ANTIPROLIFERATIVE EFFECT OF BULGARIAN SPRING WATER PROBIOTICS (LAKTERA NATURE PROBIOTIC®) AGAINST HUMAN COLON CARCINOMA CELL LINE

Kaloyan Georgiev¹*, Marieta Georgieva¹, Ivan Iliev², Maria Peneva³, Georgi Alexandrov⁴

¹Department of Preclinical and Clinical Sciences, Sector Pharmacology and Toxicology, Faculty of Pharmacy, Medical University Varna, Marin Drinov str. 55, 9002 Varna, Bulgaria.
²Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria.
³Medical Centre “Evrozdrave” Sofia, Sofia, Bulgaria.
⁴Military Medical Institute Academy – Sofia, Sofia, Bulgaria.

ABSTRACT
Colorectal carcinoma is a malignant disease, from which more than 1 million people worldwide suffer. Over the past two decades, its social significance attracts more attention. Colorectal cancer is the second leading cause of death from malignancy and the third most frequently diagnosed malignancy. It has been estimated that nutrition is the main reason for the high incidence of cancer. Probiotics are live microorganisms with proven health benefits in a variety of gastrointestinal diseases, and disorders outside the gastrointestinal tract. The aim of the present study is to explore cytotoxic and anti-proliferative effects of the new Laktera Nature Probiotic® on non-cancerous cell lines (BALB/3T3 and BJ) and colon adenocarcinoma cell line – HT-29. Laktera Nature Probiotic® has weak direct cytotoxicity against BALB/3T3 and BJ cells, but inhibits their proliferation in a dose-dependent manner. On carcinoma cell line – HT-29, in low concentration range (2-125 µg/ml) Laktera Nature Probiotic® stimulates proliferation; at high concentrations (250-1000 µg/ml) we detected concentration-dependent inhibition of proliferation. Laktera Nature Probiotic® has shown
promising primary screening results, which should be clarified as mechanisms in further works.

**KEYWORDS:** probiotics, cytotoxicity, colon carcinoma, antiproliferative effect.

**INTRODUCTION**

In recent years, incidence and mortality rates of cancer have increased in less developed countries. Colon cancer is the third most frequent form of cancer in males and the fourth in females, causing nearly 500,000 deaths every year.\(^1\) Before the twentieth century, colorectal cancer was relatively uncommon however the incidence has risen dramatically especially in the last fifty years. Several risk factors have been proposed including the adoption of westernized diet, obesity and physical inactivity.\(^2,3\) The majority of colorectal cancer continues to occur in industrialized countries. It has been estimated that nutrition could account for more than one third of cancer deaths,\(^4\) and that dietary factors are responsible for 70% to 90% of all cases. Therefore, diet optimization could potentially help to reduce the incidence of this type of malignancy.\(^5,6\)

Ilya Ilyich Mechnikov, Noble Prize winner in 1908, was the first who describes the concept of probiotic, by explaining long life of Bulgarian peasants with consumption of fermented milk products.\(^7\) The term "probiotic" – derived from the Greek word, meaning "for life", was first used in 1965, by Lilly and Stillwell for describing substances secreted by one organism which stimulate the growth of another.\(^8\) Recently, a growing number of studies has shown the various roles of probiotics and has highlighted their many health benefits. Probiotics are live microorganisms (in most cases, bacteria, in rare cases yeasts) belonging mainly to the genera *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Enterococcus*, *Pediococcus* or *Saccharomyces*, as microorganisms from the genus *Lactobacillus* are the most often used. The major use of probiotics is in prevention and treatment of gastro-intestinal related disorders – infantile diarrhoea, necrotizing enterocolitis, antibiotic associated diarrhoea, relapsing *Clostridium difficile* colitis, *Helicobacter pylori* infections, inflammatory bowel disease, colorectal cancer, but more data has become available on extra-intestinal indications.\(^9\) Recent studies indicate possible antioxidant properties of probiotics.\(^10\)

For the first time in the world Daflorn Ltd has isolated 8 all natural, original strains of probiotics from spring water in Bulgaria. They are unique probiotics – *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus lactis* and 5 new strains of *Streptococcus*
thermophilus. Due to their completely natural origin all these new strains have maximum stability and survive during production, and rehydration, when passing through the gastrointestinal tract of humans and keep their number and vitality for more than 2 years as compared with the normal conditions of storage in shops and at home.[11]

In order to investigate the potential cytotoxicity and anti-proliferative effects of the newly product of Daflorn Ltd – Lactera Nature Probiotic®, we used non-cancerous cell lines – BALB/3T3 and BJ and colon adenocarcinoma cell line – HT-29.

MATERIALS AND METHODS

Reagents
Laktera Nature Probiotic® was kindly provided by the company Daflorn MLM 5 Ltd. (Sofia, Bulgaria). 1 mg of the substance contained 25 x 10⁶ live and latent CFU Lactobacillus bulgaricus DWT1, Lactobacillus helveticus DWT2, Lactobacillus lactis DWT3 and Streptococcus thermophilus DWT 4, 5, 6, 7, 8. It also contain lipids (0,18 mg), carbohydrates (0,56 mg), proteins (0,25 mg) and lactose (0,011 mg). Before instillation in the wells, the substance was dissolved in DMSO (Sigma Chemical Co.).

Cell cultures
The BALB/3T3 clone A31 (standard mouse embryonic fibroblast cell line), BJ (human skin fibroblast cell line) and HT-29 (human colon cancer cell line) cells were cultured in Dulbecco Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Austria), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Lonza, Belgium) under 5% CO₂ atmosphere at 37°C. Plastic flasks supplied by Greiner, Germany, were used to grow the cells. For experiments the cells in exponential phase of growth after treatment with trypsin-EDTA (FlowLab, Australia) were seeded into 96-well plates (Greiner, Germany) in a concentration 2x10⁴ cells/well.

