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Biotechnology production of medium for cultivation and lyophilization of lactic acid bacteria

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The aim of our work was to develop a medium for the cultivation of lactic acid bacteria and to select the optimal protective mediums for the freeze-drying of lactic acid bacteria. The objects of research were cultures of microorganisms *Lactobacillus plantarum 7* and *Lactobacillus casei 27* isolated, selected and stored in the National Scientific Center Institute of Experimental and Clinical Veterinary Medicine (NSC "IECVM", Kharkiv). Cultivation of lactobacilli was carried out on medium MRS and Blaurock for 24-72 hours at the temperature of 37 °C. To develop nutrient media for the cultivation of lactobacilli, the selected components in the main composition of the medium: lactose, sodium phosphate disubstituted, sodium citrate. As components of the protective medium, solutions of sucrose, gelatinase, sodium acetate, and aerosol® were used. Because of the studies, the optimal composition of the nutrient medium was determined (lactose 1.2 %, sodium phosphate disubstituted 0.3 %, and sodium citrate 0.6 %) for the cultivation of lactic acid bacteria on which bacteria grow and actively accumulate a significant number of viable cells from 7.1 to 8.2 × 10^6 CFU/cm^3. In the various cultivation regimes of lactobacilli, pH 7.0 is optimal, and the temperature of the inoculum incubation is 37±0,5 °C. An alternative protective medium is added with the addition of 10 % sucrose, 1.0 % gelatinase and 3.0 % aerosil, which ensures the viability of viable cells after lyophilization of strains of *L. plantarum 7* and *L. casei 27*, respectively, 96 % and 97 %.
Key words: lactobacillus; cultivation; pH; temperature; culture medium; protective medium

Introduction

Probiotics are widely used in veterinary medicine to stimulate the growth and development of young animals, prevent gastrointestinal diseases in restoring intestinal biocoenosis and stress, and as an alternative method of antibiotic therapy (Charteris et al., 2000; Prabhurajeshwar et al., 2017). Probiotics are environmentally safe drugs, they have no side effect with prolonged and regular use (Reid, 2006). They may, in some cases, be a substitute for antibiotics in the general scheme of nonspecific prevention of gastrointestinal diseases (Jamalifar et al., 2011). Currently, a significant number of probiotic drugs based on lactobacilli and bifidobacteria are known, which are one of the main protective groups of intestinal microorganisms for humans and animals (Miele et al., 2009; Lahteinen et al., 2010).

Lactic acid bacteria are widespread in nature (Del et al., 2000; Kos et al., 2003; Belletti et al., 2009; Patel et al., 2009). The study of the biological properties of lactic acid bacteria and bifidobacteria, as well as of other microorganisms, requires the ability of long-term storage of crops. This is necessary both to maintain the collections of lactic acid bacteria in a highly active state and to manufacture and store probiotic preparations based on them (Vankerckhoven et al., 2008; Ng et al., 2009).

The most common way of preserving the viability of lactic acid bacteria is their periodic and systematic passage into fresh culture medium. However, the use of this method often causes significant changes in the cultural and morphological properties of crops. Depending on the biological characteristics of the strains, their resistance, after passage is carried out once in 2-4 months. This method is labor-intensive, in addition, after four or five passages, strains of lactic acid bacteria significantly reduce their biological activity (Kovalenko, 2002).

The main factors that affect the viability of microorganisms include temperature, pH and osmotic pressure. In addition, the growth and activity of microbes is impossible without the presence of nutrient materials in their environment that are used to construct cell components, and necessary as an energy source (De Man et al., 1960; Schillinger et al., 2003; Jankowski, 2007).

In the biotechnological process of creating therapeutic and prophylactic probiotics, great attention is paid to achieving the maximum level of biomass yield of viable bacterial cells and, accordingly, the biologically active substances synthesized by them,

it is important to consider their process ability in production conditions and stability during cultivation, considering the preservation of probiotic properties (Gujvinska et al., 2018). These indicators determine the productivity, competitiveness and profitability of the technological process. Also, an urgent problem is the search for ways of long-term storage of lactic acid bacteria, the main task of which is to support their vitality, taxonomically important and biotechnological characteristics. The solution to the problem of long-term storage of microorganisms is to provide conditions for anabiosis, that is, inhibition of metabolic processes (Romanchuk, 1999).

