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INFLUENCE OF PROTECTORS ON PRESERVATION OF LACTIC ACID MICROORGANISMS

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Abstract

In this paper we studied the influence of freeze- drying with protectors on the safety of lactic acid microorganisms : Lactobacillus plantarum 53H, Lactobacillus plantarum 22, Lactobacillus plantarum 2, Lactobacillus cellobiosus 20, Lactobacillus acidophilus 27W, Lactobacillus curvatus 18d, Lactobacillus casei 139, Lactobacillus casei 173a, Lactobacillus salivarius 8e, Lactobacillus fermentium 27. Studies have shown that in all the freeze- drying of the culture maintainned a high cell viability using all three protectors (skimmed milk, skim milk + 7% glucose, 10 % sucrose + 1 % gelatin). Study antibiotic activity of lactic acid bacteria on pathogens (Bacillus subtilis, Esherichia coli, Bacillus cereus and Staphylococcus aureus) gave different results, some cultures have retained antibiotic activity and despite the safety of others is not retained viability antibiotic activity against pathogens. Of the three best preservation protectors used antibiotic activity was observed during lyophilization when used as a protective medium of skim milk.

Key words: *lactic acid microorganisms, lyophilization, protectors, sucrose, gelatin, pathogens, antibiotic activity, viability, storage.*

At the moment are increasingly being used in practice by various representatives of the microbial world. The microorganisms used in food industry, agriculture, medicine, textile production, wine-making and environmental protection. But for the successful use of microorganisms in biotechnology need to work on their storage, taxonomy and collecting. Concentration of strains in the collection gives you the opportunity to control their distribution, to regulate the acquirement and delivery of crops; standardize documentation for all strains in which the origin of the strain is fixed , the presence of other collections, distinctive properties , markers, practical value , pathogenicity, growth conditions , methods of maintaining and storage. Essential for the maintenance of the gene pool of microorganisms is to maintain for a long time viability, taxonomic properties and physiological activity of the collection strains, which requires the selection of appropriate conditions of conservation with subsequent reactivation of microorganisms [Ashwood Smith M.J., 1980, Banno J., 1981, Emtseva T. B., 1991, Adams J., 2007, Santivarangkna C., 2007]. Selecting the method of storage of biological material is extremely important when working in the field of microbiology. It should be noted that each culture is usually stored in the collection at the same time two or three methods in order to avoid crop losses in case of failure of one of the methods of preservation [Safronova V.I., 1991, Stoyanova L.G., 2000]. The most common way of maintaining collections of live cultures of microorganisms on fresh subcultures are optimal for the given organisms of medium. It is simple, accessible, has a constant ability to control of culture. But this method requires a lot of labor and money, is not reliable, as there is great danger of losing crops as a result of extinction or contamination. Method of storage under mineral oil used for conservation of large collections and in laboratories. It is simple, requires no special hardware and provides a relatively long-term preservation viability of most microorganisms. This method allows not only to preserving the culture, but in some cases increasing the energy propagation and its biosynthetic activity, stabilizing the main valuable properties [Ruban E.L., 1989].

Currently, in the collection are widely used more advanced methods of preserving living microorganisms. This is drying, lyophilization and storage at low temperatures [Sidyakina T.M., 1986 Efremenko E.N., 2007, Strasser S., 2009, Kupletskaya M.B., 2011]. Low-temperature preservation (at temperatures from -20^0 to -130^0C) used in recent years in an increasing scale in relation to the availability and accessibility of low-temperature refrigerators, capable of reliably maintain low temperature for a long time. Reduction of temperature leads to a slowdown of the biochemical reactions in the organism, to a lower level, and many microorganisms are kept at a temperature below 60^0C whith maintaining a high titer of cells. Lyophilization for many years has been used for long-term storage of many species of microorganisms if storing them without access of oxygen, moisture, and light at lower temperatures, typically at 4^0C. This method provides better stability than the method of periodic subcultures for a wide range of microorganisms and cells can survive for many years. All collection of culture inherent in storage in various ways, are unique in their properties and have not lost relevance today. Selecting a method of storage is individual for each culture of microorganism. And it must be based on the preservation of

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culture viability, morphological traits and physiological characteristics for the maximum storage time of culture, as well as the simplicity and reliability of the implementation of this method. In the collection of the Institute of Microbiology and Virology kept a large group of lactic acid microorganisms having antibiotic activity.

