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THE CREATING A NEW BIOPRESERVATIVE BASED ON FUSANT STRAIN *LACTOCOCCUS LACTIS* SSP. *LACTIS* F-116 FOR FOOD QUALITY AND ITS SAFETY

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Abstract

Biopreservation includes processes to increase shelf life of products, their nutritional and biological value as well as safety by adding a number of microorganisms and/or antimicrobial metabolites. *Lactococcus lactis* have been used by mankind for thousands of years to extend shelf-life of the foodstuffs, due to lactic acid and bacteriocins (nisin, food preservative, E234). However, nisin is not effective against enterobacteria and fungi.

Fusant strain was obtained after protoplast fusion of two related strains with low nisin-synthesizing activities. The probiotic properties of most effective strain F-116 were determined as resistance to gastrointestinal stresses and by measuring of superoxide dismutase (SOD) activity. Analytical chromatography methods were applied to determine the antimicrobial substances. Toxicological studies were performed on ciliates *Tetrahymena pyriformis*.

Fusant strain was identified as *L. lactis* ssp. *lactis* (GenBank EF100777). Fusant formed a new antimicrobial complex effective not only against Gram-positive (like nisin), but also against Gram-negative bacteria and fungi, the potential contaminants of food products. The individual antimicrobial substances differ from each other in molecular masses, electrophoretic mobility values (Rf) and biological properties. Two substances were identified as novel antibiotics. One component, contained alkyl-aromatic ketones with hydroxyl groups was responsible for antifungal activity. Strain F-116 synthesizes metabolites, increasing the shelf life of foodstuffs and its bioavailability: the amount of lactic acid was 8 mmol/L with high antioxidant value (SOD activity - 30 U/mg of protein). Freeze-dried samples of F-116 cells have no inhibitory effect on growth of ciliates *Tetrahymena pyriformis*.

Due to ability to synthesize antimicrobial agents and useful metabolites, the fusant strain F-116 with high antioxidant activity will create effective means for reducing oxidative food spoilage during storage and protection against serious diseases and early aging. Therefore, strain F-116 can be used as biopreservative and can fulfill the growing public demand for safe and healthy foodstuffs.

Key words: *Fusant, L. lactis ssp. lactis, Food-borne pathogens. Bacteriocin, Fungi, Biopreservative, Metabolites.*

1. Introduction

Increasing the shelf life of food products - a task that humanity is trying to solve for a long time already. Food products produced on an industrial scale, are exposed to microorganisms, including pathogen-forming toxins that cause serious consequences for the human body. Existing technology of cold treatment and storage of the products do not meet the modern achievements of science and many of them are outdated. New approaches are solving the number of problems with the aim of intensification of technological processes, increase retention, create new energy- and resource-saving and environmentally friendly technologies of refrigeration preservation. Chilled food raw materials have a number of advantages in the food and biological value in comparison with frostbitten and frozen ones. However, one of the serious problems expanding production of chilled products is a limited shelf life [1]. In the food industry the chemical preservatives and antibiotics are actively used. Many preservatives cause fear among the population because of their toxicity and the possibility of the suppression of

the natural microbiota of the human body. In addition they hold the acquired resistance to pathogens contaminating food and food raw materials, causing its damage and pose a threat to health [2]. Biopreservation includes processes to increase the shelf life of the products and, as a consequence, increase their overall nutritional and biological value, as well as safety for the user by adding a number of microorganisms and/or antimicrobial metabolites. Lactic acid bacteria (LAB) play a critical role in food production and health maintenance. Therefore, products with or processed with LAB are accepted as a natural way to preserve food and promote health. LABs are widely used in various food fermentations and have a long history. The genus *Lactococcus* has "GRAS"-status (Generally Recognized As Safe) accordingly to the European Commission, while the members of genera *Streptococcus* and *Enterococcus* contain some opportunistic pathogens [3].

Lactococcus strains have ability to synthesize antimicrobial agents, vitamins, lactic and volatile fatty acids, amino acids, enzymes and others. Species of *Lactococcus* are closely associated with food and one of the most important among them are some strains of human symbiotic microbiota [3]. The use of this approach to the creation of preservatives can lead to the complete replacement of chemical preservatives and antibiotics, as well as to meet the growing public demand for safe and healthy products [4]. Potential preservatives on the basis of bacteriocins and useful metabolites are considered as valuable by many experts in the framework of industrial applications.