The literature data indicate that lyophilization of biological preparations has quickly found recognition in biotechnology, dry preparations are being applied on an increasing scale. At the same time, many questions regarding the number of microorganism cells for drying, the composition of protective media, freezing technologies, and subsequent drying of preparations by different authors are treated differently (Stoyanova et al., 2000; Yamborko et al., 2007). This is due to specific microorganisms, with the conditions of lyophilization and other technological processes that can affect the quality of the drug. Quite a few questions arise also when lyophilizing new probiotic preparations containing lactic acid bacteria (Stiles, 1996; Kapraljants et al., 2016).

It is known that lactic acid bacteria can be stored at a temperature of 4 °C in the native state by periodic passage every 1-3 months in a liquid or semi-liquid MRS medium. Also, widely used is the method of lyophilization, which is the drying of the bacterial mass in a protective environment at low temperatures. Lyophilization uses protective substances that reduce the temperature threshold of crystallization of free water, affecting its structure. They can also act as carriers for microbial cells, forming a kind of skeleton for their mobilization. Bacteria can persist for quite a long time - up to tens of years (Kvasnikov et al., 1975).

To accelerate the growth and accumulation of bacterial mass, as well as a longer shelf life of crops, we have carried out work to develop a nutrient medium for the cultivation of lactobacilli. Constructed protective medium that would allow maximum preservation of lactic acid bacteria during their lyophilization and with further use has been carried out.

Materials and methods

The objects of research were cultures of microorganisms *Lactobacillus plantarum 7* and *Lactobacillus casei 27* isolated, selected and stored in the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (NSC "IECVM", Kharkiv). Cultivation of lactobacilli was carried out on media MRS and Blaurock for 24-72 hours at 37 °C (Bannikova, 1975).

Six variants of research nutrient media for the cultivation of lactobacilli were made. For this purpose, the selected components in the main composition of the medium: lactose, sodium phosphate disubstituted, sodium citric acid. The control was MRS medium (Lee et al., 2008).

To improve the technology of lyophilization of production strains of *L. plantarum 7* and *L. casei 27*, experimental series of protective media were manufactured. As components of the protective medium, solutions of sucrose, gelatinase, sodium acetate, and aerosil were used. Strains of lactic acid bacteria grown on skim milk were introduced into the protective medium in a ratio of 1:1.

To dry the production strains of microorganisms were placed in a freeze-dried plant LZ-4527, they were dried in three different processing regimes: I mode – from minus 30 °C to plus 20 °C, II mode – from minus 45 °C to plus 38 °C, III mode – from minus 72 °C to plus 26 °C. The drying time was 26 hours.

Control of physiological and biochemical properties of production strains was carried out by generally accepted methods in terms of quality indicators: appearance; contamination by extraneous bacterial and fungal microflora; typical cultures, the number of living microbial cells in 1 cm^3 of dried drug, the activity of coagulation of milk. Acid in milk was determined by the amount of acid produced in skim milk. The number of living microbial cells was determined by serial dilution of the resulting suspension in physiological saline. The rate of milk fermentation by lactobacilli was assessed according to a conventional method (Bannikova, 1975; Kapraljants et al., 2016).

To compare the quantitative viability of bacteria, their preservation was taken into account before and after freeze-drying.

All experiments were carried out in triplicate. Statistical processing of the results was carried out with traditional methods of variation statistics using Excel and Statistica 10. Differences between the values were considered reliable at $p < 0.05$.

Results and discussion

During the experiments, components (% by weight) were selected and 6 variants of experimental nutrient media for the cultivation of lactobacilli were made, as well as the level of accumulation of bacterial mass of lactic acid bacteria on them (Table 1) was determined.

