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Original article

Antioxidant and antimicrobial activity of bacteriocin-producing strains of lactic acid bacteria isolated from the human gastrointestinal tract

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Summary. *Background:* cancer patients require special attention to maintenance therapy of their organism vital activity. Distribution of nutritional deficiency in cancer patients not only limits the possibilities of application of modern treatment methods, but can also be the direct cause of life-threatening complications and premature death. *Objective:* to study the use of bioactive peptide complexes with low molecular weight and probiotic strains isolated from the human gastrointestinal tract in food products intended for patients with cancer during their treatment and rehabilitation. *Material and Methods:* 14 strains of lactic acid bacteria were pre-selected and identified for this study. They were allocated from the gastrointestinal tract of 500 healthy people and people with cancer, who presented different age groups. The study of antagonistic activity of lactic acid bacteria was carried out by using the MRS medium and Lysogeny broth (LB). Biocompatibility study was performed through the co-culture method on solid MRS medium. *Results:* Research results proved that the isolated metabolites belonged to bacteriocins, as the studied microorganisms were resistant both to their inhibitors and mutually resistant to the single-type synthesized substances. *Conclusions.* Probiotics have a positive effect on the immune system, as the gastro-intestinal flora they reduce the impact of mutagens and carcinogens.

Key words: probiotic strains, bioactive peptides, bacteriocins, gastrointestinal tract, gastrointestinal microbiome

Introduction

During the last decades, a change in approaches to managing patients, cancer patients, in particular, significantly improved the patients' quality of life. Maintenance therapy, which involves a variety of techniques to ensure the functioning of the cancer patient's body both during and after treatment, plays an important role (1-3). Changes occur in metabolic processes of cancer patients' body, which requires a special nutritional diet (4-6). Since such patients are often unable to consume food in sufficient quantities, nutritional deficiency progresses, which can lead to cachexia, anorexia syndrome.

According to the report of ESPEN (European Society for Clinical Nutrition and Metabolism, 2000), the incidence of nutritional deficiency in cancer patients ranges from 46 to 88%. A study conducted by ECOG in 2003, which included 3047 cancer patients, showed that the frequency of nutritional deficiency with tumor lesions of the gastrointestinal tract varies from 70 to 83%. Nutritional deficiencies occur most frequently with gastric cancer and pancreatic cancer (75-80%), the localization of the tumor in the lungs,

colon, prostate (54-64%), breasts, with various sarcomas, hematological malignancies (31-40%). Nutritional deficiency, reaching its peak in the form of anorexia-cachexia syndrome, could be the direct cause of death in four out of 20 cancer patients. Recent studies have shown that nutritional deficiency has a direct correlation with the survival median, especially in patients with colorectal cancer. It should be noted that the anorexia-cachexia syndrome is aggravated as it evolves into the so-called iatrogenic syndrome of anorexia-cachexia during combination therapy. Body weight loss during treatment is over 10% and may occur in 45% of patients.

For many years, oncologists have wondered whether intensive nutrition of cancer patients caused tumor growth. Research data on the effect of nutritional support on tumor growth was presented at the ESPEN Congress in 2006. The presented data and discussion of this issue by members of the ESPEN-ESMO consensus group showed that nutritional support in oncology was similar to that of any other branch of clinical medicine and had no effect on tumor growth.

The importance of monitoring the nutritional status and conducting adequate and timely nutritional support of cancer patients is supported by many studies. For example, Snegovoi et al. (2009) showed that the use of technologies in nutritional support of cancer patients in modern practice is far from routine. One of the possible conditions for a wider introduction of nutritional support is the validation of its clinical effectiveness and feasibility, as well as the lack of effect on tumor growth.

Konovalova et al. (7) presents data on the nutritional status of children with cancer in remission, according to bioimpedance analysis. The comparison of children, who recovered from cancer and were at a clinic (main group, n = 552), with a group of healthy children (control group, n = 1500) showed significant differences in body length, composition indicators of the component and the magnitude of the phase angle of impedance. The greatest changes, in comparison with the control group, were found in standardized tested values of the phase angle, which reflected the percentage of active cell mass in the lean body mass. Nutritional status disorders, determined by the occurrence of obesity and low values of the phase angle, were observed in 52.7% of children in the main group; in the subgroup of children with tumors of the central nervous system, such disorders were observed in 76.8% of cases. Due to eating disorders, reduced tolerance to chemotherapy, increased susceptibility to infections, and increased frequency of adverse outcomes, bioimpedance analysis should be used to monitor the nutritional status of children with cancer for the purpose of timely correction of disorders and prevention of delayed risks.

Vashura et al. (8) concluded that children in the early post-transplant period (up to + 100 day) had a significantly deteriorating nutritional status and a developing tissue imbalance with relative predominance of the fatty component and a decrease in the somatic protein pool. The paper substantiates the importance a comprehensive assessment and monitoring of the nutritional status for the development of a nutritional support strategy for children after hematopoietic stem cell transplantation.

The general goals of nutritional support for cancer patients are as follows: to correct malnutrition, to maintain a pool of visceral protein, to minimize the side effects of radiation and chemotherapy, to prevent and treat immunosuppression, and to improve the patients' quality of life.

There are a number of requirements to the nutrient mixture of cancer patients. Nowadays, there is a wide range of products, applicable to each individual nosology.

For enteral nutrition products, the quality and quantity of protein, included in their composition, is important. In modern mixtures for enteral support of cancer patients, the proteinaceous ingredient may be in one of three forms: native protein, whey peptides/whole cow's milk protein, and free amino acids.

The use of the native protein in mixtures for support of cancer patients is impractical because its application requires compliance with certain conditions and, above all, the normalization of cavity and parietal digestion. In addition, the large mass of the native protein reduces the level of tolerance and significantly slows the rate of absorption.

