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STUDY OF THE BIOTECHNOLOGICAL POTENTIAL OF SELECTED LACTIC ACID BACTERIA CULTURES

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Abstract. Fermenting microflora has been selected by biotechnological activity markers, with various methodological approaches used, namely: directional selection, selection of bacteriophage-insensitive mutants, protoplast regeneration. The experimental data show a significant biotechnological potential of the selected lactic acid bacteria. They are characterized by high milk-clotting activity and yield, the ability to form aromatic compounds and/or viscous components, excellent organoleptic qualities of clots fermented by them, antagonistic activity against pathogenic and opportunistic pathogenic microorganisms, and phage-resistance to species-specific virulent phages. The collection of industrial microorganisms of Institute of Food Resources of National Academy of Agrarian Sciences (IFR NAAS) has been supplemented with new bioactive strains, in particular, the species *Streptococcus thermophilus*, *Lactobacillus acidophilus, Lactobacillus rhamnosus* and *Lactococcus lactis.* Four bacterial compositions have been created. Three of them (Iprovit-LB-R; Iprovit-LB-A, and Iprovit-ST) are supposed to be used as functional enrichers for dry foodstuffs or as biologically active supplements. The bacterial preparation Iprovit-Bifidolux is a universal composition. It can be introduced both as a fermenting culture for milk fermentation and as an enricher for dry and liquid foodstuffs. Biotechnologies for the production of dry bacterial preparations on the basis of selected strains have been field-proven at the State Research Enterprise of Starter Cultures that belongs to the IFR NAAS. The data on a wide range of clinical and therapeutic effects of the bacterial preparation Iprovit-Bifidolux allow recommending it for manufacturing functional foods.

Key words: lactic acid bacteria, selection, bacteriophage-insensitive mutants, biotechnological potential, functional nutrition.

ВИВЧЕННЯ БІОТЕХНОЛОГІЧНОГО ПОТЕНЦІАЛУ СЕЛЕКЦІОНОВАНИХ КУЛЬТУР МОЛОЧНОКИСЛИХ БАКТЕРІЙ

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Анотація. Проведено селекцію заквашувальної мікрофлори за маркерами біотехнологічної активності з використанням різних методологічних підходів, а саме: спрямованого відбору, відбору бактеріофагнечутливих мутантів, методу регенерації протопластів. Наведені експериментальні данні свідчать про значний біотехнологічний потенціал селекціонованих молочнокислих бактерій. Вони характеризуються високою молокозсідальною активністю та урожайністю, здатністю до утворення ароматичних сполук та/або в'язких компонентів, відмінною органолептикою ферментованих ними згустків, антагоністичною активністю щодо патогенних і умовно патогенних мікроорганізмів, фагостійкістю до видоспецифічних вірулентних фагів. Поповнено колекцію промислових мікроорганізмів Інституту продовольчих ресурсів Національної академії аграрних наук (ІПР НААН) новими біоактивними штамами, зокрема видів *Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus rhamnosus* та *Lactococcus lactis.* Створено 4 бактеріальні композиції – три з яких: Іпровіт-LB-R; Іпровіт-LB-A та Іпровіт-ST призначені для використання як функціональні збагачувачі для сухих продуктів чи біологічно-активні добавки. Бактеріальний препарат Іпровіт-Біфідолюкс є універсальною композицією, придатною для впровадження як заквашувальна культура для ферментації молока, так і як збагачувач для сухих і рідких продуктів. Біотехнології виробництва сухих бактеріальних препаратів на основі селекціонованих штамів опрацьовано в промислових умовах на Державному дослідному підприємстві заквасок ІПР НААН. Враховуючи наукові дані щодо широкого спектру клініко-терапевтичних ефектів від вживання бактеріального препарату Іпровіт-Біфідолюкс, його можна рекомендувати для використання у виробництві продуктів функціонального харчування.

Ключові слова: молочнокислі бактерії, селекція, бактеріофагнечутливі мутанти, біотехнологічний потенціал, функціональне харчування.