Cell viability assay
24-hours post seeding, the cultivated cells were treated with probiotic mixture in a wide concentration range (2-1000 μg/ml, double increasing manner). Untreated cells were used as controls. Cytotoxicity was measured by colorimetric assay based on tetrazolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Chemical Co.). The assay was performed 24- and 72-hours after treatment with the probiotic as described elsewhere.[12] ELISA plate reader (TECAN, Sunrise TM, Grodig/Sazburg, Austria) was used
for reading the results. Optical density was determined at a wavelength of 540 nm and a reference wavelength of 620 nm. Cell cytotoxicity determined by MTT assay was expressed as per cent of untreated control.

**Statistical Analysis**

In all experiments, data were presented as means ±SD. One-way and two-way analysis of variance (ANOVA) was used to determine significance between tested groups. Analysis was performed using SigmaPlot 11.0 software. A probability level of 0.05 or lower was considered as statistically significant.

**RESULTS AND DISCUSSION**

In the first set of our study, we wanted to observe cytotoxic and anti-proliferative activity of the new probiotic on non-carcinoma cell lines – BJ and 3T3 cells. The concentrations we used were in the range of 2-1000 μg/ml end-well concentration. On both cell lines, the new probiotic didn’t exert significant cytotoxicity. When used at higher concentrations – 1 mg/ml, the cytotoxicity did not exceed 25% (fig.1).

![Figure 1. Cytotoxicity measured by MTT-assay](image)

**A.** Laktera Nature Probiotic® used in concentrations range of 2-1000 μg/ml on BALB/3T3 cell line.

**B.** Laktera Nature Probiotic® used in concentrations range of 2-1000 μg/ml on BJ cell line.

The same approach was used in testing for anti-proliferative activity. On both cell lines, probiotic have shown dose-dependent inhibitory effect on proliferation (fig.2). BALB/3T3
cells were less sensitive to the action of probiotic. The probiotic concentrations of 2-125 μg/ml slightly affect the proliferation of these cells. Only the highest concentrations of 250-1000 μg/ml showed a marked reduction in the proliferation of BALB/3T3 cells. The IC\textsubscript{50} value were 544.66±39.89 μg/ml. Slightly more sensitive were the BJ cells. Even low probiotic concentrations, as 30-60 μg/ml, have shown approximately 20% inhibition of proliferation. However, the IC\textsubscript{50} value – 583.96±89.23 μg/ml, was higher than that on BALB/3T3 cells.

![A 3T3/BALB cell line treated with Laktera Nature Probiotic\textsuperscript{®} used in concentrations range of 2-1000 μg/ml on BALB/3T3 cell line.](image1)

![A BJ cell line treated with Laktera Nature Probiotic\textsuperscript{®} used in concentrations range of 2-1000 μg/ml on BJ cell line.](image2)

Figure 2. Anti-proliferative action measured by MTT-assay
A. Laktera Nature Probiotic\textsuperscript{®} used in concentrations range of 2-1000 μg/ml on BALB/3T3 cell line.
B. Laktera Nature Probiotic\textsuperscript{®} used in concentrations range of 2-1000 μg/ml on BJ cell line.

In the second set of our study, we tried to explore anti-proliferative activity of the new probiotic on carcinoma cell line HT-29. In a low concentration range of Laktera Nature Probiotic\textsuperscript{®} from 2-125 μg/ml, we observed a stimulation of proliferation. Further, in the highest used concentrations – 250-1000 μg/ml, it was established a dose-dependent manner of inhibition of proliferation (fig.3). Perhaps the increased proliferation observed in low concentrations is due to the extra nutrients (e.g. amino acids, etc.), which are present in the powder of probiotic. Nevertheless we were able to calculate the value of IC\textsubscript{50}, which were 711.69±82.15 μg/ml.
The possible mechanism by which the new probiotic exert growth inhibition of BALB/3T3, BJ and HT-29 cell lines at this stage we cannot define. There are several probable mechanisms by which probiotic bacteria may suppress colon cancer development. Those mechanisms involve enhancing the host’s immune response (increased production of cytokines, which inhibit tumor growth), altering enzyme activities (NADPH-cytochrome P-450 reductase, glutathione S-transferase, COX-2) in the colon, reducing the mutagenicity by inhibiting the uptake of potential carcinogens, or producing anti-proliferative and anti-tumorigenic compounds, which are united in the following review. In an experimental *in vitro* and *in vivo* study, Ma et al. showed that the probiotic *Bacillus polyfermenticus*, suppressed colon cancer cells growth through the reduction of ErbB2 and ErbB3 receptors (known to have essential roles in tumor development). In subsequent studies we have set out to examine precisely the mentioned above biochemical markers and to get more clarity on the mechanism of action of Laktera Nature Probiotic®.

**CONCLUSION**

It has been shown that probiotics inhibit the growth of cells of colon cancer. Their effect on the provision of bowel movements also reduces the risk of colon cancer, thus reducing the time during which the intestines are exposed to carcinogens in the diet. Therefore, probiotics take part in the processes of detoxification. Laktera Nature Probiotic® has shown promising primary screening results – on one hand, low direct cytotoxicity and on the other hand dose-dependent inhibition of proliferation on colon carcinoma cell line – HT-29. Further studies are needed to clarify the exact mechanism of action, to define the proper dosage and to establish the real usefulness in colorectal cancer.
REFERENCES