Mixtures, based on free amino acids, appear to be more preferable due to the low molecular weight of the main component, which obviously indicates a

high rate of absorption. However, even under normal conditions, 2/3 of nitrogen in the intestine is absorbed in the form of peptides (peptides have distinct mechanisms of passive transport, while free amino acids are transported by a specific active transport system); under stress and starvation with developing suction, peptides are even higher, which dramatically reduces the efficiency of the nutrient mixture, based on free amino acids. Some data suggests that mixtures, based on amino acids, as well as parenteral feeding and starvation causes atrophy of the intestinal mucosa.

The main way for the intestine to receive peptides is absorption through the cells via the membrane, or the use of lint-carrying molecules, or diffuse through the lipid portions in membranes. Furthermore, it is noted that peptides can be absorbed by passing intestine villi (including points with mucosa defects). In addition, the absorption of peptides does not require the enzyme activity of the pancreas. Peptides have great reduction potential, while the peptide diet supports the liver more efficiently and, consequently, contributes to the synthesis of visceral proteins (9-12).

Biologically active peptides play an important role in the protein diet of cancer patients. Peptide bioregulators are short chains of amino acids, originally isolated from animal organs and tissues, and then artificially recreated in the laboratory.

The biological value of short peptides is their ability to restore functional impairments and prevent the development of pathological processes in organs and tissues, from which they were originally isolated. The area of biologically active peptides is very wide. They affect the cardiovascular, immune, reproductive, endocrine, digestive, and other systems, and change the energy metabolism in the body. One of their prominent features is multi-functionality (the same peptide can modulate the work of many body systems). Moreover, their fragments, which emerge during the decay in the body, may have their own physiological activity.

In particular, there are known peptides with immunomodulatory and antitumor properties, isolated from extracts of plants used in traditional medicine to treat tumor diseases (St. John's wort, elecampane, celandine and chaga mushroom).

There is rich experience in the clinical use of peptide drugs of thymic origin (Thymalinum, Tactivinum, Thymoptinum), which are widely used in the treatment of cancer patients. The primary cell target for preparations of thymic origin are T-lymphocytes. Thymic preparations affect the proliferation and differentiation of T-cells; they are able to induce the production of substances in the body with thymosin like activity, IF and TNF. Thymus preparations are applied at all stages of antitumor treatment: during breast cancer radiation therapy, with endometrial cancer, lung cancer, during polychemotherapy (PCT) with breast cancer and lymphogranulematosis, in the postoperative period, after radiation therapy, and in the intervals between PCT courses with carcinomas of different localization. At the same time, all researchers observed an increase in stability and leuko- lymphopoiesis, preservation or restoration of the level of lymphocyte response to mitogenic stimuli and a reduction in the occurrence of complications after radiotherapy and polychemotherapy (13).

In a study, a peptide antagonist of leptin receptor (ObR) with peripheral antitumor activity was designed (14). The basic compound (Allo-aca) passed the blood-brain barrier (BBB). Redesign of Allo-aca leads to the production of a compound that prevents the occurrence of cancer.

Lin et al. (15) studied epinecidin-1, an antimicrobial peptide from fish *(Epinephelus coioides)* with an antitumor activity, similar to that of peptides in human fibrosarcoma cells. The cytotoxicity of epinecidin-1 was investigated on various cancer cells (A549, HA59T/VGH, HeLa, HepG2, HT1080, RAW264.7, and U937) and healthy cells (AML-12, NIH3T3, and WS-1) using the MTT assay. It is shown that epinecidin-1 exhibits anti-tumor activity against cells of HT1080.

At present, there are several ways to obtain biologically active peptides: chemical synthesis, isolation from animal organs and tissues, and genetic engineering (using genetically engineered microorganism-producers of biologically active peptides). However, all known techniques have disadvantages. For example, the disadvantage of chemical synthesis is the long and labor-intensive multistep purification of byproducts. The second technique is complex and multistage, with a high probability of bacterial and viral contamination of peptides. The main disadvantage of genetic

engineering is the impossibility to use genetically engineered peptides for food production, in accordance with the law and ethical standards.

The analysis of published data shows a promising source of peptides with a wide spectrum of biological activities: probiotic microorganisms that produce antimicrobial metabolites – bacteriocins of peptide origin.

A great number of Gram-positive (+) and Gram-negative (-) bacteria produce during their growth substances of protein structure (either proteins or polypeptides) with antimicrobial activities, called bacteriocins (16). Although bacteriocins could be categorized as antibiotics, but they, in fact, are not. The major difference between bacteriocins and antibiotics is that bacteriocins restrict their activity to strains of species, related to the producing species, particularly to strains of the same species; antibiotics, on the other hand, have a wider activity spectrum and even if their activity is restricted, this does not show any preferential effect on closely related strains. In addition, bacteriocins are ribosomally synthesized and produced during the primary phase of growth, although antibiotics are usually secondary metabolites. Nowadays, particular attention among the Gram-positive (+) bacteria is paid to lactic acid bacteria (LAB), especially *Lactobacilli*, due to the production of bacteriocins. These substances can be used in the food industry as natural preservatives. The use of LAB and their metabolic products is generally considered safe (GRAS, Grade One). The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven efficient. Nisin is the only bacteriocin that has been officially applied in the food industry, its use having been approved worldwide. Bacteriocins can be applied in purified or crude form or by using a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing or incorporated through a bacteriocin-producing strain (starter culture).

Amaretti et al. studied the antioxidant properties of potentially probiotic bacteria. Thirty-four strains of lactic acid bacteria (seven Bifidobacterium, 11 Lactobacillus, six Lactococcus, and 10 Streptococcus thermophilus) were assayed in vitro for antioxidant activity against ascorbic and linolenic acid oxidation (TAA(AA) and TAA(LA)), trolox-equivalent antioxidant capacity (TEAC), intracellular glutathione (TGSH), and superoxide dismutase (SOD). Wide dispersion of each of TAA(AA), TAA(LA), TEAC, TGSH, and SOD occurred within bacterial groups, indicating that antioxidative properties are strain specific (30). The strains Bifidobacterium animalis subsp. lactis DSMZ 23032, Lactobacillus acidophilus DSMZ 23033, and Lactobacillus brevis DSMZ 23034 exhibited some of the highest TAA(AA), TAA(LA), TEAC, and TGSH values within the lactobacilli and bifidobacteria. These strains were used to prepare a potentially antioxidative probiotic formulation, which was administered to rats at a dose of 10(7), 10(8), and 10(9) cfu/day for 18 days. The probiotic strains colonized the colon of rats during the trial and promoted intestinal saccharolytic metabolism. The analysis of plasma antioxidant activity, reactive oxygen molecules level, and glutathione concentration revealed that when administered at doses of at least 10(8) cfu/day, the antioxidant mixture effectively reduced doxorubicin-induced oxidative stress. Probiotic strains, which are capable of limiting excessive amounts of reactive radicals in vivo, may help to prevent and control several diseases associated with oxidative stress.