Objective of this work was the stability of some important physiological and biochemical properties of cell viability and antibiotic activity in lactic acid microorganisms after lyophilization.

MATERIALS AND METHODS

Object of study is the lactic acid bacterias : *Lactobacillus plantarum* 53H , *Lactobacillus plantarum* 22, *Lactobacillus plantarum* 2, *Lactobacillus cellobiosus* 20, *Lactobacillus acidophilus* 27W, *Lactobacillus curvatus* 18d , *Lactobacillus cas*ei 139 , *Lactobacillus cas*ei 173a , *Lactobacillus salivarius* 8e , *Lactobacillus fermentium* 27. Cultures were laid in storage - freeze-dried. The morphology of colonies and examined by conventional methods using a microscope. Identification of microorganisms was carried out by inoculating spore suspensions of cells after pasteurization. Cultures were grown for 5 days and then 2 ml of the suspension was transferred to sterile tubes and pasteurized for 10 min. at 80^0C. Over time, the suspension was plated on a sloped surface of the agar medium and placed the tubes in an incubator for 2-3 days, and then observed the growth or its absence. Number of viable cells of lactic acid bacteria was determined by the turbidity of the same titer for all storage methods, then method serial of dilution, followed by plating on agar media, grown microcolonies are counted. Antibiotic activity of lactic acid bacterias studied were determined by the agar diffusion to the measurement of the inhibition test-culture. As the test cultures were used: *Bacillus subtilis*, *Esherichia coli* 113, *Bacillus cereus and Staphylococcus aureus*. Lyophilization of cultures was done as follows: young culture of microorganisms from the exponential growth phase, grown in media appropriate to their physiological needs, washed in medium and added sterile lyophilization vials of 0.3 ml of slurry pervial. As protective media used: 1 - skim milk, 2- skim milk to 7% glucose, 3-1 % gelatin to 10 % sucrose. Lyophilization carried out in the apparatus «Leobeta» at a residual pressure of 0.07-0.1 mm Hg at a temperature of 35^0C with a pre - freezing at 600 for 15 hours and subsequent drying for 18 hours. Finished vials were stored in a freezer at -40C. For check the number of viable cells was poured into ampoules in 2 ml of sterile water and allowed to stand for 1-2 hours to fully suspend the pellet. Then carried out with the seeding dilutions on medium corresponding physiological properties and the number of microorganism colonies formed (CFU). All experiments to determine the number of viable microbial cells were performed in triplicates from one vial at seeding cells on appropriate media required in reconstituted suspension. The results of calculations were processed statistically.

RESULTS AND DISCUSSION

One of the main indicators reflecting the ability of microorganisms to restore is the survival of cultures.

Freeze-dried lactic acid bacteria: *Lactobacillus plantarum* 53H, *Lactobacillus plantarum* 22, *Lactobacillus plantarum* 2, *Lactobacillus cellobiosus* 20, *Lactobacillus acidophilus* 27W, *Lactobacillus curvatus* 18d, *Lactobacillus casei* 139, *Lactobacillus casei* 173a, *Lactobacillus salivarius* 8e and *Lactobacillus fermentium* 27 tested for viability. Table 1 presents the results of studies on the impact on the survival of freeze-drying of lactic microorganisms. When selecting protectors for preserving the viability of the lactic acid microorganisms was found that the most suitable for the tread of all the microorganisms studied lactic acid is skim milk and for six lactic acid of microorganisms is skim milk + 7 % glucose. When using a tread consisting of 10 % sucrose + 1% gelatin best vitality was manifested two cultures: *Lactobacillus casei* 139 and *Lactobacillus fermentium* 27.

A study on the survival of lactic acid microorganisms after leofilled drying with the use of protectors showed that all three protectors used viability suitable for preservation of lactic acid microorganisms.

STUDY ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA AFTER FREEZE-DRYING.

Cultures of lactic acid bacteria after storage in freeze- dried state were tested for preservation of antimicrobial activity against pathogens: (*Bacillus subtilus*, *Escherichia coli* 113, *Bacillus cereus*, *Staphylococcus aureus*). As protective media used: skim milk +7 % glucose, 10 % sucrose + 1% gelatin. Results of antimicrobials aactivities for *Bac.cereus* shown in таble 2.

