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Technological aspects of probiotics obtaining

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Abstract: Current groups of drugs using for correction of the humans normal microflora were reviewed. The types of problems and promising directions for improving probiotics were shown. Different probiotic's drug-forms were considered, and the possible aspects of the efficiency of bacterial agents were shown. Technological methods to improve technologies of obtaining and extending the shelf life of probiotic were analyzed. The technology of polystrain substance of probiotic with high therapeutic properties and spread spectrum of therapeutic action were shown.
Keywords*: Probiotic, probiotic microorganisms, multyprobiotic, technology.*

1. Introduction. The term "probiotic" is widely used for over 50 years. Defining it clarified during the accumulation of experimental data. The latest was proposed by Canadian professor of microbiology and immunology Gregor Reid (2003): Probiotics – is living microorganisms, using of which in adequate doses leads to improving the health of the host [4, 9].

Modern methods for correcting violations in human microbial ecosystem based on the use of a wide range of bacterial products and functional food enriched with probiotic microorganisms.

Despite a broad range of imported and domestic probiotics, problems remain in their improvement, which are as follows:

- ✓ study of the physiology of perspective industrial strains to match nutrient media for their cultivation;
- ✓ determining the sorption processes of probiotic bacteria as general biological process;
- ✓ study of the role of metabolic products and biologically active substances of microbial cells to determine the nature of adhezyns, as mechanism of antagonistic activity;
- ✓ development of technology of integrated products based on consortium of bacteria with a wide range of antagonistic activity;
- ✓ study of synergistic and inhibitory effects of various species and strains of probiotic bacteria;
- ✓ development of optimal drug release forms (powder, tablet with protective coating, capsules, granules, suppositories, ointments, gels, etc.) that would ensure the preservation of the biological properties of probiotics and ease of use;
- ✓ improvement of methods of determing antagonistic activity of preparations containing viable microbial cells and developing methods for the determination of living organisms [8, 9, 11].

Among the scientific and practical areas related to microbial ecology, promising for development and implementation are:

- ✓ development of express molecular methods for determing of the composition and activity of the humans and animals microflora;
- ✓ search for new functional prebiotic substances;
- ✓ research and detailed molecular, biochemical and other mechanisms of the effectiveness of probiotics, prebiotics and synbiotic medications in the prevention, treatment and increase in terms of remission caused by various diseases associated with imbalance of the microbial ecology of the digestive tract;
- ✓ in-depth assessment of harmlessness probiotic preparations and functional food enriched by probiotic microorgansms;
- ✓ explore the possibility of using representatives of normal microflora as carriers for constructing various bacterial and viral vaccines;
- ✓ creation of modern biotechnology companies for production probiotics, prebiotics and synbiotic medications, antibiotics, immunomodulators, vitamins, peptides, biosensors and others from representatives of normal anaerobic microflora of humans and animals [6-9].

Presently probiotics are available in following forms: freeze-dried biomass in vials or ampoules, lyophilized biomass in gelatin capsules, rectal and vaginal suppositories with lyophilized biomass; freeze-dried biomass pressed in tablets, coated with soluble in gut membrane substances, lingual tablets that dissolve under tongue [1-5, 10, 11].

Improving efficiency of domestic probiotics is an actual problem, whose solution requires development of elements of technological unification. The main stages of probiotic technologies associated with accumulation of microbial biomass and its stabilization is the object of intense research. Development and practical application of the same type culture media for cultivation of industrial strains of bacteria and protective media for lyophilization products reflect the current level of harmonization of technology of probiotics.

Microbiological practice shows that effective medium for the cultivation of industrial strains of bacteria can be prepared using nutrient bases with a fairly wide range of interchangeable substrates of animal, plant or other origin. Nutritional basis that contains the necessary nutrients for the metabolism of various microorganisms can be used as a universal base component in the design of culture media for various purposes. It is possible to develop standardized systems of nutrient media for industrial applications. Culture media as a structural unit of a unified set shall consist of two parts: constant (universal), which includes the base substrate and the variable (specific), depending on the specific needs of the production strain of bacteria. Making such medium may include separate prepering of both parts, and their mixing can be carried out immediately before or during the cultivation of microorganisms [4, 8, 13].

As an example of this approach in the practice of receiving probiotics are casein-yeast medium. It is due to the fact that they largely meet the requirements of industrial production on the set of biological, technological and economic parameters [8].