The study investigates the potential of lactic acid bacteria metabolites in functional food products for cancer patients during rehabilitation, based on bioactive peptide complexes of low molecular weight. Used probiotic strains were previously isolated from human gastrointestinal tract; 14 strains of lactic acid bacteria were identified.

Materials and Methods

Lactic acid bacteria isolates, cultivation conditions

14 strains of lactic acid bacteria, designated as K1 – Bifidobacterium bifidum, K2 – Bifidobacterium breve, K3 – Bifidobacterium longum, K4 – Bifidobacterium adolescentis, K5 – Lactobacillus plantarum, K6 – Lactobacillus acidophilus, K7 – Lactobacillus rhamnosus, K8 – Lactobacillus paracasei, K9 – Lactobacillus fermentum, K10 – Lactobacillus salivarius, K11 – Lactobacillus casei, K12 – Lactobacillus reuteri, K13 – Streptococcus agalactiae, K14 – Enterococcus faecium were preselected and identified for this study. They were

isolated from the gastrointestinal tract of 500 healthy people and people with cancer of different age groups (Russian Federation). The study of the contents of the human gastrointestinal tract does not require registering special permits, because work on isolated microorganisms was performed on human feces, hence ethical issues in this case were uninvolved.

Pure cultures were stored at 4°C ± 2 in lyophilized form. Before determination, the strains were precultured twice in anaerobic conditions (Biostat A plus MO fermenter, Sartorius, USA) in an MRS-medium (HiMedia Laboratories Pvt. Limited, India) for 24 hours at 37°C. The cultivation of bifidobacteria, streptococci, and enterococci was performed in a bifidum medium (HiMedia Laboratories Pvt. Limited, India) on selective agar for Streptococcus (HiMedia Laboratories Pvt. Limited, India) and in a medium-Slate Bartley for enterococci (HiMedia Laboratories Pvt. Limited, India), respectively.

At night, the cultures of microorganisms were collected by centrifugation (3500 r/min, 30 min, 4°C) and washed twice with phosphate buffered saline (PBS). The cell concentration was adjusted to 105 CFU/ml; cells were prepared by heating the bacteria up to 95°C for 1 hour. After the heat treatment, the cells were washed with PBS and resuspended in a suitable medium for cell viability assays. For fractionation, cell cultures were centrifuged (3500 r/min, 30 min, 4°C). The cell pellet was washed and resuspended in 100 ml of PBS for 20 min (with minute intervals); sonication was performed in cold water (4°C). Cell debris were removed by centrifugation (14,000 r/min, 1 h); supernatants were sterilized by filtration (pore size of 0.22 nm; Sartorious, Goettingen, Germany).

Exponential LAB cultures were used as inoculum for antibacterial and anti-cancer tests as follows: test or cancer cells were grown with suspension cells containing the harvested cells of lactic acid bacteria (~10^5 CFU/ml of log phase culture of each strain consortium) for 24 hours and then transferred into the chamber.

The test cultures, the medium and cultivation conditions

Escherichia coli B-6954, Bacillus fastidiosus B-5651, Pseudomonas fluorescens B-3502, Pseudomonas aeruginosa ATCC 9027, Leuconostoc mesenteroides B-8404, Candida albicans ATCC 885-653, and Staphylococcus aureus ATCC 25923 were taken as test strains from the collection of the Biotechnology Research Institute laboratory of the Kemerovo Institute of Food Science and Technology (University). The slurry of night broth cultures of test strains grown in a standard nutrient medium were used for the work. The number of microorganisms (titer) in the suspension was determined by optical density (OD) at a wavelength of 595 nm.

The test culture was cultivated in meat-peptone agar (MPA) for 24 hours at 37°C; cells were collected from the agar surface by microbiological loop and resuspended in a 109 NaCl solution. Bacilli were grown in MRS-broth for 24 hours at 37°C, the culture fluid was then centrifuged at 2000 rev/min for 10 min and the supernatant was separated. For cell separation, the supernatant was filtered through Millex-GV (0.22 mu.m, Nihon «Millipore», USA). 180 ul of each culture of Bacillus genus were added to the plate; then, 20 ul of test culture strains were added to each culture. The plate was incubated at 37°C for 24 hrs. 200 ul of sterile MRS-broth and 180 ul of clean medium with 20 ul solution of each pathogen were used as controls. Bacterial growth was monitored by measuring the optical density during culturing (31).

MRS medium and Lysogeny broth (LB) were used to examine the antagonistic activity of lactic acid bacteria. Ingredients of culture media were dissolved in 1 dm^3 of distilled water, heated until the agar melted completely, distributed into tubes or flasks, and sterilized for 1 hour at a temperature of 121°C.

Isolation of bacteriocins

Isolation and purification of bacteriocins was performed by the technique, described in Russian patent No. 2492231 (32): culture liquid is concentrated on hollow fibers, then dry NaCl is added, and mixed in a shaker; the suspension is centrifuged, supernatant is adjusted to pH 3.0, the resulting suspension is centrifuged, water is added to the precipitate, then the precipitate is suspended and alcohol is added, incubated for 30 min at 0°C, centrifuged, then alcohol is removed from the solution by evaporation, water is added and activated charcoal is centrifuged to remove impurities adsorbed on carbon, the aqueous solution is passed through the membrane.