Table 1 - Viability of lactic acid microorganisms after lyophilization

Title crop	Viability CFU ml			
	protectors			
	outgoing	skim milk	skim milk + 7% glucose	10% sucrose + 1% gelatin
Lactobacillus casei 139	10,5 x10^{10}	3x10^{10}	6 x 10^{9}	8 x 10^{10}
Lactobacillus plantarum 2	11 x10^{10}	7 x10^{10}	8 x 10^{9}	6 x 10^{8}
Lactobacillus casei 173a	8 x10^{10}	3 x10^{10}	5 x 10^{10}	4 x 10^{7}
Lactobacillus plantarum 53H	7 x10^{10}	6 x10^{10}	8 x 10^{9}	7x 10^{8}
Lactobacillus salivarius 8g	10 x10^{10}	7 x10^{10}	8 x 10^{8}	9 x 10^{7}
Lactobacillus cellobiosus 20	9 x10^{10}	13 x10^{8}	10 x 10^{9}	6 x 10^{8}
Lactobacillus acidophilus 27W	10 x10^{10}	5 x10^{10}	8 x 10^{8}	4 x 10^{8}
Lactobacillus plantarum 22	11 x10^{10}	8 x10^{10}	1 x 10^{9}	8 x 10^{8}
Lactobacillus fermentium 27	2 x10^{10}	8 x10^{9}	3 x 10^{8}	11 x 10^{9}
Lactobacillus curvatus 18g	10 x10^{10}	5 x10^{9}	8 x 10^{8}	8 x 10^{7}

Table 2 - Antimicrobial activity of the lactic acid microorganisms in relation to *Bac.cereus* after lyophilization

Title crop	Diameter growth suppression zones *Bac.cereus*			
	Protectors			
	outgoing	skim milk	skim milk + 7% glucose	10% sucrose + 1% gelatin
Lactobacillus plantarum 2	12±0,1	-	-	-
Lactobacillus plantarum 22	13±0,3	-	-	-
Lactobacillus plantarum 53H	12±0,4	-	-	-
Lactobacillus acidophilus 27W	10±0,1	-	-	-
Lactobacillus curvatus 18g	10±0,7	10±0,1	10±0,1	12±0,1
Lactobacillus casei 139	10±0,4	-	-	-
Lactobacillus casei 173a	11±0,5	11,5±0,3	10,5±0,1	12,5±0,5
Lactobacillus salivarius 8g	12±0,6	-	-	-
Lactobacillus fermentium 27	11±0,3	-	-	-
Lactobacillus cellobiosus 20	13±0,5	17,5±0,6	19,5±0,3	20,5±0,.2

After twelve months of storage antimicrobial activity against *Bac.cereus* preserved in three cultures: *Lactobacillus curvatus* 18g, *Lactobacillus casei* 173a, *Lactobacillus cellobiosus* 20. Most antimicro-bial activity of the lactic acid microorganisms was observed when used as the tread 10 % sucrose + 1% gelatin. In of *Lactobacillus cellobiosus* 20 diameter antimicrobial activity reached 20.5 mm.

In relation to *E.coli* (table 3) of the ten tested cultures retained after freeze-drying activity eight cultures, two lactic acid bacterias: *Lactobacillus plantarum* 22, *Lactobacillus plantarum* 53H lost the antimicrobial activity

Table 3 - Antimicrobial activity of the lactic acid microorganisms in relation to *E.coli* after lyophilization

Title crop	Diameter growth suppression zones *E.coli*			
	Protectors			
	outgoing	skim milk	skim milk + 7% glucose	10% sucrose + 1% gelatin
Lactobacillus plantarum 2	14±0,5	11±0,1	11±0,3	12±0,1
Lactobacillus plantarum 22	13,5±0,2	-	-	-
Lactobacillus plantarum 53H	15,5±0,8	-	-	-
Lactobacillus acidophilus 27W	14±0,1	12±0,4	10,5±0,5	11±0,2
Lactobacillus curvatus 18g	13±0,5	10±0,3	10±0,1	12±0,2
Lactobacillus casei 139	12±0,7	11±0,2	11±0,3	11±0,1
Lactobacillus casei 173a	12±0,5	12±0,4	11,5±0,2	14,2±0,3
Lactobacillus salivarius 8g	15±0,1	10,5±0,6	10,5±0,5	12±0,2
Lactobacillus fermentium 27	18±0,4	11,5±0,2	10,5±0,1	10,0±0,3
Lactobacillus cellobiosus 20	20±0,6	18,2±0,2	24±0,3	20±0,5