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Introduction. Formulation of the problem

Much attention is paid worldwide to the issues of selection of biologically active lactic acid bacteria cultures and to the search for effective methodological solutions for their industrial application. Research institutions of the Netherlands, Denmark, France, Sweden, and Germany are commonly recognized as leaders in researching the physiology and genetics of lactic acid bacteria. The best known enterprises developing and manufacturing bacterial preparations for dairy industry are Chr. Hansen, as well as Danisko, Danon, Rhodia, and others [1].

However, domestic dairy industry needs to be substantially updated as for introducing effective bacterial preparations to broaden the products assortment – in particular, to manufacture new and authentic fermented milk products and cheeses. This certainly requires a deep scientific background of the problem, fundamental changes in the selection and evaluation of microorganism cultures, new methodological solutions for the selection of lactic acid bacteria by markers of valuable industrial characteristics, principles for constructing starter preparations based on them. All of the above indicates how important and promising the problem is.

Introducing a scientifically based strategy for the search for, selection, and rotation of highly active strains of lactic acid bacteria of different taxonomic groups, establishing the conditions for combining them effectively in complex bacterial compositions will expand the range of domestic bacterial preparations, improve the productivity of the fermentation of the milk base, prevent great material and energy losses when manufacturing starter cultures and products based on their use in the presence of phage contamination.

Analysis of recent research and publications

According to experts' estimates, about 80 % of food production is somehow related to microbiological processes (the production of cheese and fermented milk products, bread baking, winemaking, beer brewing, rye beer and vinegar making, fermentation and pickling fruit, vegetables, meat, and fish).

The species of bacteria used for these industries generally belong to the genera *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Lactobacillus* and are isolated from their natural sources, namely plants or fermented dairy and meat products. As a result of the consumption of nutrients, lactic acid bacteria produce various metabolites and organic acids that do not only have a preservative effect due to suppressing the growth of microorganisms that cause food spoilage, but also improve the sensorial qualities and texture of foodstuffs [2].

The main task of the dairy industry is constant exploring new ways to improve the taste and texture of products in order to meet consumers' demand, or reducing additives, sugar, fat, and the total calorie content, or introducing probiotic microflora that has functional, healing effects on the human body. This requires the constant creation of new starter cultures with improved properties [3].

Analysis of scientific information shows that production technologies for bacterial preparations based on strains of lactic acid bacteria and other microorganisms are being developed in the following aspects: improving the texture-forming ability of strains (in particular, optimizing the metabolic pathway of exopolysaccharides (EPS), bacteriophage-resistant mutants, etc.) [4]; increasing a product's stability (low postoxidation strains of *Lactobacillus*) [5]; elimination of traits undesirable in any food fermentation (for example, elimination of resistance to antibiotics) [6]; increasing stress resilience (*Lactobacillus* and *Bifidobacterium* strains with probiotic properties, with increased resistance to phenol or bile) [7]; changing the acid-producing properties of strains by adjusting carbohydrate metabolism [8]; increasing the productivity of fermenting cultures (growth rate, yield, functionality). Scientists of the whole world put a lot of effort and use various methodologies and techniques to obtain phage-resistant sntrains of lactic acid bacteria. Although lactic acid bacteria have natural mechanisms of resistance to bacteriophages, it is necessary to select bacteriophage-resistant strains constantly due to the high adaptability and biodiversity of bacteriophages [9].

Industrial strains can be improved using classical methods based on natural strain-improving strategies, such as: screening wild-type strains from natural sources [10], mutagenesis [11], adaptation [12], and directional selection [13]. The above listed classical methods can be combined with natural gene transfer mechanisms (such as conjugation, transduction, and transformation) to create industrial strains, including phage-resistant ones, with improved properties. The use of genetic engineering methods, which have great potential for developing anti-phage mechanisms based on specific point mutations in bacterial DNA, and the creation of phage-resistant mutants are currently limited because the industrial use of genetically modified microorganisms is illegal [14].

Selected strains, as starter cultures, or cocultures, can be used in biotechnological processes of fermentation of livestock raw materials can help to achieve the desired properties *in situ*, with the product remaining perfectly natural and healthy. Examples are strains of lactic acid bacteria, which are capable of producing antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, beneficial enzymes (dietary supplements), or cultures with health-improving properties, so-called probiotic strains. Probiotic strains are used in functional starter cultures to produce fermented foods [15].