The leading role in explaining the phenomenon of antagonism of lactic acid bacteria is given to specific antibiotic substances of proteineous nature, bacteriocins. Evolutionary the synthesis of bacteriocins arose as an adaptation of microbes to survival in a competitive environment - this is one way to take a suitable ecological niche and is a natural for most, if not all, species. The most studied and approved for use as a biological preservative bacteriocin is nisin, the only antibiotic valid for «GRAS»-status and which is used as a food additive (E 234 code) in many countries around the world [5].

Nisin is the basis for the industrial preparation "Nisaplin", which is produced by the British company "Aplin & Barrett", Ltd. This preparation contains 2.5% of the active ingredient (nisin A), its activity is estimated as 1,000 IU/mg (IU - International Unit). Nisaplin obtained by fermentation medium with selective strain *Lactococcus lactis* ssp. *lactis.* Safe use of nisin is due to the fact that having a polypeptide structure, it breaks down into amino acids quickly by digestive enzymes [6, 7].

One of the very important properties of nisin is the activity against Gram-positive bacteria and bacterial spores of clostridia and bacilli, other non-spore forming bacteria, many species of pathogenic streptococci, staphylococci, listeria, but it is not effective against Gram-negative bacteria and fungi [1, 6, and 7]. Fungal spoilage of food products and raw food is a global problem today. Fungi can grow in many food products: cereals, meat, fruit, vegetables, etc. It has been estimated that global losses due to spoilage of food by molds grow annually by 5 - 10%.The potential production of toxins by fungi are of particular health concern [8].

Synthesis of bacteriocins in the majority of a population of cells can be induced by different methods of selection. The selection includes different approaches to achieve this goal. This is the isolation from natural substrates, a comparative assessment of the activity of producers and the selection of the most promising of them, the increasing the activity of experimental strains, including genetic engineering manipulations. In studies conducted in recent years the fundamental possibility was detected to obtain the biologically active substances by the use of protoplast fusion method, allowing to combine the whole genomes of parent strains and the cytoplasm of others in order to transfer the genetic information. Protoplasts in the merger process play the role of the system, given the transforming genetic material. Merger of protoplasts is possible in nature as a spontaneous process [9].

For this reason lactococci can be considered as potential producers of different antimicrobials with wider activity spectrum than nisin. In recent years, the concept of probiotic bacteria has also stimulated work on bacteriocins. In the light of the increased antibiotic resistance among pathogens, bacteriocins have attracted attention as an alternative means to prevent infection caused by pathogens [6, 9].

At present, the ways of targeted synthesis of antimicrobial substances by lactococci are studied in order to obtain new ones with more valuable properties for application them as food preservative. The interest in the use of bacteriocin producer strains has increased tremendously.

The aim of this investigation was to create a new biopreservative based on fusant strain *Lactococcus lactis* ssp. *lactis* F-116 for increasing of food quality and its security.

2. Materials and Methods

2.1 Media and conditions for isolation of lactococci strains

We used recombinant strain *L. lactis* ssp. *lactis* F-116 obtained by protoplast fusion of related strains *L. lactis* ssp. *lactis*, with low productivity of nisin [10]. The morphology of the isolated strains was examined with an MBI-15 microscope equipped with a KF-4 phase contrast condenser (LOMO, MicMed, St.Petersburgh, Russia).

2.2 Cultural properties

These properties of the isolated strains were examined by classical microbiological methods of identification and determination of the cultures [3, 11].

2.3 Physiological and biochemical features

New strains were studied and compared to the nisin-producing strain *L. lactis* ssp. *lactis* MSU. The MSU strain was obtained by adaptive selection to the low doses of nisin, and it is a reference strain which produces nisin A, identical to that in the commercial preparation Nisaplin (Aplin & Barrett, UK).

The strains were stored as lyophilized cells in a household refrigerator. The lyophilized cultures were reconstituted by incubation in sterile non-fat (skimmed) milk. To obtain an inoculum, the cultures from skimmed milk were re-inoculated into the inoculation medium, which contained glucose (1 g/L), yeast autolysate (35 mg% of ammonium nitrogen), and tap water (pH 6.8 - 7.0). Thereafter, the inoculum (OD_{540} 0.14 - 0.19) was introduced in an amount of 5 vol.% into the base fermentation medium, which contained (g/L): KH_2PO_4, - 20.0; glucose - 10.0; NaCl – 1.0; $MgSO_4$ - 0.2 and yeast autolysate (35 mg% of ammonium nitrogen), pH 6.8 - 7.0. The cultures were grown under steady-state condition at 28 ^{0}C.

Biomass was determined by measuring the absorbance (optical density (OD) at 540 nm (l = 0.5 cm), using a FEK-56 colorimeter-nephelometer (Russia). The dynamics of growth and bacteriocin accumulation in the culture liquid of strains were followed for 24 h.

