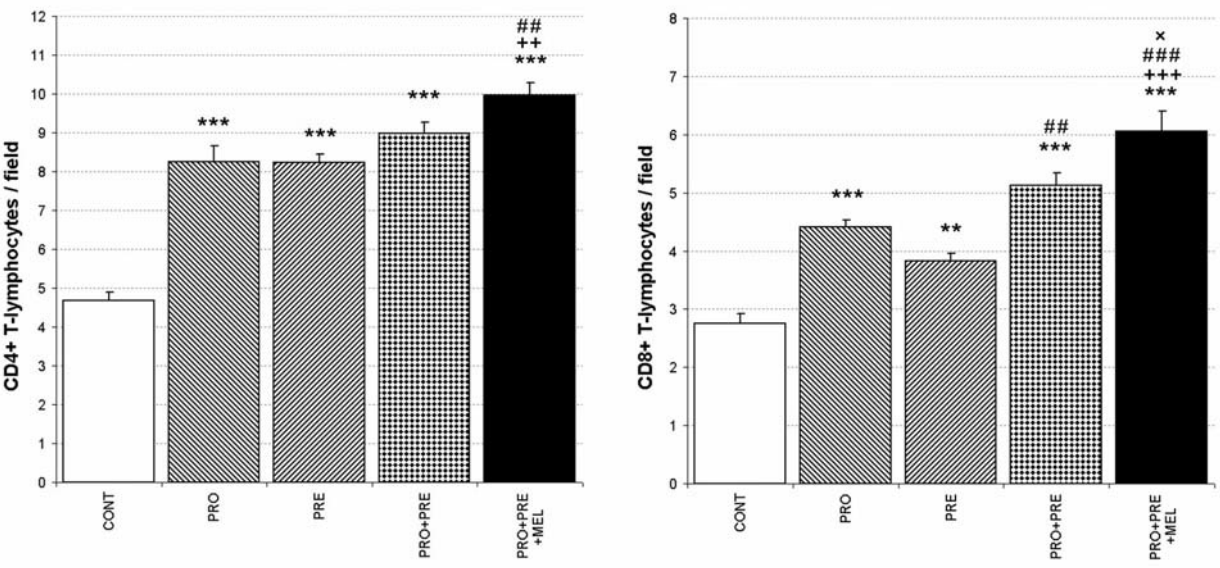

Figure 2. *Effect of Lactobacillus plantarum LS/07, oligofructose-enriched inulin and melatonin on CD4- and CD8-positive T-lymphocytes in tumor tissue. Data are expressed as mean±S.E.M. Significance versus CONT (**p<0.01; ***p<0.001) versus PRO ($^{++}p<0.01$; $^{+++}p<0.001$) versus PRE ($^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$) and versus PRO+PRE ($^{\times}p<0.05$).*

In tumor tissue, a significant increase in $CD4^+$ T-lymphocytes was observed in all treated groups compared to controls; the combination PRO+PRE+MEL was more effective than other treatments. Similar changes were seen in $CD8^+$ T-lymphocytes where PRO+PRE+MEL administration elicited a highly significant increase in $CD8^+$ T-lymphocytes as compared to all other experimental groups (Figure 2). The strong effect of PRO+PRE+MEL combination was found also on $CD25^+FoxP3^+$ regulatory T-lymphocytes where a distinct rise was detected in comparison with all groups except PRE (Figure 3).

In blood, no significant changes in the percentage of either $CD4^+$ or $CD8^+$ T-lymphocytes were registered. $CD4^+$ T-

lymphocyte values ranged from 37.51±1.68% in PRO group to 42.99±1.86% in PRE group. A similar trend was noted in $CD8^+$ T-lymphocytes; values ranged between 14.12±0.95% in PRO group and 16.69±0.84% in PRE group.

The serum TGFβ1 levels increased non-significantly in PRO (89.83±7.40 ng/ml) and PRO+PRE+MEL (83.32±6.54 ng/ml) groups compared to controls (73.78±4.47 ng/ml). No significant changes in serum IL-6 concentration were detected, with an absolute concentration ranging from 78.16±3.10 pg/ml in controls to 84.21±2.76 pg/ml in PRO+PRE+MEL group.

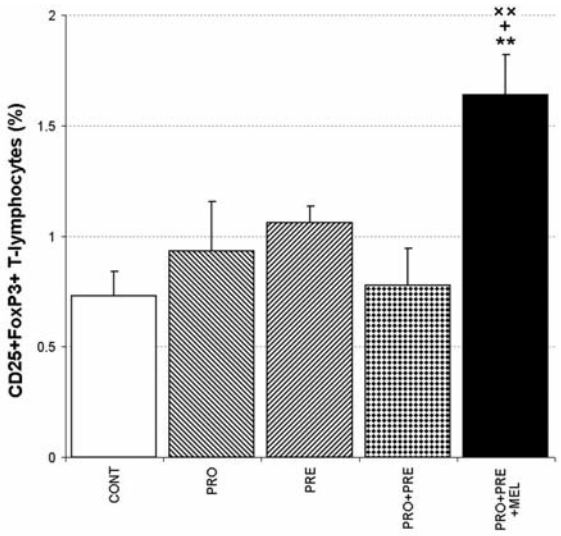

Figure 3. *Effect of Lactobacillus plantarum LS/07, oligofructose-enriched inulin and melatonin on percentage of CD25+FoxP3-positive T-lymphocytes in tumor tissue. Data are expressed as mean±S.E.M. Significance versus CONT (**p<0.01) versus PRO (+p<0.05) versus PRO+PRE (××p<0.01).*