The lipid bilayer structures and their oxidation products

Liposomes were prepared by injection (33). Five ml of distilled water or an appropriate buffer solution with constant vigorous stirring were rapidly injected by syringe into 0.25 ml of the desired concentration of phospholipid in ethanol. The liposomes were further exposed to spontaneous and induced oxidation at a temperature of 37°C.

Lipid peroxidation products were labeled: carbonyl compounds – PR1, substances, which react to 2-thiobarbituric acid – PR2, conjugated diene – PR3, crotonaldehyde – PR4.

The concentration of carbonyl compounds was determined with N-(2,4-dinitrophenyl) hydrazine. 0.05 ml of the liposome suspension was added with 0.2 ml of 5 mM solution of (2,4-dinitrophenyl) hydrazine (34). After 10 minutes, 1 ml of 0.75 M NaOH was added to the reaction mixture. After 10 minutes, the absorbance of the reaction mixture was measured with a UV 1800 spectrophotometer (Shimadzu, Japan) at a wavelength of 460 nm.

The concentration of substances in the suspension of liposomes reacting with 2-thiobarbituric acid was determined by the technique, described in (34) (Esterbauer H an Cheeseman KH, 1990). 0.5 ml of 0.92 M of trichloroacetic acid and 1 ml of 49 mM 2-thiobarbituric acid was added to 0.5 ml of liposome suspension, heated for 15 minutes in a water bath, and centrifuged for 10 minutes at 3000 g. The supernatant was subjected to photometric measurements at wavelengths of 452 and 532 nm with a UV 1800 spectrophotometer (Shimadzu, Japan).

Conjugated dienes in a suspension of liposomes were determined by the technique, suggested in (35). 0.2 ml of liposome suspension was extracted with 2 ml of heptane - 2-propanol (1: 1) for 1 min with vigorous shaking, after which 0.2 ml of water was added to the system. After 10 minutes, 0.2 mL was collected from the upper heptane phase, diluted with 0.2 ml ethanol, and subjected to photometric measurements at 232 nm with a UV 1800 spectrophotometer (Shimadzu, Japan).

The concentration of crotonaldehyde in the liposome suspension was determined by photometry of the heptane extract at 220 nm.

Disc diffusion method

Antimicrobial activity of lactobacilli strains was determined by agar disk diffusion assay (36). The antimicrobial activity was assessed by measuring the inhibition zones against the microorganism culture test (37).

LAB antioxidant properties

The efficiency of the chemical compound antioxidant activity in each series of experiments and for each duration of liposomes oxidation in the model system of microorganisms, isolated from the gastrointestinal tract, is determined by the following formula:

$$EAA = \frac{C_0 - C_1}{C_0} \cdot 100\%$$

where C0 is the concentration of carbonyl compounds in the liposome suspension that does not contain test organisms (Control), C1 is concentration of carbonyl compounds in the liposome suspension containing test microorganisms (Experiment). If the calculated value > 0, it is considered that the studied microorganism inhibits lipid peroxidation, if < 0, then the studied bacteria reinforces lipid oxidation, i.e. they are a pro-oxidant.

LAB biocompatibility

The study of biocompatibility was carried out by the co-culture technique in a solid MRS medium. The overnight culture grown in a liquid medium and standardized by the turbidity standard was applied on the surface of a dense nutrient medium by bacteriological loop 3 mm in diameter. After the drop is absorbed, a drop of another test culture is applied on the surface of the same medium 1-2 mm from the edge of the previous one in the same volume, which, after spreading, covers about half of the first drop. In the overlay of the developed cultures in mutual presence (co-cultivation), the cultures were competing with each other. After the second drop dried, cups with crops were turned upside down and incubated at 37-39°C in air with increased carbon dioxide concentration. Each experiment was repeated, changing the position of crops (to avoid the impact of successive layers of crop drops on the growth pattern in the co-cultivation area).

The control group included drops of the same culture, layered on each other as described above. Results were interpreted 24 and 48 hours after the start of the incubation. When the growth retardation of the studied crops occurred, the relationship between them was regarded as antagonistic, while the crops themselves belonged to the category of bio-incompatible ones. Cultures were considered biocompatible if a full "merger" of spots was observed or the growth of strains in the area of co-culture enhanced (mutualism, synergy, satellitism). If one of the cultures in the area of co-culture "went up", inhibiting the growth of the second culture, regardless of the order of their application, such an option was considered weak antagonism. The presence of a well-defined zone of inhibition (growth retardation) of one culture by another peripheral spot test culture was regarded as a sign of strong antagonism.

Statistical analysis

All experiments were performed three times. Data processing was performed by standard methods of mathematical statistics. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \pounds 0.05$).

Results

The important properties of probiotic strains with regards to their use in the technology of functional foods for the rehabilitation of cancer patients are antioxidant and antimicrobial activity.

The antioxidant activity of the studied microorganisms was assessed by oxidation of liposomes over time. During the reactions of lipid peroxidation, the formation of PR1 – carbonyl compounds, PR2 – substances, which react to 2-thiobarbituric acid, PR3 – conjugated dienes, and PR4 – crotonaldehyde was studied. The results are shown in Fig. 1. All investigated strains of microorganisms reduce the amount of liposomes in the suspensions, exposed to induced oxidation, products of lipid peroxidation; hence, the mechanism of antioxidant activity of probiotic microorganisms is the inhibition of lipid peroxidation me-