The aim of the work is to search and select cultures of lactic acid bacteria with high biotechnological potential, and to study the prospects of industrial use of the selected cultures.

The objectives of the research are:

1) to screen biotechnologically active strains of various taxonomic groups;

2) to create effective bacterial compositions based on them;

3) to field-test the biotechnologies developed;

4) to study the functional effect of the selected strains.

Research materials and methods

The objects of the research were newly selected cultures of lactic acid bacteria, microorganism compositions, bacterial preparations, and fermented dairy products. During the work, traditional and novel microbiological, technological, and biochemical research methods were used. Selection studies of strains were carried out in accordance with the *Bergey's Manual of Determinative Bacteriology* [16]. The number of microorganisms was determined by the number of colony forming units (CFU) in an agarized medium, using the tenfold dilutions method. The bacteriophages ratio was determined by the double agar method with the addition of 10 mM $CaCl_2$ [17]. By the method of co-cultivation of lactic acid bacteria with test cultures in a liquid medium, the antagonistic activity titre was defined as the maximum dilution of the bacterial mixture after 24 hours' cultivation, which the test culture was plated from. The lactose content was determined by high performance liquid chromatography on a LC-5 chromatograph (Shimadzu). To obtain dry bacterial preparations, the biomass of the sample cultures was accumulated in industrial reactors with the working and total volumes 700 and 1000 dm^3, respectively. The biomass obtained was centrifuged from the culture fluid, and mixed with a cooled protective medium. The drying was carried out on a TG15 freeze dryer, with the initial temperature minus (30 ± 2)°C, final temperature plus (20 ± 2)°C, residual pressure not more than $13.3 \cdot 10^3$ Pa (0.133 kgf/cm^2, drying time 24–28 h).

Results of the research and their discussion

A number of biotechnological properties of strains selected with different techniques have been studied. The strains selected are listed in Table 1.

Table 1 – Species and selection technique of the lactic acid bacteria strains considered

Genus, species	Number of selected strains	Origin	Method of obtaining
Lactococcus lactis ssp. lactis	5	Curdled milk	*Directional selection*
Lactococcus lactis ssp. cremoris	2	Sour cream	*Directional selection*
Lactobacillus rhamnosus	2	Cheese	*Directional selection*
Streptococcus thermophilus	14	Cheese, sour cream, yoghurt	*Directional selection, selection of bacteriophage-insensitive mutants*
Lactobacillus delbrueckii ssp. *bulgaricus*	2	Yoghurt	*Directional selection*
Lactobacillus acidophilus	4	Yoghurt	*Directional selection, protoplasts regeneration method*

Each of them was characterized by high milk-clotting activity (MCA) and yield, ability to form aromatic compounds and/or viscous components, excellent sensorial qualities of fermented clots. Most of them were phage-resistant to species-specific phages. The parameters of the main technological properties of the strains are given in Table – 2.

Table 2 – The technological properties of the lactic acid bacteria strains considered

Genus, species	Number of selected strains	MCA, h	Yield in milk, $\times 10^8$ CFU/cm^3	Number of phage-resistant strains	Number of strains able to synthesize	
					viscous polymers	diacetyl, acetoin
L. lactis ssp. *lactis*	5	7–12	7.4–20.0	3	0	2
L. lactis ssp. *cremoris*	2	6	1.0–6.3	1	1	0
L. rhamnosus	2	19–22	6.7–8.2	2	2	2
S. thermopilus	14	3–9	6.4–15.0	11	11	2
Lb. delbrueckii ssp. *bulgaricus*	2	5–6	6.8–7.8	2	2	0
Lb. acidophilus	4	5–7	5.6–7.4	4	4	0

Cultures of *L. rhamnosus* and some strains of *L. lactis* ssp. *lactis* had a high level of accumulating aromatic compounds, such as diacetyl (DC) and acetoin (AC). During the fermentation, most DC was formed by *L. rhamnosus* strains (up to 600% of the initial level) (Table. 2). The ability of thermophilic streptococci to synthesize DC and AC was determined only in 2 of the 12 strains studied and was significantly less intensive – the diacetyl content was (0.03–0.05) mg/100 g.