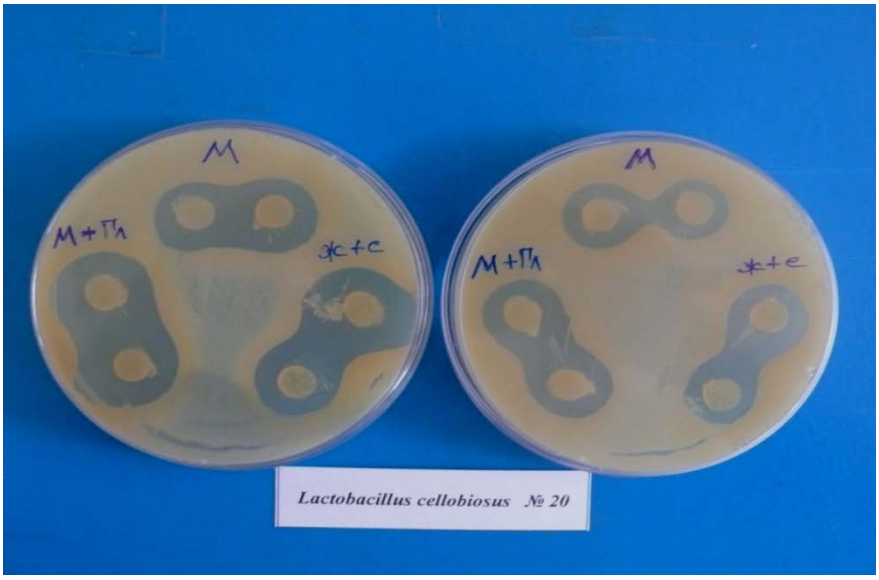
Antimicrobial activity to *Staphylococcus* aureus after freeze-drying retained only two lactic cultures: *Lactobacillus casei* 139, *Lactobacillus fermentium* 27.

Table 4 - Antimicrobial activity of lactic acid microorganisms in relation to *Staphylococcus aureus* after lyophilization

Title crop	Diameter growth suppression zones *Staphylococcus aureus*			
	Protectors			
	outgoing	skim milk	skim milk + 7% glucose	10% sucrose + 1% gelatin
Lactobacillus plantarum 2	13±0,3	-	-	-
Lactobacillus plantarum 22	16±0,4	-	-	-
Lactobacillus plantarum 53H	12±0,6	-	-	-
Lactobacillus acidophilus 27W	18±0,5	-	-	-
Lactobacillus curvatus 18g	15±0,3	-	-	-
Lactobacillus casei 139	14±0,4	12±0,3	12±0,4	12±0,3
Lactobacillus casei 173a	17±0,2	-	-	-

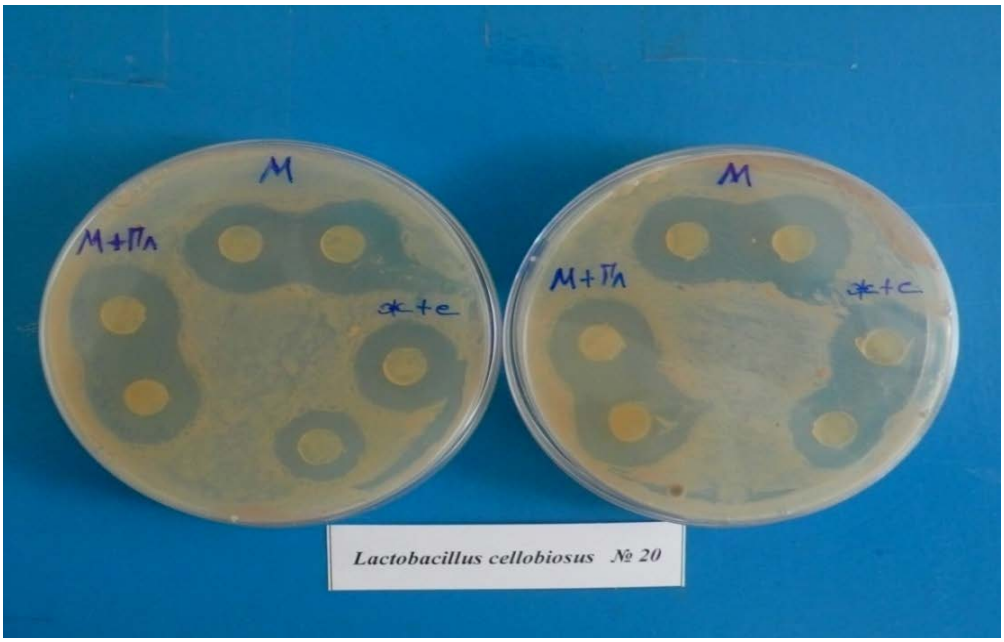
Lactobacillus salivarius 8g	12±0,1	-	-	-
Lactobacillus fermentium 27	13±0,3	24±0,2	19,5±0,4	20,5±0,2
Lactobacillus cellobiosus 20	14±0,4	-	-	-