2.4 Taxonomic description

To confirm the taxonomic status molecular genetic studies were conducted. Computer processing of the results of 16S rRNA gene sequencing and comparative analysis of our data with the sequences of the type strains revealed high similarity between them.

2.5 Antimicrobial testing

The antimicrobial activity of lactic acid bacteria was determined by the diffusion into agar by measuring of the growth inhibition zone of test cultures in mm [10]. The bacteriocin-synthesizing activity was assessed as nisin production. The test culture for nisin determination activity was *Bacillus coagulans* 429, a thermophilic spore-forming, acid-resistant bacteria.

2.6 The spectrum of antimicrobial action

These spectrum of the strains was studied by growing cultures under steady-state conditions in the fermentation medium of the above composition. The test cultures (contaminants of canned and preserved food products and food raw materials) used in these experiments were from the Collection of Microbes of the Department of Microbiology of Moscow State University: 6 strains of Gram-positive bacteria*: Bacillus subtilis* 2, *B. coagulans* 429, *B. cereus* 31, *Micrococcus luteus* 128, *Staphylococcus aureus* 144; 4 strains of Gram-negative bacteria: *Escherichia coli* 52; *Pseudomonas aeruginosa* 21, *Proteus vulgar*is 206, *Salmonella gallinarum* 32; and five strains of microscopic fungi, including yeasts: *Aspergillus repens* de Bary 9, *Penicillium chrysogenum* 32, *Fusarium oxysporum* 9, *Candida guilliermondii* 17, *Rhodotorula aurantiaca* 226.

The bacilli were grown in an organic medium containing (g/L): glucose - 10.0, peptone - 5.0, NaCl - 5.0, and agar - 25.0; the medium was supplemented by Hottinger's broth (25 mg% of amine nitrogen), fungi were grown in Saburo's medium containing (in g/L): glucose - 40.0; peptone - 10.0; agar - 20.0; chloramphenicol - 0.025.

The bacteria were cultured at 28 - 55 ^{0}C: the temperature used in the case of bacilli, staphylococci, and micrococci was 37 ^{0}C; *E. coli* was grown at 42 ^{0}C; *B. coagulans* at 55 ^{0}C, and fungi at 28 ^{0}C. Petri dishes were inoculated with a 24h-old cultures of test- microorganisms (0.1 mL cell suspension in physiological saline per dish; the concentrations were adjusted to 10^9 cells per 1 mL using a bacterial turbidity standard).

Quantitative determination of the antimicrobial activity was performed by measuring the zones of growth suppression with subsequent calculation involving a calibration plot for standard nisin solutions. Solutions of the preparations served as the standards: "Nisaplin (activity 1 mln. IU/g, "Aplin & Barrett", Ltd., UK) - for Gram-positives; chloramphenicol (HiMedia Laboratories Ltd, Mumbai) - for Gram-negative bacteria; nystatin (4670 U/mg, Sigma) - for fungi. The fungicidal activity was assessed with indicator culture *Aspergillus repens* de Bary 2, which was previously isolated from the surface of smoked sausage [12].

2.7 The isolation and purification of the antimicrobial complex

Antimicrobial substances were extracted from the cells and culture liquid using a mixture of acetone, acetic acid, and water as 4 : 1 : 5 (55 ^{0}C, 1.5 h). The obtained samples were purified by solid-phase extraction methods and by thin-layer chromatography [13]. Purification of the valuable compounds from the impurities was carried out by RP-HPLC on a chromatograph «Milihrm A-02» («Econova», Russia) and purified by using the C_8 cartridges Diapak («BioKhimMak ST», Russia). Individual antimicrobial fractions were analyzed by IR and mass spectrometry, and by their spectrum of antimicrobial action. UV-VIS-spectra were recorded on a spectrophotometer Shimadzu UV-1601 PC (Japan). The analysis were performed using the properties of bioactive compounds BNPD computer database [14].

2.8 The amount of lactic acid was

It was determined by titration of the difference between the volumes of 0.1N NaOH solution used for the titration of culture medium before and after the growth of bacteria using a lactic acid standard curve [15].

2.9 Model experiments

Model experiments on the effects of adverse conditions of the gastrointestinal tract on the fusant strain were carried out. Resistance to action of hydrochloric acid and bile acids were studied by culturing cells in a culture medium of the above composition with the addition of different concentrations of bile acids up to 20% ("Samson-med", Russia) and hydrochloric acid from 0.2 to 0.6%. Survival was assessed by measuring the optical density at λ = 540 (l = 0,5 cm), and direct recalculation on 1 mL of culture liquid.