The strains studied differed in the level of lactose consumed. In particular, *Lb. acidophilus*, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* fermented 20 to 50% lactose after 18-h cultivation, and mesophilic lactic acid cocci and bacilli of *Lb. rhamnosus* species – about 18–37% (Table 3).

It should be noted that two strains of *Lb. acidophilus* had a low acid-producing ability – the value of the AFT parameter did not exceed 180°T. This resulted from the selection of strains by the protoplast regeneration method [18].

Table 3 – The amount of fermented lactose and the acid production level of lactobacilli during their growth in milk (after 18 h of fermentation)

Genus, species	Number of selected strains	Uilized lactose, %	Acid formation threshold (AFT), °T
		$M_{min} – M_{max}$	$M_{min} – M_{max}$
L. lactis subs. *lactis*	5	18–25	88–110
L. lactis ssp. *cremoris*	2	19–22	100–106
Lb. rhamnosus	2	28–37	140–160
S. thermophilus	14	40–48	108–116
Lb. acidophilus	4	20–45	180–350
Lb. delbrueckii ssp. *bulgaricus*	2	39–50	365–370

Note: M_{min}, M_{max} – minimal and maximal values of a parameter

The antagonistic activity of the cultures was investigated by the method of co-cultivation with test cultures of pathogenic and opportunistic pathogenic microorganisms. It has been found that in a pure culture, the number of test cultures was 10^8 CFU/cm^3, and during co-growth with lactobacilli, the cells of the test cultures were thousands and millions of times fewer (Table 4).

The study of the antagonistic activity of the cultures has shown that the lactobacilli, compared to mesophilic cocci and thermophilic streptococci, more actively suppressed the development of test cultures. Besides, it has been found that the lactic acid bacteria studied had a greater inhibitory effect on all test cultures compared to the control (pH 3.8). So, it can be assumed that their inhibitory effect is associated with the production of not only acids, but, possibly, of certain antimicrobial agents as well.

Table 4 – Titre of the antagonistic activity of lactic acid bacteria, lg CFU/cm^3 *(co-cultivation)*

Strains	Test-cultures			
	E. coli	*St. aureus*	*Pr. vulgaris*	*Bac. subtilis*
L. lactis ssp. *lactis* L 1	4	5	5	5
L. lactis ssp. *cremoris* П1	4	5	5	5
S. thermophilus 2/14л	4	4	3	4
S. thermophilus ET-1	5	5	3	5
S. thermophilus CT-гр.	4	4	3	4
S. thermophilus O-4	5	5	3	5
Lb. acidophilus R 18/H	2	3	2	2
Lb. acidophilus Lb. K_9	2	1	less than 1	1
Lb. rhamnosus 3333	2	2	less than 1	2
Control 1, pH 3,8	5	6	6	6
Control 2, pure test-culture	8	8	8	8

Note: mean error – 3–5 %, $p < 0.05$

The studies have shown high antagonistic activity of lactic acid bacteria, thus allowing determination of their antimicrobial action intensity and the breadth of the spectrum of pathogenic and opportunistic pathogenic microorganisms sensitive to them.

Generally, the studies allowed selecting, by a complex of traits, these strains (out of the newly selected cultures) as promising: *L. lactis* subsp. *lactis* L 1, *L. lactis* ssp. *cremoris* P 1; *Lb. acidophilus* R 18/H and *Lb. acidophilus* K9 and 3333. Among the thermophilic streptococci, preference was given to strains of *S. thermophilus* 2/14l, ET-1, CT-gr. and O-4, which are included in the collection of industrial strains of Institute of Food Resources of National Academy of Agrarian Sciences (IFR NAAS).

Recently, starter cultures have begun to be employed more widely: if earlier, they were only used for the production of fermented dairy products, now they often become functional enrichers for dry and liquid products, as well as biologically active supplements (BAS).

So, we have created four varieties of bacterial compositions. Three of them (LB-R, LB-A, and ST) are intended to be used as functional enrichers for dry products, or as BAS, while Bifidolux is a universal composition that can be used as a milk fermenting culture as well as an enricher for dry and liquid products, a BAS, etc.