Table 1. Accumulation of bacterial mass of lactobacilli on experimental environments

Type of medium	The ratio of components in the medium, mass, %			Number of microbial cells, 10^6 CFU/cm^3
	Lactose	Sodium phosphate disubstituted	Sodium citric acid	
1	1.0	0.20	0.50	7.4±0.23*
2	1.1	0.25	0.55	7.7±0.07
3	1.2	0.30	0.60	8.2±0.11*
4	1.3	0.35	0.65	7.1±0.09
5	1.4	0.40	0.70	7.4±0.21
6	1.5	0.45	0.80	7.3±0.21
Control medium				6.1±0.07

Notes: * – $p < 0.05$.

From the Table 1, on the developed nutrient medium, lactic acid bacteria grew and actively accumulated a significant number of viable cells (from 7.1 to 8.2 × 10^6 CFU/cm^3). Comparing the variants of the nutrient medium with each other, it was established that when cultivating lactobacilli on medium No. 3, the yield of biomass increases by 1,3 times in comparison with other media and control media. So, according to the results of the studies, the optimal composition of the nutrient medium for the cultivation of lactic acid bacteria was determined: lactose – 1.2 %, sodium phosphate disubstituted – 0.3 %, sodium citrate – 0.6 %.
In the process of studying the properties of the developed nutrient medium, it is established that the growth of microorganisms depends on its pH. So, the higher the pH of the medium, the less the quantity of lactic acid cells develops in it (Table 2).

Table 2. Influence of the pH of the nutrient medium on the growth of lactic acid bacteria (M±m, n=3)

Initial Ph	Number of microbial cells, 10^6 CFU/cm^3 M±m	
	control	Experiment
5.5	13.70±0.15	14.10±0.14
6.0	14.40±1.16	14.50±1.10
6.5	23.20±1.70	24.0±1.16*
7.0	24.30±1.30	25.0±1.14*
7.5	22.50±1.10	23.0±1.01*
8.5	10.10±0.50	12.0±4.10

Notes: * – $p < 0.05$.

Based on the results presented in Table 2, it was found that the pH of the research medium varies from 5.5 to 8.5 units. Because of statistical processing of the obtained results, it was found that intensive growth of microorganisms was observed at pH 6.5-7.5. It should be noted that the maximum accumulation of cultures was observed at pH 7.0 (control 24.3 ± 1.30 × 10^6 CFU/cm^3, experiment 25.0 ± 1.14 × 10^6 CFU/cm^3). At pH 5.5-6.0, the number of microbial cells was 14.1 ± 0.14 × 10^6 CFU/cm^3 and 14.5 ± 1.10 × 10^6 CFU/cm^3, respectively. The lowest concentration of microorganisms in the culture medium was observed at pH 8.5.
Having obtained positive results for the determination of optimal pH conditions, optimal temperature regimes for culturing lactic acid bacteria on the proposed medium were studied. In the experiments, the incubation of the culture inoculation was carried out at a temperature of 30 °C (minimum) and 50 °C (maximum) (Table 3).

Table 3. Influence of growing temperature on the growth of lactic acid bacteria (M±m, n=3)

Temperature, °C	Number of microbial cells, 10^6 CFU/cm^3	
	control	Experiment
30	14.50±1.77	1.25±0.15*
37	24.70±0.36	25.0±0.34*
40	23.50±0.92	24.0±0.87*
45	8.05±0.92	9.0±0.87
50	9.05±0.06	1.20±0.05