Most probiotics come in lyophilized form (powders, tablets, capsules, suppositories). The dry form is characterized by a high shelf life, easy of transportation and storage, requires strict adherence to temperature. More efficient use of hardware freeze equipment in the traditional production of probiotics in the form of dry biomass in vials and ampoules involves using protective mediums, allowing maintaining cell viability to provide the necessary structure (appearance) of dry product in a short and intense mode of drying. Practice of developing protective mediums suggests to minimize cell death and waste products in the physical properties of cryoprotectant composition for each species of bacteria must include qualitatively and quantitatively balanced set of components [4, 6, 8, 11].

Unification of protective mediums used in the production of probiotics includes limiting the number of components required in the cryoprotectants for "hard" freeze mode. Under these modes of drying negative biological effects and defomation of structure are decreases by offset increasing concentration of cryoprotectant in the microbial suspension. At the same time achieve a better structure of dry biomass is much more complicated than getting the required number of living cells in a dry preparation. Working out the indicated problems was succeeded by using sucrose-gelatin-milk protective medium that are currently using in the production of most probiotic products [8, 13].

However lyophilized forms of probiotics have several disadvantages, including long term release of microbial cells from a state of anabiosis (8-10 hours in optimal conditions of cultivation, which can be achieved only in the laboratory). In the gastrointestinal tract (GIT) during this period of time much of probiotic cells can eliminated, failing to activate. Therefore, the production of probiotics in dry form has more to do with business interests of manufacturers than to providing high quality products. In humans, much of lyophilized microorganisms are killed before reactivation in harsh conditions of GIT [4, 8].

Technological methods that are administering prebiotics to stimulate probiotic flora, can not always make a difference. Firstly, the amount of prebiotics that can be entered into the dose is too small for the display of significant effect. Secondly, during transit through the proximal GIT habitats in most cases prebiotic metabolized [4, 10].

Use acid-soluble capsule does not solve the problem of increasing the effectiveness of oral probiotics as high acidity of the GIT tract is only one of obstacles. And the significance of this barrier disappears if oral probiotic taken with food, which is a powerful factor in protecting microorganisms from gastric juice. Noteworthy and are increasingly being used rectal probiotics [4].

Much more effective is "live" probiotics in the form of a liquid suspension in a special protective environment. In these preparations the bacteria are in physiologically active form and can act immediately after ingestion. Probiotic microorganisms in a liquid form is active, viable in harsh conditions of GIT, do not require long-term reactivation, showing its effect upon entry into the body. In addition, this

form of probiotics is best for children [8-10].

Innovation dosage form of probiotics is lingual (porous instant) tablets are prepared by freeze-forming technology. This allows to obtain bacterial preparations with highly internal surface (porosity). Advantages of freeze-forming technology is one-step formation of a probiotic tablet form with high biological activity, while traditional technology is multistage and includes drying and growing of biomass, mixing biomass powder with excipients (fillers, baking powder, binders, dyes etc) and compaction under pressure. Technology of tablet form bacterial drug is reduced to one operation – freeze-drying cell suspension into matrices with complex protective medium adding to it at least 7 – 9% of ballast substances (structuremakers, bioprotectors). Production of porous tablets requires no special equipment for release from forms as a result of biomaterial compression during drying under vacuum is its detachment from the walls of shape matrix, which allows removing the dry tablet by shaking [4, 10].

Development of a method of designing probiotics in the form of strong mutualistic multysymbiozis represents significant progress in improving treatment of bacterial drugs. When receiving other complex probiotics a certain strains mixed in certain proportions under one condition – no antagonism between the strains. These combinations of strains grown under standard laboratory conditions, is not the rule but the exception to the existence of microorganisms. Getting in human biotops in the highly competitive conditions with other well-adapted microflora, they either die, turning into an edible substrate, or significantly reduce its activity [13].

Necessary condition in the development of technology and production of probiotics is to keep them stable for a long time. Bacterial drugs containing live microorganisms, is the least stable, since their activity may decrease as a result of cell death. Microorganisms because of low levels of biological organization remains viable even with complete dehydration, in which case only in cells inversely slowing or stopping metabolism. To prolong the viability of bacteria is advisable to freeze drying, which occurs at low temperature and high vacuum. Due to the hygroscopic sealing dry biologics engaged under vacuum or in inert gas flow [4, 8, 12].

Factors affecting the survival of microorganisms in dry probiotics during storage is regulated residual moisture content, the presence of protective mediums, storage drugs in dry oxygen-free atmosphere. In order to protect probiotics from stomach acid into tablets and encapsulated forms applied acidresistant coatings or immobilizate bacteria on sorbent [4, 8].