Table 5 shows the makeup of the bacterial compositions created: 1) LB-R – *Lb. rhamnosus* single-species preparation containing one selected strain; 2) LB-A – a preparation of two newly isolated *Lb. acidophilus* strains; 3) ST – a preparation of 3 selected *S. thermophilus* strains; 4) Bifidolux – a complex preparation: *Lb.. rhamnosus* (a newly isolated strain), *Lb. acidophilus* (a strain with low acid production energy, obtained by protoplast regeneration), 4 strains of *S. thermophilus* (one is a bacteriophage-insensitive mutant, obtained by the spontaneous mutagenesis method; the other three are isolated from self-fermented authentic products such as yoghurt), bifidobacteria (three strains from the IFR NAAS collection).

Table 5 – The main biotechnological parameters of the dry bacterial preparations

Biotechnological parameters	Iprovit-*LB-R*	Iprovit -*LB-A*	Iprovit -*ST*	Iprovit - Bifidolux
Lb. rhamnosus content in g, lg CFU	10.7±0.2	-	-	10.1±0.3
Lb. acidophilus content in g, lg CFU	-	10.0±0.1	-	9.0±0.1
S. thermophilus content in g, lg CFU	-	-	10.5±0.2	10.2±0.1
Bifidobacteria content in g, lg CFU	-	-	-	10.9±0.3
Yield of raw biomass, g	2000±50	1200±50	1500±50	2300±50
Yield of dry preparation, g/dm^3	15.5±0.5	11.9±0.5	12.6±0.5	18.0±0.5

Practical evaluation of the research results. The bacterial preparations production biotechnologies have been field-tested at the State Research Enterprise of Starter Cultures that belongs to the IFR NAAS. The biotechnological parameters of the dry bacterial preparations are presented in Table 5.

The functional effect of the bacterial preparation Iprovit-Bifidolux has been clinically tested at Ivano-Frankivsk Children's Clinical Hospital (Ch. Doctor R. Y. Koturbash, Honoured Doctor of Ukraine), Ivano-Frankivsk Regional Clinic (A. L. Shapoval, PhD (Medicine), Assoc. Prof.; Ya. Lyakhnovsky, PhD (Medicine), Assistant) and Horodenka Central District Hospital of Ivano-Frankivsk Region (Ch. Doctor A. V. Kosovets; D. V. Verezhak, Board Certified in dental surgery). The doctors have found that the bacterial preparation Iprovit-Bifidolux and dairy products based on it are well tolerated by patients and have no negative consequences. A positive effect on patients' microflora has been noted: an increase in the number of lactic and bifidobacteria with normal enzymatic activity, the elimination of pathogenic and opportunistic pathogenic bacteria, and of fungi of the genus *Candida*. The immunomodulatory effect of these products has been recorded. It consisted in an increase in the number of leukocytes and lymphocytes after their preceding decrease, and, conversely, in a decrease in their pool during leuko- and lymphocytosis. A normalized CD^{4+} CD^{8+} ratio of immunoregulatory lymphocyte subpopulations has been noted, which means an increase in the subpopulation of inducer T-lymphocytes. Changes in the humoral link consisted in the normalized parameters characterizing the anti-infective resistance and the tendency to a decrease in inflammatory processes.

Conclusion

A methodology has been developed for obtaining biotechnologically active lactic acid bacteria strains with the use of various selection techniques: directional selection, selection of bacteriophage-insensitive mutants and the protoplast regeneration method. The active strains were screened by their biotechnological characteristics: milk-clotting activity level, bacterial yield, acid production energy, resistance to aggressive intestinal compounds, ability to reduce lactose levels during milk fermentation, ability to form aromatic compounds and viscous components, good sensorial qualities, antagonistic effect against technically harmful and opportunistic pathogenic microflora.

The results obtained have established the prospects for the practical use of the selected lactobacilli strains in the new bacterial preparations not only for traditional products, but also for those of functionally directed action. It has been clinically proved that the use of the bacterial preparation Iprovit-Bifidolux and dairy products based on it is reasonable and promising to restore the homeostasis, normalize the microbiocenosis, correct the immune system, increase the body's nonspecific defence, and accelerate the healing of erosions and intestinal ulcers.

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