Unusually manifested itself after the freeze-drying of *Lactobacillus cellobiosus* 20.



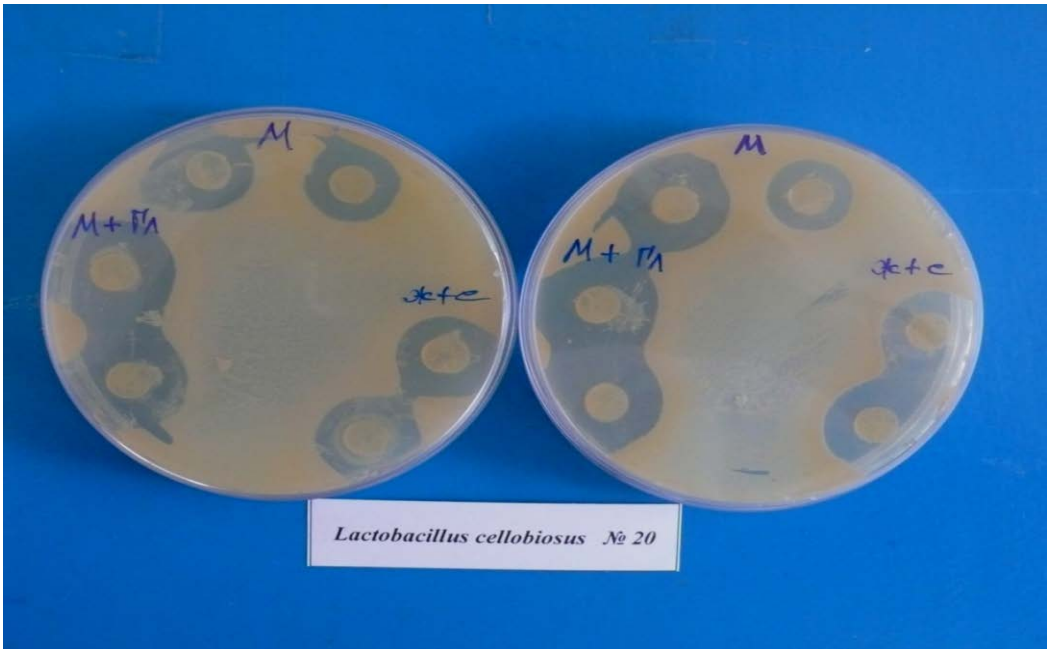
Protectors: м- skim milk , м+гл- skim milk +7% glucose, ж+ с- 1% gelatin+10% sucrose

Figure 1 - Antimicrobial activity of *Lactobacillus cellobiosus* 20 to test culture *Bac.cereus* after freeze-drying