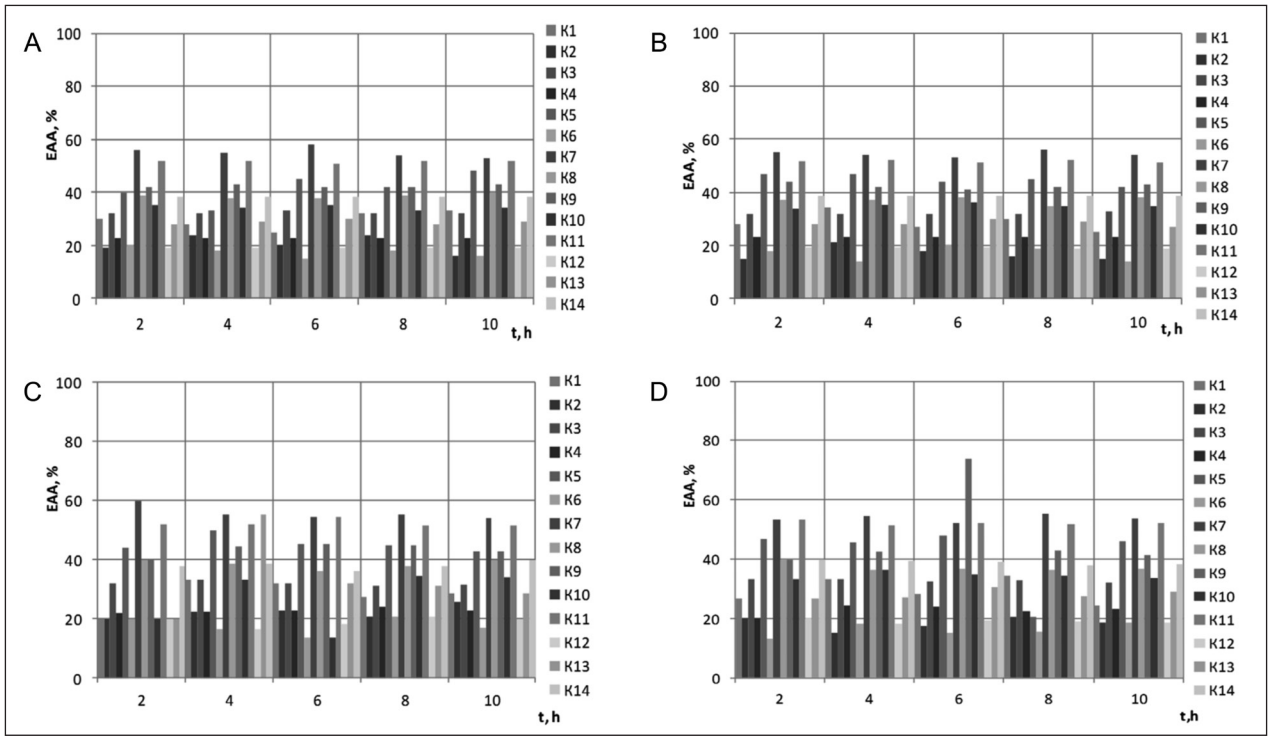

Figure 1. The effectiveness of the antioxidant activity of a product, relative to strains of LAB (A) PR1, (B) PR2, (C) PR3, (D) PR4, depending on the duration of oxidation.

tabolites that make up cell membranes. This is likely caused by the binding of free radicals and the inhibition of the lipoxygenase enzyme.

The antimicrobial activity of microorganisms, isolated from the human intestinal tract, was studied by the inhibitory action of the test culture. The results are shown in Fig. 2. Metabolites, produced by microorganisms of the *Bifidobacterium* genus, exhibit antimicrobial activity against the test strains of *E. coli* B-6954, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, and *Klebsiella pneumoniae* B-7001; those of the *Lactobacillus* genus – against *E. coli* B-6954, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, *Listeria innocua* LMG, *Clostridium tyrobutyricum* LMG, and *Klebsiella pneumoniae* B-7001; those of the *Streptococcus* genus – against *E. coli* B-6954, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, and *Klebsiella pneumoniae* B-7001; those of the *Enterococcus* genus – against *E. coli* B-6954, *Salmonella enterica* ATCC 14028, *Listeria innocua* LMG, *Clostridium tyrobutyricum* LMG, and *Klebsiella pneumoniae* B-7001.

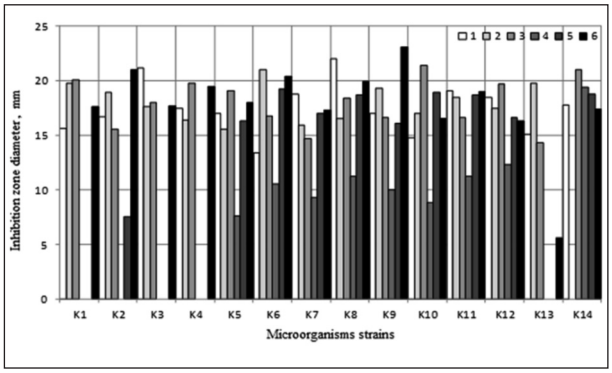

Figure 2. The antimicrobial properties of bacteriocin-like complexes, synthesized by microorganisms, isolated from the human gastrointestinal tract: 1 – E. coli B-6954; 2 – Staphylococcus aureus ATCC 25923; 3 – Salmonella enterica ATCC 14028; 4 – Listeria innocua LMG; 5 – Clostridium tyrobutyricum LMG; 6 – Klebsiella pneumoniae B-7001.

Figure 2 near here

To confirm the belonging of LAB inhibitor substances isolated from the human gastrointestinal tract to bacteriocins, their antibacterial effect on closely producing microorganisms was studied. The results are shown in Table 1. All studied microorganisms are resistant to both their own inhibitors and mutually resistant synthesized substances of one kind, which

Table 1. The study of bacteriocin-producing strains' cross-sensitivity of microorganisms by the inhibition zone diameter (mm).

