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Selection of technological regime and cryoprotector for lyophilization of lactobacteria (*Lactobacillus* spp.)

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Despite the success achieved in the comprehensive study of probiotic cultures, today there are a number of problems associated with the low viability of lactic acid bacteria during their processing and long-term storage in probiotics. Our work aimed to select the optimal technological regime and cryoprotectant to preserve the viability of lactic acid bacteria *Lactobacillus* spp. during their lyophilization. According to the results of the conducted researches, it is established that for freeze-drying of probiotic cultures *Lactobacillus* spp. in the facility LZ-45.27 (Frigera, Czech Republic) the most optimal is the mode which provides a rise of temperature within 45 hours from minus 70.0±1.0 °C to plus 26.0±1.0 °C with a speed of 2.2±0.1 °C/hour. It is effective to use protective media for lactobacilli, which consist of: skim milk (90%) and sucrose (10%); skim milk (90%) and lactose (10%); skim milk (90%), glucose (2.5%), sucrose (2.5%), lactose (5.0%) ($P \leq 0.05$). Freeze-drying of lactic acid bacteria under optimal conditions and the addition of cryoprotectants will avoid the problems associated with a significant reduction in the number of microbial cells. The results of research can be used for long-term storage of cultures of lactobacilli by their lyophilization.

Keywords : lactobacilli, temperature, cryoprotectants, freezing, freeze-drying.

Introduction

Today in veterinary medicine in the general complex of preventive and therapeutic measures much attention is paid to the use of environmentally friendly, natural means of animal protection (Paliy et al., 2018; Nazarenko et al., 2020; Rodionova et al., 2020; Kasianenko et al. , 2020), which include probiotic drugs. Different species of lactobacilli are found in nutrient-rich habitats associated with food, feed, plants, animals and humans (Duar et al., 2017).

The genus of lactic acid bacteria *Lactobacillus* spp. has more than 130 different species and is represented by non-pathogenic, gram-positive obligate or facultative anaerobes with high enzymatic activity (Talib et al., 2019). Most lactobacilli are part of the normal microflora of the gastrointestinal tract and are represented mainly by *Lactobacillus acidophilus, L. casei, L. bulgaricus, L. plantarum, L. salivarius, L. reuteri* and *L. rhamnosus* (Paliy et al., 2020b). Lactobacilli represent a smaller part of the intestinal microflora, but perform no less important metabolic functions than the main representatives of the normoflora - bifidobacteria (Gujvinska & Paliy, 2018). Due to the synthesis of lactic acid, lysozyme, bacteriocins, etc. lactobacilli inhibit the growth of putrefactive pathogenic and opportunistic microflora (Pithva et al., 2014; Karami et al., 2017).

Thus, it is reported that *L. plantarum* has a wide range of activity against gram-positive (*Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus*) and gram-negative (*Pseudomonas aeruginosa, Shigella sonnei, Enterobacter sakazakii, Salmonella tychericholium, Escherichia coli*) bacteria (Jiang et al., 2016). The antimicrobial action of lactic acid bacteria against rotaviruses (Liévin-Le Moal & Servin, 2014), yeast (Coman et al., 2014), food and feed filamentous fungi (Prema et al., 2010) has been established. Lactobacilli have also been shown to activate cellular immunity and inhibit the production of immunoglobulins, which have been linked to the presence of peptidoglycans and teichoic acids in their cell wall (Wells, 2011; Kolling et al., 2018). Most strains of *L. plantarum* produce β-galactosidase and bile salt hydrolase, which improve the overall condition of the macroorganism (Shekh et al., 2016).

The ability of lactobacilli to produce different end products directly depends on their species, strain, genetic ability, expression of active enzymes, environmental conditions (Tseng & Montville, 1993; Li & Cao, 2010). Lactic bacteria are characterized by antibiotic resistance (Sukmarini et al., 2014; Anisimova & Yarullina, 2018), but it varies depending on the type of lactobacilli and their origin (Prabhurajeshwar & Chandrakanth, 2017, 2019). These microorganisms survive under the action of gastrointestinal juice, bile (García-Ruiz et al., 2014; Casarotti et al., 2017), phenol (Boricha et al., 2019), grow well in acidic environment (Prabhurajeshwar & Chandrakanth, 2019).