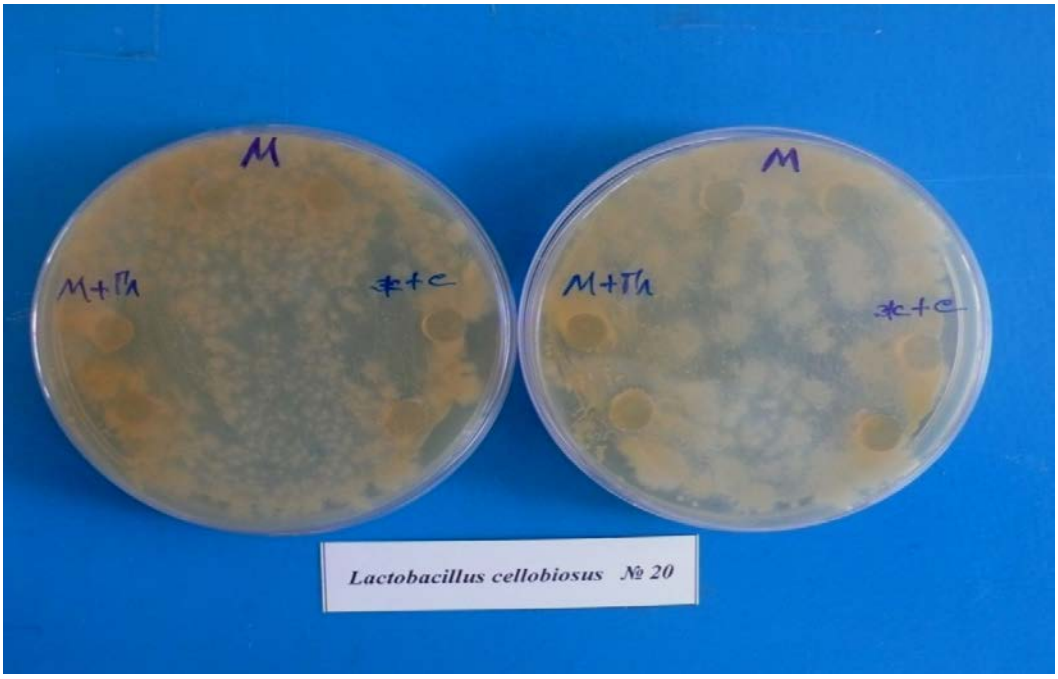
Protectors: м-milk , м+гл- skim milk +7% glucose, ж+ с- 1% gelatin+10% sucrose

Figure 2 Antimicrobial activity of *Lactobacillus cellobiosus* 20 to test the culture of *Bac. subtilus* after freeze-drying.



Protectors: м- skim milk , м+гл- skim milk +7% glucose, ж+ с- 1% gelatin+10% sucrose

Figure 3 - Antimicrobial activity of Lactobacillus cellobiosus 20 to test the culture of E.coli after freeze-drying



Protectors: м- skim milk, м+гл- skim milk +7% glucose, ж+ с- 1% gelatin+10% sucrose

Figure 4 Antimicrobial activity of Lactobacillus cellobiosus 20 to the test culture St. aureus after freeze-drying

Antimicrobial activity of *Lactobacillus cellobiosus* 20 towards *Bac.cereus, Bac. subtilus, E.coli* was quite high and exceeded the original. This can be clearly traced in figures 1, 2, 3, and did not manifest antimicrobial activity against St. aureus (figure 4).

Table 5 - Antimicrobial activity of the lactic acid microorganisms in relation to *Bac. subtilus* after lyophilization.

Title crop	Diameter growth suppression zones *Bac. subtilus*			
	Protectors			
	outgoing	skim milk	skim milk + 7% glucose	10% sucrose + 1% gelatin
Lactobacillus plantarum 2	-	-	-	-
Lactobacillus plantarum 22	23±0,4	12,5±0,3	11±0,2	11±0,1
Lactobacillus plantarum 53H	17±0,6	10,5±0,4	10,5±0,5	12±0,6
Lactobacillus acidophilus 27W	25±0,5	10±0,3	10±0,2	12±0,2
Lactobacillus curvatus 18g	-	-	-	-
Lactobacillus casei 139	22±0,6	12±0,1	12±0,3	13±0,4
Lactobacillus casei 173a	21±0,2	11±0,5	12±0,6	13±0,1
Lactobacillus salivarius 8g	-	-	-	-
Lactobacillus fermentium 27	23±0,1	11±0,3	11±0,2	10,5±0,4
Lactobacillus cellobiosus 20	11±0,2	21,7±0,1	22,2±0,5	21,5±0,2

By Bac. subtilus all seven cultures retained antimicrobial activity. All three have the same tread pro-tective effect on the safety of the activity (Table 5).

From these results we can conclude that by freeze drying of the lactic acid culture is not retain all the antimicrobial activity, even though the viability is retained at a rather high level.

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