2.10 Antioxidant activity

These activity was measured as the superoxide dismutase (SOD) activity in cell extracts by spectrophotometer using xanthine-cytochrome method (λ = 550 nm, HITACHI 200-20). One unit of SOD activity is taken as the amount of enzyme which inhibits cytochrome *c* reduction rate by 50% at pH 7.8 and temperature of 25 ^{0}C. SOD activity was recalculated on the protein content. Protein was determined by the Bradford method with Coomassie Brilliant Blue G-250. As a standard for the calibration curve bovine serum albumin was used [16].

2.11 LGS preparation

It was obtained by freeze-drying the cultural broth of fusant strain F-116 grown in a fermenter LKB company (Sweden). The culture was grown under steady-state condition at 28 ^{0}C for 10 h and then the whole cultural broth was freeze-dried. Drying parameters were as follow: freezing temperature of -40 ^{0}C, sublimation - at -30 ^{0}C, the maximum temperature of the final drying of the product was -60 ^{0}C. The dried LGS preparation was packaged under vacuum in a three-layer polymer film.

2.12 Toxicity assessment

Toxicological studies of F-116 strain and the LGS preparation were carried out on the culture of ciliates *Tetrahimena pyriformis* in accordance with the methodological guidelines [17].

All experiments were replicated three times. Standard deviations were calculated and included in the graphical representation of the data. The obtained data were statistically analyzed using Excel software.

3. Results and Discussion

Although bacterial interaction by whole genomes during hybridization is a rare event, however, at the confluence of the protoplasts many microorganisms can overcome this barrier. The genomes of the fused protoplasts typically fragmented and multiple cross-overs occur between them, which leads to haploid recombinants, and the number of the fusion recombinant progeny are much more than during the transformation or conjugation [9]. Physiological stress, caused by the formation and regeneration of the protoplast fusion, can lead to changes in various types of genetic rearrangements which cause the changes in the metabolic pathways, gene expression regulation and expression of the "silent" genes. By comparison of the physiological and biochemical properties of the breed new strain with the type strains, the acquisition of new properties can be found.

3.1 Morphology

The morphology of the fusant strain demonstrated that the cultures was represented by cocci assembled in pairs or short chains of various lengths: two, four or 10 cells. The bacteria are non-motile and Gram-positive. They did not differ in their cultural features from the bacteria belonging to the genus *Lactococcus* [3].

3.2 Cultural properties

Growth was absent in the meat–peptone broth containing 6.5% NaCl and at pH 9.6. The pH from 6.6 to 7.2 was optimal for the growth and development of these strains. The optimal incubation temperature was 28 ^{0}C; at 10 ^{0}C the growth was minimal and was completely absent at 45 ^{0}C. Uniform growth of bacteria along the entire inoculum's length in a stab of an agar medium is characteristic of facultative anaerobes [11].

3.3 Biochemical testing

Ability to consume of various carbohydrates, including sugars, alcohols and organic acids is the basis of the distinguishing features in the identification of lactic acid bacteria. Studies have found that fusant strain F-116 differ from parents on utilization of: arabinose, sucrose, maltose, rhamnose, raffinose, but also the sorbitol, dulcitol and mannitol. All the lactococci strains did not hydrolyze starch.

The hallmark of lactic acid bacteria is a great need for complex nutrients: purines, pyrimidines, amino acids and vitamins, especially of group B. Amino acids are essential for the construction of the cell, and for the bacteriocin formation. It is known that threonine, serine, cysteine, lysine, and aspartic acid are precursors of lanthionine and β-methyllanthionine and part of the nisin molecules [7].

The results of the experiments for determination of the needs of strains in amino acids and other growth components revealed some features of the strain F-116 showed good growth in media, indicating the specific needs in glutamine, asparagine, uracil, aspartic acid and arginine. Strain MSU does not require adenine and phenylalanine, Strain 729 not need the following growth components: thymine, valine, proline, glycine. Studied lactococci grew equally well in media containing a mixture of amino acids in combination with vitamin B_1 and biotin, as well as with uracil. In control experiments in the absence of growth factors in the environment lactococci did not grow, confirming that they belong to auxotrophic microorganisms. Strains F-116 and MSU need the presence of glycine in the medium. All strains were in need of arginine, that is a differentiating feature of *Lactococcus lactis* ssp. *lactis* from ssp. *cremoris.*

Based on the phylogenetic distance of nucleotide substitutions in 16S rRNA genes it was calculated that strains show a high degree of DNA homology (98.9 - 100%) relative to the reference strains of *L. lactis* ssp. *lactis* AB100798, AJ419572, AB118034. The level of genetic similarity (in %) of all the strains studied in relation to closely related strains *L. lactis* ssp. *cremoris* AB100802 and AB100792 were 95.4 - 96.6%. The nucleotide sequences of the 16S rRNA genes of studied strains were deposited to the GenBank database under following accession numbers: strain MSU - DQ255952, 729 – EF102814, 1605 – EF102815 and F-116 - EF100777.