Today, lactobacilli are widely used in clinical practice as part of various probiotics and dietary supplements (Yadav et al., 2016; Lukic et al., 2019), the use of which prevents the emergence of antibiotic-resistant bacterial isolates (Hadzevych et al., 2019). The study of the biological properties of lactobacilli, as well as other microorganisms, requires the ability to long-term preservation of cultures (Brizuela et al., 2001; Otero et al., 2007). Maintaining collections of lactic acid bacteria in a highly active state, preserving their biological properties are important conditions in the production of probiotic drugs (Dekker et al., 2007; Di Cerbo et al., 2016). It is known that bacterial cultures, in order to increase the shelf life, are subjected to various methods of preservation (Tan et al., 2018; Ukhovskyi et al., 2019). The most effective method of storage is freeze-drying (Gehrke et al., 1992). In this rather "soft" way, microorganisms are exposed to factors such as low temperatures and dehydration in a vacuum.

When freezing microorganisms, much attention is paid to the composition of the protective medium, which contains cryoprotectants (Elliott et al., 2017). The following substances can be used as cryoprotectants: glycerin, dimethyl sulfoxide, sucrose, lactose, glucose, sorbitol and others. These substances are selected according to certain rules and regulations that further ensure the high viability of bacteria and the preservation of their cultural and biological properties (Jofré et al., 2015; Gujvinska et al., 2018). Despite the success in the study of probiotic cultures, there are still a number of problems associated with the low viability of lactic acid bacteria during their processing and long-term storage in probiotics (Anal & Singh, 2007). Thus, freeze-drying can be used for the production of probiotics on a large scale, but this method involves a significant impact on microbial cultures of extreme environmental conditions.

Methods for the production of dried probiotic cultures should be such as to ensure a sufficient number of bacteria in the final product and their high biological activity (Meng et al., 2008). Lyophilized preparations have advantages over preparations made by other techniques in terms of long-term storage, combined with ease of use, storage, marketing and use (Carvalho et al., 2004). The aim of our work was to select the optimal technological regime and cryoprotectant to preserve the viability of lactic acid bacteria *Lactobacillus* spp. during their lyophilization.

Materials and methods

The research was conducted using 9 cultures of microorganisms that were isolated, selected and stored at the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (NSC "IECVM") (Kharkiv) (Table 1).

Table 1. Culture of microorganisms

№	Culture of microorganisms	Strain
1	*Lactobacillus plantarum*	7
2	*Lactobacillus delbrueckii*	8
3	*Lactobacillus casei var. rhamnosus*	9
4	*Lactobacillus casei var. rhamnosus*	14
5	*Lactobacillus plantarum*	15
6	*Lactobacillus plantarum*	19
7	*Lactobacillus plantarum*	22
8	*Lactobacillus casei*	27
9	*Lactobacillus plantarum*	7-317

Pre-cultivation of lactobacilli was performed on skim milk for 24 hours at a temperature of 37.0±0.5°C (Desai et al., 2006). The viability of lactobacilli was determined by comparing the number of colony-forming units (CFU) in 1 cm^3, before and after freeze-drying, and the use of cryoprotectants by serial dilutions of the resulting suspension in saline followed by seeding on agar medium MPC-4 (de Man et al., 1960). Glucose, sucrose, and lactose were used as cryoprotectants for the protective medium, separately at concentrations of 10.0%, and their composition at a concentration of 2.5%; 2.5%; 5.0% of each ingredient, respectively, which were added to skim milk. At the first stage of research, there was determined the viability of cultures *Lactobacillus* spp. which were grown on skim milk during their lyophilization without the use of protective media.

The experiments used vials with a capacity of 10 cm^3, in which cultures of lactobacilli were introduced separately in the amount of 5.0±0.1 cm^3. 10 vials were used for each culture of lactobacilli. Lyophilization was performed in the unit LZ-45.27 (Frigera, Czech Republic) (Table 2).

Table 2. Technical characteristics of the LZ-45.27 unit (Frigera, Czech Republic)

Parameters	Value
Year of production	1986
Sublimation chamber capacity	0.93 m^3
Residual humidity / for plasma /	not more than 1.0%
Productivity of the desublimator at -50°C	≈1.8 kg/hour
The maximum temperature of baskets and racks	+79°C
he minimum temperature of the desublimator	-70°C
Supply voltage	380 V / 50 Hz

Mathematical processing of digital data included determination of arithmetic mean (M), arithmetic mean error (m). The hypothesis was tested through the degree of differences between the compared objects using Student's t test (t) using Excel.