Notes: * – $p<0.05$

The results presented in Table 3 show that the best growth and development of lactobacilli takes place at a temperature of 37-40 °C. At a temperature of 30, 45, and 50 °C, the growth of microbial cells was low and was from 9.0 ± 0.87 up to 1.20 ± 0.05 × 10^6 CFU/cm^3.
Thus, experimental studies have shown that, under different cultivation regimes of lactobacilli, pH is 7.0, and the temperature of incubation of inoculum is 37 °C. The results of the conducted studies indicate that on the developed medium, the accumulation of microbial cells is higher than when applied common (control) environment. So, growing lactobacilli on the developed medium makes it possible to receive 15-20 % more microbial cells at the same time, which means that from the same number of sown reservoirs with a nutrient medium, it is possible to obtain 15-20 % more probiotics than in normal cultivation on the MRS medium.
To test the lyophilization of lactic acid bacteria, 8 variants of protective media were investigated. As protective media, various components were used separately and in combination: solutions of sucrose, gelatinase, sodium acetate, aerosil. The results of determining the concentration of aerosil A-300 (1.0 %, 2.0 %, 3.0 %, and 6.0 %) as a component of the protective medium for freeze drying of lactic acid bacteria are presented in Table 4.
The results of the studies showed that a protective medium with the addition of 3,0 % aerosil ensures high safety of vital activity of lactic acid bacteria. In this case, the number of microbial cells of *L. plantarum 7* before lyophilization were (7,7 ± 0,21) × 10^6 CFU/cm^3, *L. casei 27* – (8,3 ± 0,17) × 10^6 CFU/cm^3; after lyophilization – (7,5 ± 0,15) × 10^6 CFU/cm^3 (97 % safety) and (8,2 ± 0,11) × 10^6 CFU/cm^3 (98 % safety), respectively.
Thus, an alternative protective medium was developed with the addition of 10% sucrose, 1,0 % gelatinase and 3,0 % aerosil, which can be effectively used for the freeze-drying of lactobacilli.

Table 4. Viability of bacteria after freeze-drying with the addition of Aerosil A-300 (M±m, n=3) to the protective medium

Aerosil. %	*L. plantarum 7*				*L. casei 27*			
	Number of bacteria, 10^6 CFU/cm^3		Duration of milk coagulation, h		Number of bacteria, 10^6 CFU/cm^3		Duration of milk coagulation, h	
	b.l.	a.l.	b.l.	a.l.	b.l.	a.l.	b.l.	a.l.
1	7.7±0.21	6.1±0.17	24	24	8.3±0.17	7.4±0.23*	24	48
2	7.7±0.21	6.0±0.13	24	24	8.3±0.17	6.2±0.07	24	24
3	7.7±0.21	7.5±0.15	12	12	8.3±0.17	8.2±0.11*	24	24
6	7.7±0.21	6.8±0.07	24	48	8.3±0.17	7.1±0.09*	24	24
control	7.7±0.21	5.4±0.18	24	24	8.3±0.17	4.4±0.21	24	24

Notes: * – $p < 0.0$; b.l. - before lyophilization, a.l. - after lyophilization

Subsequent studies were aimed at manufacturing different variants of protective medium for the long-term storage of industrial cultures of bacteria. Table 5 presents data on the effect of protective medium on the reserve of viability of lactic acid bacteria after freeze-drying.

Table 5. Effect of the protective medium's composition on the vital activity of bacteria after freeze-drying (M ± m, n = 3)

Composition of protective medium	Number of bacteria, 10^6 CFU/cm^3			
	L. plantarum 7		*L. casei 27*	
	b.l.	a.l.	b.l.	a.l.
sucrose 10 %	7.7±0.21	6.2±0.13*	8.3±0.17	7.2±0.12*
sucrose 10 % gelatinase 5 %	7.7±0.21	6.3±0.14*	8.3±0.17	7.7±0.15
glucose 5 %	7.7±0.21	6.8±0.17	8.3±0.17	7.9±0.08
glucose 5 % gelatinase 5 %	7.7±0.21	6.9±0.19	8.3±0.17	7.8±0.12
aerosil 3 %	7.7±0.21	6.8±0.08	8.3±0.21	7.4±0.11*
sucrose 10 % aerosil 3 %	7.7±0.21	7.1±0.33	8.3±0.17	7.9±0.13
sucrose 10 % gelatinase 5 %	7.7±0.21	7.4±0.20	8.3±0.17	8.1±0.15
aerosil 3 % sodium acetate 5 %	7.7±0.21	6.2±0.15*	8.3±0.17	6.3±0.31*
control (medium without protective components)	7.7±0.21	3.2±0.09	8.3±0.17	4.5±0.21