3.4 A study of the growth dynamics

Parental and fusant strains in question in the fermentation medium with glucose and yeast autolysate demonstrated that their growth was characteristic for mesophilic lactococci that demonstrated on Figure 1. An experiment to study the F-116 strain of dynamics of the biosynthetic showed that the increase antimicrobial activity was paralleled to growth cultures: for 9 h incubation the biomass level has reached the maximum value (OD_{540} = 1.37), and the nisin activity increased to 4200 IU/mL. It was 10 - 12 times more active than parent strains.

After 9 h of incubation, the cells passed from the exponential growth phase to the stationary phase, which continued for 5 h. After 12 h, a further decrease in pH, antimicrobial activity, and optical density of the culture liquid were observed due to cell lysis.

Bacteriocins are microbial proteins with antimicrobial activity, which differ from antibiotics in the kinetics of the synthesis, being formed in parallel with the growth of producer microorganisms, DNA and protein synthesis [7, 10]. As secondary metabolites, antibiotics are largely synthesized during the second (steady-state) phase of microorganism growth and subsequently, when the cells start to die. The strain MSU differed in its growth characteristics from F-116 strain: the amount of the cells are slowly increased during the first 12 h, which was paralleled by a gradual increase in the antibiotic activity of the culture liquid up to 2000 IU/mL.

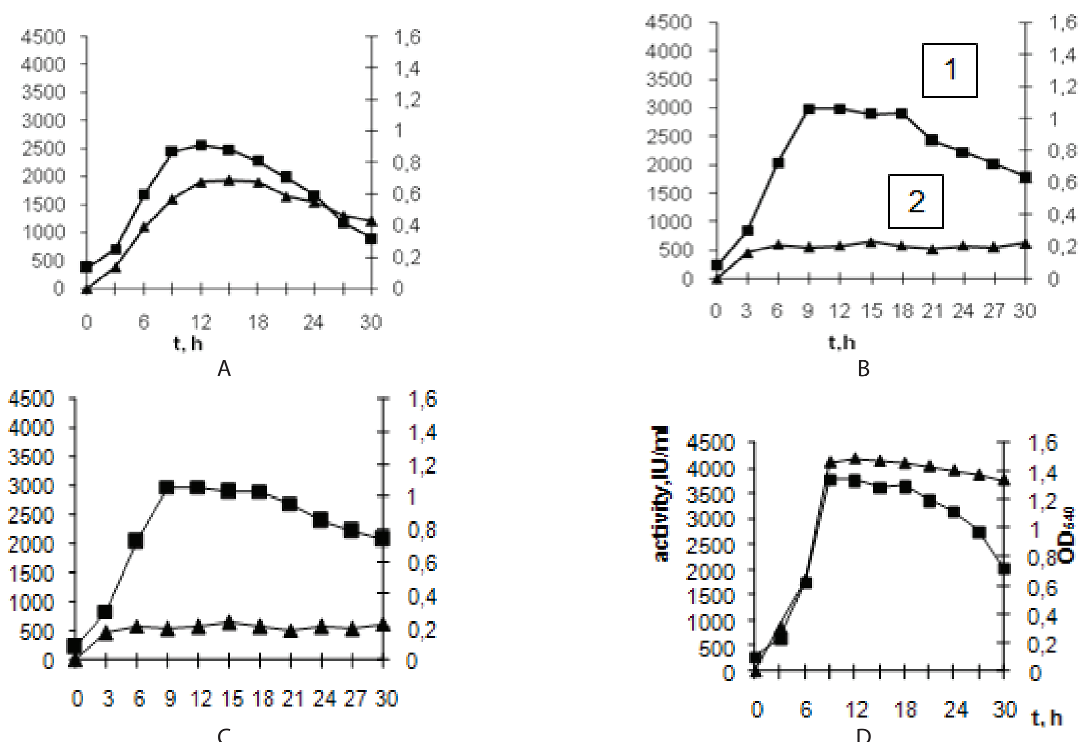

Figure 1. Dynamic of growth (1) and antimicrobial activity (2) of strains *Lactococcus lactis* ssp. *lactis*: A - strain MSU; B - 729; C - 1605; D - F-116

The results of our study of the spectra of antimicrobial action of culture liquids of the studied strains of *L. lactis* subsp. *lactis*, grown in the fermentation medium, are summarized in Table 1.