2. Results. As an example of probiotics technology can lead technology, developed by author, of substance of polystrain bacterial probiotic with therapeutic properties and wide spectrum therapeutic effects through additional beneficial activities: antimutagenic, hypocholesterolemic, proteolytic action, sorption of heavy metals, immunomodulation and antiviral properties. Substance of polystrain probiotic developed on the basis of five pre-selected high-probiotic strains of bacteria genus *Lactobacillus*. Technology of the substances is include such stages:

- ✓ A culture first generation (all five strains of lactic acid bacteria cultured separately in flasks on MRS medium at 37 ± 1 ºC during 48 hours);
- ✓ A culture of second generation (the first generation culture used as inoculum for obtaining appropriate cultures of the second generation, increasing the volume of culture medium in 10 times, after 24 hours carrying out microbiological control and determine the number of live cells of lactic acid bacteria - should be at least 10^9 CUO / ml);
- ✓ Biosynthesis (carried separately each strain in fermenter on casein-yeast medium with parameters: temperature 37 ± 1 °C; pH 6.8 – 7.0 (regulated 5% solution of ammonia) overpressure 0.03 – 0.04 MPa, duration of 8 – 10 hours, periodically (every hour for 10 minutes) include mixing device (70 rev/min) and twice (after 2 and 5 – 6 h of cultivation) in fermentor served sterile glucose solution to final concentration in the medium 1.5 – 1.7%. Cultivation process is stopped when concentration of lactic acid bacteria - 10^9 CUO / ml.)
- ✓ Stabilization of culture broth (culture fluid of five strains of bacteria of genus Lactobacillus, obtained under industrial biosynthesis are combined in a ratio of 1:1:1:1:1 (in terms of optical density of cell suspension) and add milk-sucrose-gelatin protective medium;
- ✓ Freeze-drying [12].

Developed substance consist of – *Lactobacillus delbrueckii subsp. VKPM*

bulgaricus LB86-B-5788: *Lactobacillus delbrueckii subsp. delbrueckii* DSM20074: *Lactobacillus rhamnosus* LB3 IMB B-7038: *Lactobacillus rhamnosus* V ®: *Lactobacillus acidophilus* (C) – 1:2:1:1:1, the final concentration of strains in substance – 1×10^9 CFU / ml. This ratio of strains responsible for high rates of probiotic properties developed substance. All strains isolated from healthy people, resistant to harsh conditions of the gastrointestinal tract of human (gastric juice, low pH, digestive enzymes), and have a high probiotic properties listed in tabl. 1.

Tab. 1 Probiotic properties of strains genus *Lactobacillus* as base of polystrain substance

Property	Strains of genus *Lactobacillus*				
	L. rhamnosus LB3 IMB B-7038	*L. acidophilus* (C)	*L. delbrueckii subsp. bulgaricus* LB86 BKПM-B-5788	*L. delbrueckii subsp. delbrueckii* DSM20074	*L. rhamnosus* V®
Antagonistic activity, mm					
E. coli	42.3±0.51	36.1±1.05	37.9±1.23	36.6±1.21	31.2±1.25
P. fluorescens	30.4±1.0	24.2±0.83	27.8±0.94	268±0.97	29.8±1.03
A. colcoaceticus	35.3±0.73	32.3±0.99	20.9±0.71	21.2±0.83	31.3±1.18
S. marcescens	36.4±1.12	19.2±0.52	38.2±0.99	27.8±0.79	33.4±1.22
B. linens	30.7±1.09	28.8±0.74	22.8±0.85	23.1±0.67	34.2±1.07
B. mycoides	30.5±0.95	35.1±1.22	34.7±1.13	32.4±1.18	30.3±1.19
B. megaterium	33.2±1.21	20.1±1.07	26.1±0.97	34.3±1.21	24.3±0.98
B. subtilis	34.1±1.18	28.4±0.98	20.9±0.81	29.8±0.99	30.7±1.14
B. cereus	37.7±2.07	33.4±0.83	37.8±1.13	38.3±1.22	30.4±1.17
S. citreus	37.5±1.17	34.6±1.15	34.0±1.15	36.1±1.25	36.4±1.15
S. aureus	37.6±1.32	33.7±1.20	34.7±1.24	32.4±1.18	32.5±1.23
N. carollina	22.3±1.16	27.4±0.85	31.1±1.18	20.3±0.76	30.3±1.26
S. flava	20.3±0.92	28.6±0.91	34.1±1.11	33.9±1.08	33.4±1.29
Acid formation, °T	210.8±4.75	193.8±5.81	170.0±7.81	178.5±6.33	197.2±5.45
Lyzocime formation, mm	0	5	0	0	0
Adchesive activity					
Native erithrocyte	5.08±0.23	3.23±0.11	4.93±0.17	5.09±0.25	6.57±0.28
Formalize erithrocyte	4.21±0.18	3.34±0.08	4.16±0.18	4.93±0.22	6.0±0.29
Resistant for antibiotics, zone of growth delay, mm					
benzylpenicillin	2±0.07	0	0	11±0.52	3±0.12
ampicillin	0	0	0	0	0
amoksicillin	0	0	4±0.11	13±0.59	7±0.35
cefazollin	0	0	0	11±0.55	5±0.21
amiacin	0	0	0	6±0.28	0
gentamicin	4±0.13	0	9±0.44	11±0.49	7±0.36
streptomicin	7±0.34	0	3±0.08	7±0.31	0
linkomicin	17±0.75	5±0.22	9±0.24	25±1.14	13±0.54
doksiciklin	0	0	0	9±0.38	7±0.32
levoflocin	4±0.17	0	2±0.06	5±0.24	0
levomicitin	0	3±0.14	0	4±0.18	4±0.17
Antimutagenic activity,%	99.03±2.87	58.43±1.99	90.38±3.12	95.90±4.05	81.22±3.98
Proteaze, OD/mg	637.04±17.13	339.13±15.01	74.82±2.34	492.5±9.81	70.84±3.42