Producer strain	**Indicator strain**													
	K1		K1		K1		K1		K1		K1		K1	
K1	0	K1	0	K1	0	K1	0	K1	0	K1	0	K1	0	K1
K2	0	K2	0	K2	0	K2	0	K2	0	K2	0	K2	0	K2
K3	0	K3	0	K3	0	K3	0	K3	0	K3	0	K3	0	K3
K4	0	K4	0	K4	0	K4	0	K4	0	K4	0	K4	0	K4
K5	4.7	K5	4.7	K5	4.7	K5	4.7	K5	4.7	K5	4.7	K5	4.7	K5
K6	2.9	K6	2.9	K6	2.9	K6	2.9	K6	2.9	K6	2.9	K6	2.9	K6
K7	1.5	K7	1.5	K7	1.5	K7	1.5	K7	1.5	K7	1.5	K7	1.5	K7
K8	1.8	K8	1.8	K8	1.8	K8	1.8	K8	1.8	K8	1.8	K8	1.8	K8
K9	5.3	K9	5.3	K9	5.3	K9	5.3	K9	5.3	K9	5.3	K9	5.3	K9
K10	4.5	K10	4.5	K10	4.5	K10	4.5	K10	4.5	K10	4.5	K10	4.5	K10
K11	3.3	K11	3.3	K11	3.3	K11	3.3	K11	3.3	K11	3.3	K11	3.3	K11
K12	2.7	K12	2.7	K12	2.7	K12	2.7	K12	2.7	K12	2.7	K12	2.7	K12
K13	4.5	K13	4.5	K13	4.5	K13	4.5	K13	4.5	K13	4.5	K13	4.5	K13
K14	3.0	K14	3.0	K14	3.0	K14	3.0	K14	3.0	K14	3.0	K14	3.0	K14

confirms the selected metabolites' belonging to bacteriocins and defines the action mechanism of these probiotic microorganisms.

Discussion

Probiotics have a positive effect on the immune system, as the gastrointestinal flora reduces the impact of mutagens and carcinogens. The biological action mechanisms of microorganisms, isolated from the human intestinal tract (*Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacteriu longum, Bifidobacterium adolescentis, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus fermentum, Lactobacillus salivarius, Lactobacillus casei, Lactobacillus reuteri, Streptococcus agalactiae, Enterococcus faecium*), and their metabolites were investigated. In vitro experiments demonstrated that the studied strains of microorganisms reduce the amount of liposomes in suspensions, exposed to induced oxidation, lipid peroxidation products; hence, the mechanism of antioxidant activity of probiotic microorganisms is the inhibition of lipid peroxidation metabolites that make up cell membranes, which is probably determined by the binding of free radicals and the inhibition of the lipoxygenase enzyme.

Saraniya et al. (17) purified and studied the properties of bacteriocins, produced by a strain of *Lactobacillus pentosus* SJ65, isolated from a fermented batter Uttapam. Bacteriocin was purified by various techniques, such as precipitation with acetone, gel permeation and hydrophobic chromatography. Bacteriocin was resistant at acidic and neutral pH up to 100°C and exhibited a broad spectrum of activity against clinically relevant gram-positive and gram-negative pathogens. However, gel electrophoresis in polyacrylamide gel and activity against *Listeria monocytogenes* have shown that purified bacteriocin consists of two individual peptides with molecular weights of 3.9 and 1.6 kDa.

Das et al. (18) purified and characterized non-toxic bacteriocin produced by the probiotic *Lactobacillus plantarum* DM5, as a potential biological preservative. *Lb. plantarum* DM5 shows in vitro probiotic properties such as high resistance to gastric acid and bile salts, adhesion to human adenocarcinoma cells (HT-29), and the ability to assimilate cholesterol. Furthermore, *Lb. plantarum* DM5 exhibits bactericidal activity against major food-borne pathogens. Analysis of zymography of the purified bacteriocins (plantaricin DM5) allowed determining its molecular weight ~ 15.2 kDa. Plantaricin DM5 was sensitive to proteolytic enzymes, but was stable in a pH range of 2.0-10.0, thermally stable (121°C for 15 min), and retained activity during the processing of surfactants and detergents. Analysis of cytotoxicity of plantaricin DM5 on human embryonic kidney 293 (HEK 293) and cell lines of human cervical cancer (HeLa) showed its nontoxicity and biocompatibility.

The growth, metabolism, and production of bacteriocins was studied with seven strains of *Lactobacillus*, including five commercial probiotic strains, during fermentation in an MRS-medium and milk medium at constant pH (19). These strains are *Lactobacillus acidophilus* ACC, *L. acidophilus* IBB 801, *L. casei imunitas*, *L. casei* YIT 9029, *L. gasseri* K7, *L. johnsonii* La1, and *L. rhamnosus* GG. The introduction of yeast extract (0.3-1.0% w/v) in the dairy medium increased growth and production of bacteriocins for all test strains. Production of bacteriocins was observed in yeast extract, added to milk, for strains *L. acidophilus* IBB 801, *L. johnsonii La1*, and *L. gasseri* K7. *L. acidophilus* IBB 801, the only strain of dairy origin, showed the best growth (10.5 log CFU mL-1) and production of bacteriocins (3200 AU mL^{-1}).

Das et al. (20) studied probiotic properties and antagonistic activity of *Lactobacillus plantarum* DM5 isolate, extracted from fermented national drink "Marcha" of the North-Eastern Himalayas. The new DM5 isolate is identified as Lactobacillus plantarum, which demonstrates probiotic properties and antimicrobial activity in vitro. It showed an appropriate level of survivability in severe conditions of the gastrointestinal tract and low pH of 2.5 for 5 hours. Model gastric juice and intestinal juice reduced initial population of viable DM5 cells by only 7% and 13%, respectively, whereas lysozyme (200 μg/ml), and bile salt (0.5%) increased the growth of the strain. The ability of the strain to cleave taurodeoxycholic acid was found, which makes it a potential agent for reducing hypercholesterolemia. DM5 isolate showed hydrophobicity of the cell surface (53%) and autoaggregation (54%), which is promising

for the epithelial cell adhesion and colonization of the organism. Bactericidal activity of isolate 6400 AU/ml was found, which inhibits the growth of food pathogens *Escherichia coli*, *Staphylococcus aureus*, and *Alcaligenes faecalis*.