The strain MSU suppressed the growth of Gram-positive bacilli and micrococci in a manner similar to that of nisin. The study of the antimicrobial spectrum of action showed that the fusant strain also suppressed the growth of Gram-positive bacteria, including *Bacillus coagulans*, *Listeria monocytogenes, Staphylococcus aureus* as well as some Gram-negative cultures, such as: *Salmonella gallinarum, Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and also possessed fungicidal action - suppressed the growth of microscopic fungi: *Fusarium oxysporum, Penicillium chryzogenum, Aspergillus repens* de Bary, what is rare biological property for the natural strains of lactococci. The strain F-116 was effective against *Rhodotorula aurantiaca and Candida guilliermondii* too.

The inhibited activity of F-116 against Gram-negative bacteria consisted of 370 U/mL (as compared with chloramphenicol), and its antifungal activity (with nistatin as a standard and test-culture *A. repens*) was 1700 u/mL. The strain MSU did not inhibited the growth of Gram-negative bacteria and fungi.

It should be noted that the parent strains had a different spectrum of activity: 729 strain significantly inhibited the growth of micrococci, but not inhibited *S. aureus*, but as a strong acidifier suppressed the growth of *E. coli*, and strain 1605 acquired inhibitory activity against *S. aureus*, selectively inhibited the growth of yeast and fungi.

Lactic acid - one of the main end metabolites formed by *L. lactis* ssp. *lactis* as homofermentative LAB in the fermentation process [2, 4]. The amount of lactic acid (in mmol/L) formed by fusant strain F-116 was 8.35, nisin-producing strain MSU - 8.0, strain 729 as a strong acidifier - 16.5, strain 1605 - 10.4. Lactic acid has antimicrobial action, but this is not determinative. For example, at pH below 5.0 lactic acid inhibits the growth of bacterial spores without affecting the development of the microscopic fungi and yeast, at pH below 4.0 the strong antimicrobial activity was observed as in some scientific publications [2, 15, 18]. These results suggest that the lactic acid is not a determining factor in lactococci antimicrobial activity.

The strain F-116, the result of protoplast fusion, has a broad spectrum of antimicrobial action. It has been revealed, that the strain F-116 produced antibiotic complex which differed from nisin. Differences in biological and physicochemical properties of bacteriocins formed by the strains were identified with thin layer chromatography (TLC). Bioautography on Silufol plates with *B. coagulans* as the test organism in system: MeOH-H_2O (96 : 4) shown that two of these components are uncharged and hydrophobic, one component was positively charged. It has been elucidated that one component was a peptide like nisin by electrophoretic migration and biological activity. A parent strain 729 revealed a small fraction of activity with R_f = 0, and in strain 1605 biologically active fraction with R_f = 0 is detected, which was confirmed by the lack of inhibition zones of test-cultures, but is weaker activity fractions with R_f = 0.7. The strain MSU synthesis in salt medium nisin only.

Table1. Antimicrobial action of strains of *Lactococcus lactis* ssp. *lactis* in comparison with "Nisaplin"

Test-microorganisms	**Strains**				
	729	**1605**	**F-116**	**MSU**	**Nisaplin 3000 IU/mL**
	Diameter of inhibition zone, mm				
Bacillus coagulans	10.0	10.0	**26.0**	15.0	17.0
Bacillus subtilis	10.0	10.0	**24.0**	16.0	18.0
Micrococcus luteus	8.0	9.0	**27.0**	18.0	21.0
Bacillus cereus	8.5	10.0	**18.0**	16.0	18.0
Staphylococcus aureus	10.5	10.0	**19.0**	19.0	25.0
Listeria monocytogenes	0	9.0	**16.0**	12.0	15.0
Salmonella gallinarum	0	0	**17.0**	0	0
Escherichia coli	10,0	0	**18.0**	0	0
Proteus vulgaris	9.0	0	**16.0**	0	0
Pseudomonas aeruginosa	0	0	**12.0**	0	0
Fusarium oxysporum	0	0	**14.0**	0	0
Penicillium chryzogenum	0	0	**16.0**	0	0
Aspergillus repens	0	0	**17.0**	0	0
Rhodotorula aurantiaca	0	10.0	**14.0**	0	0
Candida guilliermondii	0	9.0	**16.0**	0	0