Results and discussion

The first technological operation of lyophilization is the freezing of biological material. For this purpose, the cultures of lactobacilli in the vials are exposed to a temperature of minus 40±1.0°C for 30 minutes, and then a temperature of minus 30±1.0°C is applied for 48 hours. After that, the vials are placed in a refrigerator at a temperature of minus 55±1.0°C for 2 hours to "harden" the frozen product. Lyophilization was directly performed in the unit LZ-45.27 (Frigera, Czech Republic), using three different technological modes:

mode I – the temperature of the sublimator was increased from minus 30.0±1.0°C to plus 20.0±1.0°C at a rate of 1.1±0.1°C/hour;
mode II – the sublimator temperature was increased from minus 45.0±1.0°C to plus 38.0±1.0°C at a rate of 1.8±0.1°C/hour;
mode III – the temperature of the sublimator was increased from minus 70.0±1.0°C to plus 26.0±1.0°C at a rate of 2.2±0.1°C/hour.

Vials with cultures of lactobacilli were gradually heated to the appropriate temperature by heating the shelves on which they were placed for 45 hours. The experiments were performed in triplicate (n=3). Cultures that were not exposed to low temperatures were used as a control, and their cultivation was performed on skim milk for 24 hours at a temperature of 37.0±0.5°C. Residual moisture in all vials after lyophilization did not exceed 3.0% (Table 3).

Table 3. Influence of temperature regimes at lyophilic drying on the viability of lactobacilli (n=3)

Culture of lactobacilli	The number of bacteria (× 10^6 CFU/cm^3)			
	Mode I	Mode II	Mode III	Control
L. plantarum 7	6.3±0.19*	5.9±0.25*	7.1±0.33*	7.7±0.21
L. delbrueckii 8	6.1±0.22*	5.2±0.27*	6.8±0.29*	7.4±0.33
L. casei var. rhamnosus 9	5.8±0.27*	5.7±0.19*	7.2±0.23*	8.3±0.25
L. casei var. rhamnosus 14	4.3±0.24*	5.0±0.18*	6.8±0.31*	7.9±0.34
L. plantarum 15	4.2±0.21*	5.9±0.25*	7.7±0.27*	8.5±0.27
L. plantarum 19	4.4±0.17*	5.6±0.21*	6.5±0.32*	7.3±0.19
L. plantarum 22	4.6±0.22*	4.7±0.24*	7.4±0.33*	8.2±0.25
L. casei 27	5.5±0.18*	6.4±0.23*	8.0±0.29*	9.1±0.22
L. plantarum 7-317	6.8±0.19*	7.1±0.24*	7.6±0.18*	8.7±0.24

Notes: * – the level of probability between the averages after lyophilization of cultures using different technological modes and control, the reliability of the difference P≤0.05.

The results of lyophilic drying of lactobacilli (Table 3) showed the least impact of the technological mode III on the viability of cultures of all studied strains of microorganisms has. The safety of cultures *L. plantarum 7* was 92.2%, *L. delbrueckii 8* – 91.8%, *L. casei var. rhamnosus 9* – 86.7%, *L. casei var. rhamnosus 14* – 86.0%, *L. plantarum 15* – 90.5%, *L. plantarum 19* – 89.0%, *L. plantarum 22* – 90.2%, *L. casei 27* – 87.9%, *L. plantarum 7*-317 – 87.3%. Along with this, it is proved that in the first technological mode the preservation of cultures of *L. plantarum 7* was 81.8%, *L. delbrueckii 8* – 82.4%, *L. casei var. rhamnosus 9* – 69.8%, *L. casei var. rhamnosus 14* – 54.4%, *L. plantarum 15* – 49.4%, *L. plantarum 19* – 60.2%, *L. plantarum 22* – 56.0%, *L. casei 27* – 60.4%, *L. plantarum 7-317* – 78.1%, and in the second mode this figure was for *L. plantarum 7* – 76.6%, *L. delbrueckii 8* – 70.2%, *L. casei var. rhamnosus 9* – 68.6%, *L. casei var. rhamnosus 14* – 63.2%, *L. plantarum 15* – 69.4%, *L. plantarum 19* – 76.7%, *L. plantarum 22* – 57.3%, *L. casei 27* – 70.3%, *L. plantarum 7*-317 – 81.6%.