Notes: * – $p < 0.05$, b.l. - before lyophilization, a.l. - after lyophilization

From the materials presented in Table 5, immediately after lyophilization, the number of viable cells of *L. plantarum 7* has changed, namely the proportion of bacteria that survived was 80 to 96 %, and for *L. casei 27*, 81 to 97 %. The maximum number of viable cells after lyophilization was recorded for the strain *L. plantarum 7* – 96 % and for the *L. casei 27* – 97 % using a protective medium supplemented with 10 % sucrose, 1.0 % gelatinase and 3.0 % aerosil.

Compared with the control (medium without protective components), the safety of cells was low (41-54 %). A composition of an alternative protective medium that can be effectively used for the freeze-drying of lactobacilli is proposed.

The next step in our work was to determine the rate of milk coagulation with production strains, an important indicator for lactic acid bacteria (Table 6).

According to the results presented in Table 6, after lyophilization of bacteria with protective media, the rate of milk coagulation for *L. plantarum 7* was (12-24) hours, and for *L. casei* strain *27* – (24-48) hours. In the control experiment (medium without protective components), the *L. plantarum 7* culture has a low rate of milk coagulation in lyophilization for 12 hours, and after lyophilization – 36 hours; respectively, strain *L. casei 27* – before lyophilization 24 hours, after lyophilization – 56 hours.

It has been established that cultures of lactic acid bacteria have a high rate of coagulation of milk after the improvement of lyophilization technology by applying the developed medium.

Table 6. Coagulation activity of milk with strains of *L. plantarum 7* and *L. casei 27*

Composition of protective medium	Duration of milk coagulation, h			
	Lactobacillus plantarum 7		*Lactobacillus casei 27*	
	b.l.	a.l.	b.l.	a.l.
sucrose 10 %	24	24	24	24
sucrose 10 %, gelatinase 5 %	24	24	24	36
glucose 5 %	12	12	24	24
glucose 5 %, gelatose 5 %	24	24	48	48
aerosil 3 %	12	12	24	24
sucrose 10 %, aerosil 3 %	12	12	24	24
sucrose 10 %, gelatinase 5 %, aerosil 3 %	12	12	24	24
sodium acetate 5 %	12	12	24	48
control (medium without protective components)	12	36	24	56

b.l. - before lyophilization, a.l. - after lyophilization

The next stage of the research was the study of the influence of temperature regimes during the lyophilic drying of lactobacilli developed by the protective environment for the preservation of bacteria (Table 7).

Table 7. Influence of temperature regimes at lyophilic drying of lactobacilli on their safety (M±m, n=3)

Technological regime	Number of bacteria, 10^6 CFU/cm^3			
	Lactobacillus plantarum 7		*Lactobacillus casei 27*	
	b.l.	a.l.	b.l.	a.l.
I	7.7±0.21	3.6±0.19*	7.1±0.33	1.5±0.22*
II	7.7±0.21	2.1±0.23*	7.1±0.33	2.7±0.27*
III	7.7±0.21	7.1±0.33	7.1±0.33	6.4±0.23

Notes: * – $p < 0.05$, b.l. - before lyophilization, a.l. - after lyophilization

When analyzing the results given in Table 7, it was found that the optimum freeze drying was recorded in the III technological regime with an initial temperature in desublimator minus 72 °C, final – plus 26 °C for 26 hours. The bacterial count of *L. plantarum 7* before lyophilization was 7.7 ± 0.21 × 10^6 CFU/cm^3; after lyophilization – 7.1 ± 0.33 × 10^6 CFU/cm^3, (safety 92 %). The number of *L. casei 27* bacteria before lyophilization was 7.1 ± 0.33 × 10^6 CFU/cm^3; after lyophilization – 6.4 ± 0.23 × 10^6 CFU/cm^3 (safety 90 %).