Delgado et al. (21) studied the technological and probiotic properties of *Lactobacillus spp.* strains, isolated from the healthy human stomach, due to the possibility of their use as potential agents for preventing dysbiosis of gastrointestinal tract. Among 19 isolates, obtained from a biopsy sample of gastric juice, 10 different strains were identified, based on rep-PCR and PFGE fingerprinting. These strains belong to five types: *Lactobacillus gasseri* (3), *Lactobacillus reuteri* (2), *Lactobacillus vaginalis* (2), *Lactobacillus fermentum* (2), and *Lactobacillus casei* (1). All 10 strains were subjected to a series of in vitro tests to assess their functional and technological properties, including resistance to acids, resistance to bile, adhesion to the epithelial cells of the stomach, production of antimicrobial compounds, inhibition of Helicobacter pylori, antioxidant activity, antibiotic resistance, fermentation of carbohydrates glycosidic activity, and ability to grow in the milk. As expected, given their origin, all strains showed high stability at low pH (3.0). Species and strain-specific differences with respect to the production of antimicrobial compounds, antagonistic effects with respect to *H. pylori*, antioxidant activity and adhesion to epithelial cells of the stomach were found. None of the studied strains showed uncharacteristic resistance to the series of 16 antibiotics of clinical and veterinary importance. Two strains of *L. reuteri* are suggested as the most suitable candidates for use as potential probiotics for preventing dysbiosis of gastrointestinal tract. They showed a high survival degree in the gastrointestinal tract, reproduced *in vitro*, had high activity against *Helicobacter* and antioxidant activity. In this regard, two strains of *L. reuteri* are recommended for use as functional cultures when creating fermented dairy products.

The antifungal activity of lactic acid bacteria isolated from human microbiota was investigated (22). Fifteen strains of lactic acid bacteria were isolated, nine of which have shown a wide spectrum of activity against fungal strains. *Lactobacillus fermentum* was recognized as the most promising strain, cell-free supernatant maximally inhibited the growth of *Penicillum sp.* and *Fusarium oxysporum* for 72 hours.

The use of bacteriocins and other bioactive compounds of probiotic lactic acid bacteria as a biological weapon against *Neisseria gonorrhoeae* (23) was studied. The inhibitory effects of biologically active substances, such as organic acids, hydrogen peroxide and other bacteriocin-like inhibitory substances (BLIS) L23 and L60 were studied. Different non-treated and treated cell-free supernatants of two probiotic lactobacilli containing these metabolites were used. The objectives of the work included the evaluation of the antimicrobial activity of metabolites, produced by the two strains of probiotic lactobacilli, the determination of the proportions, in which each of them is responsible for the inhibitory effect, the determination of the minimum inhibitory concentrations (MICs), and the assessment of the potential interaction between biologically active compounds in relation to *N. gonorrhoeae*. The main anti-microbial metabolites were BLIS-es L23 and L60. In proportion, their contribution to the growth inhibition of N. gonorrhoeae was 87.28% and 80.66%, respectively. MIC values of bacteriocins were promising as these substances when diluted showed significant inhibitory activity. The study of interactions between bacteriocins showed 100% synergy. The obtained results allow using both substances (L23 and L60) to create bioproducts that suppress *N. gonorrhoeae*.

Monteagudo-Mera et al. (24) studied in vitro the physiological properties of different probiotic strains of lactic acid bacteria of dairy and human origin. Seven strains were isolated from the milk of sheep and cows (*Enterococcus faecalis* – five, *Lactococcus lactis* and *Lactobacillus paracasei*). Four were obtained from American Type Culture Collection (ATCC), isolated from cheese (Lactobacillus casei 393), human feces (L. paracasei 27092 and Lactobacillus rhamnosus 53103) and used in cheese making (L. lactis 54104). Although none of the strains was able to degrade mucin, all E. faecalis showed at least one transferable antibiotic resistance, which excluded them as candidates for adding to foods. Of the remaining six safe strains, L. lactis strains were more tolerant to low pH than Lactobacillus spp.; all were tolerant to pancreatin and bile salts and showed antibacterial activity. The highest level of adhesion to Caco-2 cells was observed with L. lactis

660, even higher than L. rhamnosus ATCC 53103 (recognized probiotic and used as control). The physiological probiotic properties of these strains, mainly isolated from dairy sources, are interesting in view of their use in cheese production as starter and non-starter cultures.

The in vitro use of a variety of selected commercial and potential lactic acid bacteria probiotics was investigated, such as transit tolerance in the upper human gastrointestinal tract, adhesion capacity to human intestinal cell lines, and the effect on the epithelial barrier function. The selected bacteria include strains of Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus farciminis, Lactobacillus sakei, Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus reuteri, and Pediococcus pentosaceus. Viable counts after simulated gastric transit tolerance showed that L. reuteri and P. pentosaceus tolerate gastric juice well, without reduction of viability, whereas L. pentosus, L. farciminis and L. sakei strains lost viability over 180 min. All tested strains tolerated the simulated small intestinal juice well. The bacterial adhesion capacity to human intestinal cells revealed major species and strain differences. Overall, L. plantarum MF1298 and three L. reuteri strains had a significantly higher adhesion capacity, compared to other tested strains. All strains, both living and UV-inactivated, had little effect on the epithelial barrier function. However, living L. reuteri strains revealed a tendency to increase the transepithelial electrical resistance (TER) from 6 to 24 h.

Tejero-Sariñena et al. (25) examined the antimicrobial properties of fifteen strains of microorganisms belonging to the genera *Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus*, and *Bacillus* against Gram-positive and Gram-negative pathogenic bacteria. The result revealed that the majority of the selected strains are capable of producing biologically active compounds in a solid medium with antagonistic properties towards *Salmonella typhimurium, Escherichia coli, Enterococcus faecalis, Staphylococcus aureus*, and *Clostridium difficile*. These results were also confirmed by using the cell-free supernatant (CFCS) of hypothetical probiotics in diffusion. The neutralization of alkaline cell supernatants reduced the antagonistic effect. These experiments confirm the ability of the potential probiotics to inhibit the growth of investigated pathogens. One of the principal mechanisms of inhibition is the production of organic acids during the fermentation of glucose and subsequent reduction of pH. Lactic and acetic acid were found to be the main products of probiotics metabolism.