Thus, for freeze drying of the lactobacilli cultures for further work technological mode III of the unit LZ-45.27 (Frigera, Czech Republic) was selected. Subsequent studies were aimed at studying the effect of cryoprotectants on the viability of *Lactobacillus* spp. after lyophilization. Glucose (10.0%), sucrose (10.0%), lactose (10.0%) separately, and their composition: glucose (2.5%) + sucrose (2.5%) + lactose (5.0%), were used as cryoprotectants. Variants of protective media were prepared on skim milk. Table 4 shows the data on the effect of cryoprotectants on the viability of lactic acid bacteria after freeze-drying.

Table 4. The effect of freeze-drying with different cryoprotectants on the viability of lactobacilli (n=3)

Culture of lactobacilli	The number of bacteria (× 10^6 CFU/cm^3)				
	10% glucose	10% lactose	10% sucrose	composition	control
L. plantarum 7	7.3±0.31**	7.4±0.28*	7.1±0.33*	7.5±0.29*	7.7±0.21
L. delbrueckii 8	7.0±0.27**	7.1±0.31*	7.1±0.27*	7.2±0.27*	7.4±0.33
L. casei var. rhamnosus 9	7.4±0.21**	7.8±0.24*	7.5±0.25*	7.9±0.24*	8.3±0.25
L. casei var. rhamnosus 14	7.1±0.30**	7.5±0.31*	7.3±0.29*	7.7±0.31*	7.9±0.34
L. plantarum 15	7.9±0.26**	8.1±0.32*	8.0±0.27*	8.3±0.28*	8.5±0.27
L. plantarum 19	6.8±0.32**	7.1±0.32*	6.9±0.22*	7.2±0.33*	7.3±0.19
L. plantarum 22	7.5±0.29**	7.8±0.27*	7.9±0.31*	8.0±0.29*	8.2±0.25
L. casei 27	8.1±0.28**	8.4±0.19*	8.5±0.27*	8.8±0.25*	9.1±0.22
L. plantarum 7-317	7.7±0.17**	8.0±0.18*	7.9±0.21*	8.2±0.18*	8.7±0.24

Notes: * – reliable probability level is absent; ** – reliability of the difference P≤0.05.

Research have shown (Table 4) that, when freezing cultures with the addition of 10% glucose, the safety of cultures *L. plantarum 7* was 94.8%, *L. delbrueckii 8* – 94.5%, *L. casei var. rhamnosus 9* – 89.1%, *L. casei var. rhamnosus 14* – 89.8%, *L. plantarum 15* – 92.9%, *L. plantarum 19* – 93.1%, *L. plantarum 22* – 91.4%, *L. casei 27* – 89.0%, *L. plantarum 7-317* – 88.5%; with the addition of 10% lactose, the safety of cultures was: *L. plantarum 7* – 96.1%, *L. delbrueckii 8* – 95.9%, *L. casei var. rhamnosus 9* – 93.9%, *L. casei var. rhamnosus 14* – 94.9%, *L. plantarum 15* – 95.2%, *L. plantarum 19* – 97.2%, *L. plantarum 22* – 95.1%, *L. casei 27* – 92.3%, *L. plantarum 7-317* – 91.9%; with the addition of 10% sucrose, the safety of cultures *L. plantarum 7* was 92.2%, *L. delbrueckii 8* – 95.9%, *L. casei var. rhamnosus 9* – 90.3%, *L. casei var. rhamnosus 14* – 92.4%, *L. plantarum 15* – 94.1%, *L. plantarum 19* – 94.5%, *L. plantarum 22* – 96.3%, *L. casei 27* – 93.4%, *L. plantarum 7-317* – 90.8%.

Along with this, it was found that when adding the composition of these ingredients, this figure for *L. plantarum 7* was 97.4%, and for *L. delbrueckii 8* – 97.2%, *L. casei var. rhamnosus 9* – 95.1%, *L. casei var. rhamnosus 14* – 97.4%, *L. plantarum 15* – 97.6%, *L. plantarum 19* – 98.6%, *L. plantarum 22* – 97.5%, *L. casei 27* – 96.7%, *L. plantarum 7-317* – 94.2%.