3.5 Identification of the components of antimicrobial complex of the strain *L. lactis* ssp. *lactis* F-116

Chromatographic and electrophoretic study of complex synthesized by F-116, revealed the presence of three fractions differing in magnitude of R_f, the chemical nature and the biological properties. These fractions were named LGS-B, C-LGS, LGS-H (Table 2). The main fraction is a fraction antibiotic complex LGS-H, obtained from the aqueous fraction after separation of LGS-B, it was active against Gram-positive bacteria. The results obtained by TLC and paper electrophoresis, mass and IR spectrophotometry showed that the components of the complex physico-chemical properties differ from nisin A, having basic properties. Faction LGS-H_1 has a peak at λ_{max} 215 nm in the UV spectrum (in H_2O) with mol. mass (MALDI-MS) = 3161.6; IR spectrum (in KBr) with peaks at V_{max}, cm^{-1}: 3600-3400, 3300-3200, 2940, 1700-1650, 1600-1560, 1470-1420, 1350 1250-1230 1160, 1130, 1060, 1020, 950.850, 750, 660; TLC (on SiO_2) with R_f = 0,0 in MeOH-H_2O system (96 : 4); on paper electrophoresis in electrolytes with a pH = 2.4 and 1.1 (550 V, 2 h.) the fraction migrated to the cathode with a mobility higher than the standard nisin.

Faction LGS-H is a polypeptide, disintegrating in the acidic environment in the two subunits with molecular masses of 3353 and 3161.6 Da. Based on the physico-chemical data LGS-H antibiotic attributed to a group of antibiotics, polypeptides having basic (alkaline) rather than acidic properties, i.e., it can be attributed to nisins. Faction LGSH_1 is a nisin A and LGSH_2 - a new form of nisin. Bi -peptide bacteriocins were isolated and identified from the LAB by other authors [13, 18]. For example, wild-type strain *L. lactis* ssp. *lactis* -K isolated complex, one of which was with nisin A, a second peptide having a lower molecular weight, was a new biologically active substance of peptide nature with a broad-spectrum bactericidal activity (inhibited and Gram-negative bacteria), but not classified as lantibiotic nisin [13]. From fraction LGSB by column chromatography and preparative TLC chromatography the pure component B was obtained. Fraction LGS-B in the UV spectrum (in C_2H_5OH) has a peak with λ_{max} at 260 nm, with mol. mass (MALDI-MS) = 506,9; TLC (on SiO_2) gives a R_f = 0.75 in MeOH-H_2O system (96 : 4); on paper electrophoresis in electrolytes with a pH = 2.4 and 1.1 (550 V, 2 hr.) the fraction remains at the start. Spectrum IR (in $CHCl_3$) has the peaks with following V_{max}, cm^{-1}: 3670, 3360, 2850, 1720, 1665, 1450 1380, 1220-1200, 1120, 1090.

The antibiotic LGS-B assigned to the group of alkyl-aromatic ketones containing hydroxyl group. This component is a low molecular mass hydrophobic compound. Results of the study of the spectra of antimicrobial action of selected components shown, that the component B has a broad spectrum of antimicrobial activity: active against most Gram-positive organisms, including heat-resistant *B. coagulans*, Gram-negative bacteria and fungi *Aspergillus repens, Candida guilliermondii*, which expands the range of applications and the importance of it as biopreservative for sausages, fresh vegetables and raw mushrooms undergoing deterioration [7, 8, and 18].

Faction LGS-C is a minor component, its biological activity is negligible.

Table 2. Physico-chemical and biological properties of the components of antimicrobial complex of the strain *L. lactis* ssp. *lactis* F-116

Properties	Components				
	LGS -B	**LGS -C**	**LGS-H_1**	**LGS –H_2***	**Nisin** A
$(M+H)^+$, m/z, (MALDI-MS), Da.	506.9	-	3161.6	3353	3353**
UV-VIS spectrum , λ_{max}, nm (solvent)	260 (C_2H_5OH)	215; 274 (C_2H_5OH)	215 (H_2O)	215 (H_2O)	215 (H_2O)
TCX (SiO_2), R_f MeOH–H_2O (96 : 4)	0.75	0.43	0	0	0
Electrophoresis on paper in an electrolyte:** **1. E_1, pH = 2.4, 550 V, 2 h**	0	0	11.0	9.5	9.5
2. E_2, pH = 1.1, 250 V, 3 h	0	0	4.3	3.3	3.3
The biological activity spectrum	Gram+, Gram- bacteria, fungi	Weak effect on Gram+ bacteria	Gram+ bacteria, including thermostable *B. coagulans*		

*Fractions obtained from LGS-H by preparative electrophoresis on paper at E_1 electrolyte 550V, 2.5 h. Manifestation of Pauli reagent, test-organisms *B. coagulans*.

**Submitted by the distance traveled by a substance from the starting line to the cathode, to see "0 cm" (on the start line).