It should be noted that the lyophilized bacterial mass that has been dried with a protective medium had the appearance of a crumbly dry mass of white or cream color and was readily soluble in the solvent. Cultures that were dried without protective media looked like a loose dry mass, but dissolved in the solvent more slowly, in some cases had small insoluble flakes.

Most authors are convinced that the preservation of microorganism's strains of depends on many factors, among which one of the most important is their cultivation under optimal conditions (Kovalenko, 2002, Basjul et al., 2014). Despite this, when developing a culture medium for the cultivation of lactobacilli, it is very important that they contain nutrient and growth substances in an accessible form (Khilko et al., 2004; Gujvinska, 2014), and do not act bactericidally on the probiotic culture (Bujalance, 2006). In the specialized literature it is reported that the collection can be stored in frozen condition and under vaseline oil. However, the efficiency of these storage methods is not high - the number of viable cells decreases by 60-70 % by the end of the first year of storage (Labinskaya, 1978).

Even earlier, a number of scientists came to the conclusion that lyophilization is the best way to preserve lactobacilli (Bannikova, 1975; Kvasnikov et al., 1975). It is established that lactic acid bacteria after lyophilization stored their properties for 40 months. Lyophilization of microorganisms is a method of reverse conservation, which allows a long time to preserve their biological properties. In vaseline oil, *L. casei* and *S. cremoris* cultures are stored for a period of 8 months at a temperature of minus 30 °C (Arkad'eva et al., 1983). The lyophilization is widely used in the storage of collection crops, but in the industrial production of vaccines, serums, probiotics, bacteriophages, etc. (Kovalenko, 2002). Also important is the composition of the drying medium. In most cases, it uses skimmed milk, albumin, gelatin, peptone, sugar solutions (Schillinger et al., 2003). The maximum number of viable cells after lyophilization was recorded for strain *L. plantarum 7* (96 %) using a protective medium supplemented with 10 % sucrose, 1.0 % gelatose and 3.0 % aerosil.

The protective medium, in addition to its main purpose – to ensure the maximum preservation of the biological properties of microorganisms in the process of lyophilization, must be technological, that is, to have the optimum viscosity to exclude the dissolution of the suspension prior to freezing and at the same time ensure its high permeability through various cell systems. The composition of the medium for lyophilization should not affect the properties of the biological preparation and should not have toxic or inhibitory properties. The components of the medium must be available for industrial production (Yamborko et al., 2007).

The results of our studies have shown that a protective environment with the addition of 3.0 % aerosil ensures high safety of vital activity of lactic acid bacteria. At the same time, to lyophilization, the quantity of microbial cells of *L. plantarum 7* was 7.7 ± 0.21 × 10^6 CFU /cm^3, *L. casei 27* – 8.3 ± 0.17 × 10^6 CFU/cm^3; after lyophilization – 7.5 ± 0.15 × 10^6 CFU/cm^3 (97 % safety) and 8.2 ± 0.11 × 10^6 CFU/cm^3 (98 % safety) respectively.

Conclusions

A culture medium was proposed (lactose 1.2 %, sodium phosphate disubstituted 0.3 %, sodium citrate 0.6 %) for cultivation of lactobacilli on which bacteria grow and actively accumulate a significant number of viable cells (7.1-8.2) × 10^6 CFU/cm^3). Under different temperature regimes and pH of the medium, the best is that it has a pH of 7.0 and the incubation of the inoculum passes at a temperature of 37 ± 0.5 °C. The maximum preservation of viable cells after lyophilization of strains *L. plantarum 7* and *L. casei 27*, respectively, 96 % and 97 % were observed using a protective medium supplemented with 10 % sucrose, 1.0 % gelatose and 3.0 % aerosil. The addition of protective media according to the given formulation with the freeze-drying of lactic acid bacteria and the proposed drying regime have technological advantages and ensure the preservation of the basic biological properties of microorganisms.

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