Five newly isolated lactic acid bacteria, identified as Weissella cibaria, Enterococcus faecium, and three different strains of Lactobacillus plantarum were studied by 16S rRNA sequencing (26). Essential probiotic requirements of these isolates, such as phenol tolerance, low pH, high sodium chloride, and bile salt concentration were studied. Antimicrobial activities against some pathogens were tried; the sensitivity of these strains against 25 different antibiotics was investigated. Further studies revealed *Weissella* and *Enterococcus* as substantial producers of folic acid. Folate is involved in many metabolic reactions as a cofactor and has to be an essential component in the human diet. The folate level in the fermented samples was determined by microbiological assay using *Lactobacillus casei* NCIM 2364 as the indicator strain. The three strains of *L. plantarum* showed significant inhibitory activity against various fungi that commonly contaminate foodstuffs, which indicates their potential as a biopreservative for food material.

Kazemipoor et al. (27) screened the antibacterial activity of lactic acid bacteria isolated from fermented vegetables toward food pathogens. The antagonistic properties of these isolates against Escherichia coli, Staphylococcus aureus, Yersinia enterocolitica, and Bacillus cereus were examined with agar well diffusion. Four LAB, namely, MF6, MF10, MF13, and MF15, identified as Lactobacillus animalis, Lactobacillus rhamnosus, Lactobacillus fermentum, and Lactobacillus reuteri, respectively were effective against all selected pathogenic strains. Amongst the four isolates, MF6 exhibited the highest antibacterial activity against all tested indicator pathogens except Y. enterocolitic. Its activity was maximal against E.coli with a Zone of Inhibition (ZOI) ranging from 18.7 to 21.3 mm and least for Y. enterocolitica (10±1.1 mm). Isolate MF13 also showed antimicrobial properties against all tested pathogens, showing the highest activity against Y. enterocolitica (14 ± 1.7 mm) and lowest activity against E.coli (8 ± 1.4 mm), which was in direct contrast to isolate MF6. Isolate MF15 showed the highest activ-

ity against E.coli (12 ± 0.8 mm) and lowest activity against S. aureus (8 ± 1.7 mm). The lowest antimicrobial property was observed in isolate MF10, with a ZOI of 2.5-7 mm. The level of the antimicrobial property among the isolates was in the following order: MF6>MF13>MF15>MF10. Overall, the isolated LAB showed a remarkable inhibitory effect against both Gram-positive and Gram-negative pathogenic strains. However, the inhibition spectrum was different for tested isolates. These results suggest that these potent isolates could be used as natural bio-preservatives in different food products.

Birri et al. (28) characterized lactic acid bacteria (LAB), isolated from feces of healthy Ethiopian infants with an emphasis on the production of bacteriocins and antibiotic susceptibility. One hundred fifty LAB were obtained from 28 healthy Ethiopian infants. The isolates belonged to *Lactobacillus* (81/150), *Enterococcus* (54/150) and *Streptococcus* (15/150) genera. *Lactobacillus* species were more abundant in breast-fed infants, while *Enterococcus* dominated the mixed-fed population. Bacteriocin-producing LAB species were isolated from eight infants. Many different bacteriocins were identified, including one new bacteriocin from *Streptococcus salivarius*, avicin A (class IIa) from *Enterococcus avium*, one class IIa bacteriocin from *Enterococcus faecalis* strains, one unknown bacteriocin from *E. faecalis*, two unknown bacteriocins from *Lactobacillus fermentum* strains, and the two-peptide gassericin T from the *Lactobacillus gasseri* isolate. Susceptibility tests performed for nine antibiotics suggest that some lactobacilli may have acquired resistance to erythromycin (3%) and tetracycline (4%) only. The streptococci were generally sensitive to antibiotics except for penicillin, to which they showed intermediate resistance. All enterococci were susceptible to ampicillin, while 13% showed penicillin resistance. Only one *E. faecalis* isolate was vancomycin-resistant. Tetracycline (51%) and erythromycin (26%) resistance was prevalent among enterococci, but multidrug resistance was confined to *E. faecalis* (47%) and *Enterococcus faecium* (33%). The structural genes of cytolysin were detected in 28% of the isolates in five enterococcal species, the majority being *E. faecalis* and *Enterococcus raffinosus*.

Antimicrobial peptides, active against gram-negative pathogens *Escherichia coli* and *Salmonella*, produced by recombinant lactic acid bacteria *Lactococcus lactis* were described (29). In an initial screening, the activities of numerous candidate antimicrobial peptides, made by solid-state synthesis, were assessed against several indicator pathogenic E. coli and Salmonella strains. Peptides A3APO and Alyteserin were selected as top performers, based on high antimicrobial activity against tested pathogens and a significantly lower antimicrobial activity against L. lactis. Expression cassettes containing the signal peptide of the protein Usp45 fused to the codon-optimized sequence of mature A3APO and Alyteserin were cloned under the control of a nisin-inducible promoter PnisA and transformed into L. lactis IL1403. The resulting recombinant strains were induced to express and secrete both peptides. A3APO- and Alyteserin-containing supernatants from these recombinant L. lactis inhibited the growth of pathogenic E. coli and Salmonella by up to 20-fold.

The study of antimicrobial activity of microorganism metabolites found that metabolites, produced by microorganisms of the *Bifidobacterium* genus, exhibit antimicrobial activity against the test strains of *E. coli* B-6954, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, and *Klebsiella pneumoniae* B-7001; those of the *Lactobacillus* genus – against *E. coli* B-6954, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, *Listeria innocua* LMG, *Clostridium tyrobutyricum* LMG, and *Klebsiella pneumoniae* B-7001; those of the *Streptococcus* genus – against *E. coli* B-6954, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, and *Klebsiella pneumoniae* B-7001; those of the *Enterococcus* genus – against *E. coli* B-6954, *Salmonella enterica* ATCC 14028, *Listeria innocua* LMG, *Clostridium tyrobutyricum* LMG, and *Klebsiella pneumoniae* B-7001.

The analysis of the antibacterial activity of inhibitory substances, produced by lactic acid bacteria isolated from the human gastrointestinal tract, proved the belonging of isolated metabolites to bacteriocins, since studied microorganisms were resistant to both their own inhibitors and mutually resistant synthesized substances of one kind.

Identified antioxidant and antimicrobial activity of 14 probiotic strains, isolated from the human gastrointestinal tract, demonstrated the feasibility of their

use for creating functional foods for the rehabilitation of cancer patients.

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