3.6 Effects of adverse conditions of the gastrointestinal tract

In our model experiments on the effects of adverse conditions of the gastrointestinal tract on the fusant strain F-116 was studied as probiotic culture. The food entering the stomach is exposed to the action of gastric juice. Pure gastric juice has hydrochloric acid in concentrations of about 0.3 - 0.5%, this corresponds to a pH of 1 to 3 in the healthy stomach [2, 6]. It was revealed that addition of 0.2% of hydrochloric acid to the cultural medium the growth rates were reduced by 30% to 49% after 3 hours of incubation. But the strain was resistant to high concentrations of hydrochloric acid (0.3% - 0.5%).The survival rate of strain F-116 was up to 90% after 1 hour of exposure, 84% - after 2 hours, and after 3 h - 82%. Lactococcus number remained at the same level when bile concentration in the medium was up to 20%.

3.7 Antioxidant activity

Growth of LAB inhabiting human macro-organism, as well as coming from outside, depends on processes occurring in it, including free radical action. Free radicals damage DNA, proteins, lipids, forming the peroxide compounds. Reactive oxygen species (ROS) trigger programmed cell death - apoptosis [19]. ROS accumulation (particularly hydrogen peroxide) in the products of fat causes rancidity and discoloration of meat products by destruction of the pigments, which leads to a loss of quality [1]. An interesting fact is that some of the LAB can survive in the presence of sufficiently high concentrations of H_2O_2. That capable not only remain viable, but also to share in a sufficiently strong oxidative stress indicates that the cells have their antioxidant defense enzymes, such as superoxide dismutase (SOD). It was found that the recombinant strain F-116 has a high-SOD activity up to 30 U/mg of protein that was 3.7, 5.3 and 6.0 times greater than the activity of the strain MGU and parental strains 729 and 1605, correspondingly. The use of the LAB with high antioxidant activity will create effective preparations for reducing oxidative food spoilage during storage, as well as the fight against serious diseases and early signs of aging [16, 20].

3.8 Characteristic LGS preparation synthesized by strain *L. lactis* ssp. *lactis* F-116

LGS preparation was obtained by freeze-drying the culture broth of the strain F-116 after 10 h growth in accordance with laboratory regulations to the process of synthesis of bacteriocin. The activity of freeze-dried sample was 1.2 mln. IU/g, based on the activity of the standard Nisaplin containing 2.5% of pure nisin A. The dried LGS preparation is a mixture of preservative, probiotic cultures of *Lactococcus* and useful metabolites. The pH of 10% aqueous solution was 3.6 - 3.8. The preparation was stable when it was heated at 100 ^{0}C for 30 min. at a pH lower, than 4.0.

The solubility of the preparation is depend on the pH: in distilled water at pH 5.9 dissolves 50 mg/mL, which is equal to 2 mln. IU/mL. In tap water having a pH of 7.0 it gives 49 mg/mL (1.96 mln. IU/mL); in 0.02 N hydrochloric acid - 118 mg/mL (4.72 mln. IU/mL); in 2% NaCl solution - 47.9 mg/mL (1.91 mln. IU/mL); in skim milk at pH 6.4 - 87.5 mg/ml was dissolved. LGS preparation in biological effect exceeds the commercial preparation of Nisaplin which acts only on Gram-positive bacteria.

According to the properties and spectrum of inhibitory activity, the isolated compounds correspond to the currently accepted definition of bacteriocins, which include thermostable peptide protein compounds. Fast display of antimicrobial effect of bacteriocins may be indicative of membrane orientation of action of inhibiting substances, characteristic of the majority of known bacteriocins [13, 19, 21].

3.9 Toxicological studies

These studies have shown that this LGS preparation has no inhibitory effect on the growth of ciliate *Tetrahymena pyriformis*. The addition of 1.0% of it in a medium with *T. pyriformis* caused the growth incensement for 17 days compared to control in the lag- and the exponential growth phases. The extend of the length of stationary phase was also observed, possibly, due to the fact that the culture of protists received additional substrate contained in a composition.

4. Conclusions

- The prospects of protoplast fusion method, ensuring the creation of active producers of broad spectrum antimicrobials with unique properties. By this method we obtained a new non-toxic preservative with broad spectrum of antibacterial and fungicidal action, produced by fusant strain of *L. lactis* ssp. *lactis* F-116.

- This biopreservative is effective against pathogenic and opportunistic bacteria developing in food raw materials during storage.

- Obvious prospects of using the preparation to increase the shelf life of products and raw materials are visible, which will help the food industry to abandon the use of expensive chemical preservatives, antibiotics, and satisfy customer requirements for food safety.

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