The survival of lactic acid bacteria under stress conditions has been confirmed by other researchers (Hill et al., 2018), who have established the resistance of certain species of *Lactobacillus* spp. to oxidative, acidic, osmotic, cold stresses, and also to long storage. However, the viability of lactic acid bacteria after freeze-drying depends on the strain and cryprotector (Montel Mendoza et al., 2014). The selection of nutrient media for lyophilization of lactobacilli was based on the results of our previous experiments (Paliy et al., 2020a) and data from other researchers (Montel Mendoza et al., 2014; Chen et al., 2015; Shekh et al., 2020), which report that the use of lactulose, sucrose and skim milk ensure the viability and safety of *L. plantarum* at the level of 98±2.8%. The efficacy of these compounds in the lophilization of lactobacilli has been obtained by other researchers (Otero et al., 2007; Juárez Tomás et al., 2009; Li et al., 2011).

A medium containing a minimum amount of sucrose (1.2%) in skim milk (6%) maintains the viability of lyophilized cultures for two years (Turuvekere Sadguruprasad & Basavaraj, 2018). With the use of sucrose and betaine, it was found that only sucrose has cryoprotective properties in the freezing of lactic acid bacteria *L. coryniformis* (Bergenholtz et al., 2012).

Along with this the addition of ascorbic acid to probiotic cultures has been shown to significantly increase their viability during 12 months of storage at 5.0°C (Zárate & Nader-Macias, 2006). Other researchers have achieved 90% survival of lactobacilli cells using a composition of skim milk (4%), glycerin (1%) and calcium chloride (0.1%) (Kang et al., 1999). The viability of *L. brevis* at the level of 67.8% during lyophilization is provided by the use of yeast extract (4.0%) and monosodium glutamate (2.5%) (Zhao & Zhang, 2005).

Maximum viability (78%) of *Lactococcus lactis* cells after freezing and thawing was obtained with a mixture of sucrose and skim milk, while the use of skim milk or MRS broth ensured the survival of cultures at the level > 60% (Berner & Viernstein, 2006). Sucrose, glycerin, sorbitol, and skim milk have been shown to be the most effective protective agents for *L. bulgaricus* during lyophilization (Huang et al., 2006). Sucrose, as a cryoprotectant, is widely used in the preservation of various biological objects (Schoug et al., 2006; Shakhova et al., 2020). However, it has been reported that the addition of trehalose or sucrose does not increase the survival of lactic acid bacteria and yeast after freeze-drying (Bolla et al., 2011).

The use of probiotics in increasing quantities, the optimization of the processes of their development and industrial production require consideration of cell viability and functionality of the final product (Jankovic et al., 2010). A promising direction in the development of probiotics are technologies that involve the creation of means in the form of tablets or capsules, which in turn increases the viability of probiotic cultures and are more convenient pharmaceutical forms for use by the end user (Zárate & Nader-Macias, 2006; de Vos et al., 2010; Burgain et al., 2011). The use of probiotics in combination with a set of veterinary and sanitary measures in livestock farms improves the culture of the industry, ensures sustainable epizootic welfare of livestock and is a cost-effective technological technique (Zavgorodniy et al., 2013; Shkromada et al., 2019; Paliy et al., 2020).

The results of the research will be used for the long-term storage by lyophilization of lactobacilli cultures in the Museum of Microorganisms NSC "IECVM".

Conclusions

For freeze-drying probiotic cultures of *Lactobacillus* spp. in the unit LZ-45.27 (Frigera, Czech Republic) the most optimum mode is the mode which provides temperature rise within 45 hours from minus 70.0±1.0 °C to plus 26.0±1.0 °C with a speed of 2.2±0.1 °C/hour. It is effective to use protective media for lactobacilli, which consist of: skim milk (90%) and sucrose (10%); skim milk (90%) and lactose (10%); skim milk (90%), glucose (2.5%), sucrose (2.5%), lactose (5.0%).

Freeze-drying of lactic acid bacteria under the optimal conditions and the addition of cryoprotectants will avoid the problems associated with a significant reduction in the number of microbial cells.

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