Also selected strains have hypocholesterolemic activity, the ability to reduce serum cholesterol. Strains with high level of accumulation of external proteolytic enzymes, indicating the possibility of their use in digestive disorders associated with lack of

appropriate enzymes in the host, and to modify the immunogenicity of foreign proteins by proteolysis. The strains have high antimutagenic properties (at 58.43–99.03%). They exhibit high desmutagenic effect. Selected strains do not exhibit antagonistic activity against to each other, but rather characterized symbiotic relationship in the mixture.

Design of the polystrain bacterial substance based on the fact that for each person who uses the bacterial drug based on it, creating favorable conditions for the selection of his representatives of those species of lactobacilli that his intestines are in short supply and polystrain probiotics concentrated in a wide range of biotherapeutic functions.

Designed substance is not toxic to cells monolayer pig testicles PTP, mouse fibroblast L-929 and splenocytes and is able to stimulate the functional activity of peritoneal exudate macrophages of mice, increase the cytotoxicity of natural killer cells and has antiviral activity. Characteristics of desined polystrain probiotic substance listed in table 2.

Tab. 2 Probiotic properties of the polystrain probiotic substance

Properties	**Characteristic of the polystrain probiotic substance**
Concentration of live bacterial cells, CFU / ml	1×10^9
Antagonistic activity (zone of growth retardation test cultures), mm *Staphylococcus aureus* *Staphylococcus citreus* *Salmonella typhimurium* TA100 *Klebsiella pneumonia* *Serratia marcescens* *Escherichia coli* *Bacillus subtilis*	 39.0±1.20 38.0±1.15 41.0±1.22 39.0±1.15 37.0±1.15 32.0±1.15 38.0±1.20
Adhesive index	5.31
Titer of serum interferon, units/ml on 6-th hour on 24-th hour	 3.7 $\log_2$ 3.0 $\log_2$
Titer of circulating interferon, units/ml	4.2±0.5 $\log_2$
Concentration of Tumor necrosis factor-α, ng/ml on 6-th hour on 24-th hour	 1.1 0.8
Phagocytic number,% on the 1st day of observation on the 3rd day of observation 5 th day of observation	 57.2±2.2 57.5±2.3 57.4±2.2
Phagocytic index, conv on the 1st day of observation on the 3rd day of observation 5 th day of observation	 5.8±1.6 5.7±1.3 5.8±1.6
Cytotoxicity index, %	39.7
Index of effectivenes in experimental herpetic meningoencephalitis in mice	50.0

3. Conclusion. Due to the results of numerous medical studies, probiotics based on human physiological flora at present regarded as an effective method of restoring normal composition and function of different biotops, and the emergence of a new science-based information on the subject creates huge opportunities for probiotics to replenish the arsenal of new effective bacterial drugs.

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Старовойтова С.А., Скроцкая О.И. Технологические аспекты получения пробиотиков

Аннотация. Рассмотрены современные группы препаратов, используемые для коррекции нарушений нормальной микрофлоры человека. Освещены проблемы и перспективные направления совершенствования пробиотиков. Рассмотрены формы выпуска пробиотиков, а также показаны возможные аспекты повышения эффективности производства бактериотерапевтических препаратов. Проанализированы технологические приемы, направленные на совершенствование технологии получения и продления срока хранения пробиотиков. Приведена технология получения субстанции полиштамового бактериотерапевтических препарата с широким спектром терапевтического действия.

Ключевые слова: *пробиотики, пробиотические микрорганизмы, мультипробиотики, технологии.*