Discussion

This is the first study evaluating the effect of co-administration of probiotic and prebiotic with melatonin in mammary carcinogenesis. Preventive and oncostatic activities of a new probiotic strain, *L. plantarum* LS/07, administered alone (PRO) or in combination with oligofructose-enriched inulin (PRO+PRE) in rat DMBA-induced mammary carcinogenesis were demonstrated in our previous study (15). In the present experiment, when mammary carcinogenesis was initiated with NMU, less extensive changes of the basic tumor growth parameters were recorded. The addition of melatonin to PRO+PRE combination did not improve these parameters. No changes were seen in tumor incidence, latency and cumulative tumor volume. Tumor frequency, the most sensitive indicator of response, was decreased in all treated groups, but only non-significantly. Since, in the present series, NMU was administered intraperitoneally, *i.e.* out of the gastrointestinal lumen, while DMBA had been applied intragastrically, we can speculate that the significant tumor growth inhibition observed in DMBA-treated animals, compared to NMU could be caused (at least partially) by the alteration of DMBA adsorption and/or metabolism by probiotic bacteria *L. plantarum* LS/07 in the intestinal lumen. Similarly, the ability of *L. plantarum* CICC 22135 strain to remove the other PAH - benzo(a)pyrene - from liquid medium under *in vitro* conditions has been reported by Zhao *et al.* (39). Carcinogen was bound to peptidoglycans of bacterial cell wall *via* physical adsorption. As binding was affected by temperature, incubation time, as well as pH, this ability could be strain-specific. To confirm, however, this hypothesis, further experiments are needed, particularly under *in vivo* conditions. From this point of view, the model selection may be important for investigation of the anticancer effects of probiotics because DMBA needs to be metabolized to an active carcinogen and probiotic bacteria could alter DMBA absorption into the blood, thereby acting as tumor anti-initiating agent. In contrast, NMU causes DNA point mutations and probiotics do not seem able to prevent them (40). This claim needs to be proven in experimental practice, too.

On the other hand, a significant impact of PRO+PRE and PRO+PRE+MEL combinations on the differentiation and proliferation of NMU-induced mammary tumors was found. The PRO+PRE administration induced a marked shift from poorly to well-differentiated carcinomas (*i.e.* the decrease in HG/LG tumor ratio), that was accompanied by a reduced percentage of tumor cells expressing Ki-67, as an endogenous cell proliferation marker. Melatonin supplementation amplified this response. Both these indicators suggest that long-term combination treatment with *L. plantarum* LS/07 and inulin administered with or without melatonin may improve prognosis of mammary tumors. The direct antiproliferative action of melatonin has been demonstrated in various estrogen receptor α (ERα)-positive and ERα-negative human breast tumor cell lines *in vitro* (reviewed in (22)) and proven by reduced Ki-67 expression in a xenograft mouse model (41). Among all groups in our experiment, the highest number of benign and *in situ* carcinomas, as well as the lowest number of invasive carcinomas, was found in PRO+PRE+MEL group. All these findings indicate that melatonin is able to enhance the antineoplastic activity of *L. plantarum* LS/07 and inulin combination in NMU mammary cancer model.

$CD4^+$ and $CD8^+$ T-lymphocytes are the key players in immune antitumor defense. $CD8^+$ cytotoxic T-cells have the potential to attack and kill cancer cells directly, whereas $CD4^+$ T-cells are a heterogeneous population of various cell types that may have different functions. Helper $CD4^+$ T-cells are involved in initiation and maintenance of adaptive antitumor immune response. Regulatory $CD4^+$ T-cells (Tregs), however, may operate as suppressors of anticancer immunity under certain conditions (42). Some clinical data have suggested that

the high tumor infiltration by helper type 1 (Th1) $CD4^+$ T-cells and $CD8^+$ T-cells has been positively associated with good prognosis for breast cancer patients (43).

In our experiment, $CD4^+$ and $CD8^+$ T-cell subsets were evaluated in two compartments, in the systemic circulation and the tumor tissue. Response of blood $CD4^+$ and $CD8^+$ T-cells to *L. plantarum* LS/07 and inulin administration seems to be dependent on the experimental model used. In our previous DMBA study, no changes in $CD4^+$ T-cells, along with the decline in $CD8^+$ T-cell count in blood, were seen after PRO was applied alone or in combination with PRE (15). In contrast, no significant differences in blood $CD4^+$ and $CD8^+$ T-cell number among groups were recorded in the present NMU experiment. These observations may be related to the aforementioned differences between DMBA and NMU tumor induction.

Within the tumor microenvironment, a marked enhancement of local immune response was observed after PRO, PRE, PRO+PRE and PRO+PRE+MEL administration in comparison with controls, which was seen as a significant rise in the number of tumor-infiltrating $CD4^+$, as well as $CD8^+$ T-cells. These findings are consistent with those from our previous DMBA study, which has shown an increased tumor $CD4^+$ and $CD8^+$ infiltration after PRO and PRO+PRE administration (15). Similar changes have been reported by Rachid *et al*. in 4T1 breast cancer cell-challenged mice treated with *L. helveticus*-fermented milk (44). Due to existence of common mucosal immune system, orally administered probiotic bacteria may improve local immunity in the mucosal/epithelial sites distant from gastrointestinal tract, *e.g*. in mammary gland (45, 46). In this context, tumor cells, occurring in mammary gland as a result of chemocarcinogen administration, may operate as a local stimulus that could possibly trigger the migration of activated immune cells (including T-cells) from Payer's patches to the breast tissue (47). The duration of probiotic treatment, as well as tumor development stage, may play a role in this process that needs to be investigated in future experiments. Inulin is known to increase the survival and activity of lactobacilli; therefore, combined PRO+PRE administration induced the stronger response in $CD4^+$ and $CD8^+$ T-cells tumor infiltration as compared to PRE and PRO administered alone. Melatonin supplementation augmented this response: the most significant rise in tumor $CD4^+$ and $CD8^+$ population was found in PRO+PRE+MEL group. Melatonin binding sites were identified in all B- and T-subsets of rat lymphocytes, including $CD4^+$ and $CD8^+$ T-cells (48). Our results have shown, for the first time, that melatonin is able to enhance the local immune response induced by combination of probiotic and prebiotic in mammary tumor tissue.

Tregs represent a special subset of $CD4^+$ cells whose hallmark is the constitutive expression of the forkhead box protein P3 (FoxP3) (49). Their role in cancer development remains controversial. Despite numerous reports indicating a positive association between Tregs and progression of solid tumors (50), a growing number of studies demonstrates that tumor-infiltrating Tregs can also be associated with a favorable prognosis (51, 52). In ER-negative breast cancer patients, a high degree of concurrent tumor infiltration by both $CD8^+$ T-cells and $FoxP3^+$ Tregs was correlated with robust antitumor immunity and good clinical outcome (53). Although NMU-induced rat mammary tumors are ER-positive (54), a significant increase in the percentage of tumor-infiltrating $CD25^+Foxp3^+$ Tregs has been observed in PRO+PRE+MEL-treated animals of our study, whereas no effect was seen after PRO, PRE and PRO+PRE administration. As melatonin possesses antiestrogenic activity (55), an inverse relationship between administered melatonin and endogenous estrogens could be linked to this effect. Considering an improved histopathological profile of PRO+PRE+MEL-treated rats, the high tumor infiltration by $CD4^+$, $CD8^+$ T-cells and $CD25^+FoxP3^+$ Tregs can be regarded as a sign of boosted immune response against tumors. The precise role of Tregs, melatonin, inulin and *L. plantarum* LS/07 in breast cancer inhibition remains to be elucidated.

TGFβ1 is a pleiotropic cytokine involved in the regulation of $CD4^+$ and $CD8^+$ T-cell proliferation (56), as well as in promotion of the peripheral Tregs' differentiation (57). In early stages of mammary carcinogenesis, TGFβ1 exerts tumor suppressive effects, inhibits epithelial cell-cycle progression and promotes apoptosis. In late stages, however, the association of TGFβ1 with increased tumor progression, cancer invasiveness and metastasis has been shown (58). Melatonin has been found to induce growth inhibition of MCF-7 breast cancer cells, which was associated to the activation of the TGFβ1 pathway (59). *L. acidophilus* administration to mammary tumor-bearing mice resulted in reduced production of TGFβ by cultured splenocytes (60). In our study, however, no significant changes in serum TGFβ1 levels were observed.

IL-6 is a proinflammatory cytokine that plays an important role in estrogen-dependent tumor growth due to stimulation of local estrogen-synthesizing enzymes in normal and malignant breast tissues (61). Moreover, the inhibitory effect of IL-6 to the development of Tregs has been reported (62). In mammary tumor-bearing mice fed with milk fermented by *L. helveticus*, the delay of tumor growth was related to a decrease of serum IL-6 levels (63). *L. plantarum* LS/07 administered alone or with inulin has been shown to inhibit IL-6 synthesis in intestinal mucosa (14). However, serum IL-6 concentrations did not differ among the groups in the present experiment.

In conclusion, our study revealed the strong potential of probiotic *L. plantarum* LS/07 strain administered with

inulin to promote differentiation, inhibit proliferation of tumor cells, as well as elicit the local immune response in tumor tissue in our rat NMU-induced breast cancer model. Melatonin amplified the antineoplastic and immunomodulatory activity of probiotic and prebiotic, although tumor growth was not suppressed significantly. This promising combination needs to be evaluated in future clinical studies.

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