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EXPERIMENTAL ARTICLES

Comparative Investigation of Different Methods of Storage of Lactic Acid Bacteria

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Abstract—The study was undertaken to elucidate how different methods of storage (immersing in mineral oil, lyophilization, and subculturing) of lactic acid bacteria belonging to the genera *Lactobacillus* and *Lactococcus* affect their viability, antibiotic activity, and ability to accumulate organic acids. Storage of the lactic acid bacterium *Lactococcus lactis* subsp. *lactis* by immersion in mineral oil proved to be ineffective. Lyophilization allowed the survival of a sufficiently large number of cells, although their antibiotic activity somewhat decreased. The resuscitation of lyophilized bacteria by subculturing them in rich nutrient media, such as skim milk, led to the restoration of their physiological activity, including the effective antimicrobial spectrum.

Key words: lactic acid bacteria, lyophilization, storage by immersion in mineral oil, subculturing, viability, titratable acidity, effective antimicrobial spectrum.

It is known that some properties of lactic acid bacteria are unstable [1, 2]. Maintenance of microorganisms by subculturing them in rich nutrient media often leads to the loss of some valuable physiological and biochemical properties due to mutation and autoselection. To avoid this, it is necessary to drastically suppress metabolism and genetic rearrangements in stored microorganisms, or, in other words, to bring them to a state close to anabiosis [3, 4].

Lactic acid bacteria are commonly maintained by subculturing or are stored either in 25% glycerol at –20°C, on agar media under a layer of mineral oil, or in a lyophilized state [5–7]. None of these methods is universal [4, 8].

The aim of the present work was to study how different storage methods may affect the preservation of some valuable physiological and biochemical properties of lactic acid bacteria, namely, their viability, fermenting capacity, and antibiotic activity.

MATERIALS AND METHODS

Twenty strains of lactic acid bacteria stored in the collection of cultures of the Department of Microbiology of the Moscow State University were studied.

Lyophilization. Stationary-phase cells of lactic acid bacteria grown in liquid medium completely satisfying their nutritional requirements were harvested by centrifugation, suspended in a preservation medium, and freeze-dried at –30°C in a Beta A lyophilizer (Cryst, Germany) to a residual moisture of 2.5–4.0%. The preservation medium was an aqueous solution of gelatin (10 g/l) and sucrose (100 g/l), which are known as efficient cryoprotectants diminishing water activity and changing the osmotic pressure of the medium [9, 10]. Lyophilized cells were kept in sealed ampules in a refrigerator (4°C). Immediately after lyophilization or after long-term storage in the lyophilized state, cultures were revived in skim milk, and the number of viable cells was determined by plating serial dilutions, prepared in physiological saline solution (0.8% NaCl), on a modified MRS medium representing nutrient broth supplemented with (g/l) yeast extract, 5; glucose, 20; $K_2HPO_4 \cdot 3H_2O$, 2; sodium acetate, 5; ammonium citrate, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4$, 0.05; cysteine, 0.1; agar, 20; and Tween-80, 1 ml/l. The pH of the medium was adjusted to 6.2–6.5. Cysteine was added in the form of a 0.4% solution.

To study the preservation of physiological and biochemical properties during storage, strains were restored in skim milk and cultivated in the respective fermentation media either immediately after lyophilization or after 5, 7, 9, 14, 15, 20, or even 35 (*Leuconostoc mesenteroides*) years of storage. The lactococci *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* were grown at 30°C in skim milk and liquid MRS medium without agar, whereas the lactic acid bacteria *Lactobacillus acidophilus, L. bulgaricus, L. casei, L. lindneri, L. plantarum, L. pentoaceticum, Lactococcus lactis* subsp. *diacetilactis*, and *Leuconostoc mesenteroides* were grown at 37°C in 8°B (Balch) wort with mash (crushed barley malt), to which sterile chalk was added to neutralize acidic metabolites excreted to the medium. Cultivations lasted 20–24 h.

Storage under mineral oil. Of all the strains studied, thirteen were stored under sterile mineral oil in a semiliquid MRS medium containing 0.15% agar. For this purpose, cultures were grown in tubes containing

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Table 1. Effect of lyophilization and long-term storage in the lyophilized state on the viability of lactic acid bacteria of the genus *Lactobacillus*

Strain	Number of viable cells in 1 ml		
	before lyophilization	after lyophilization	after 5-year storage in the lyophilized state
L. casei 153	1.4×10^{10}	9.2×10^{9}	1.1×10^{9}
L. "caucasicus" 155	1.2×10^{10}	5.8×10^{9}	8.5×10^{7}
L. lactis subsp. *cremoris* 163	1.0×10^{10}	5.4×10^{9}	4.0×10^{7}
L. lactis subsp. *lactis* 170	5.5×10^{9}	3.0×10^{9}	6.4×10^{8}
L. lactis subsp. *lactis* 283	1.8×10^{10}	9.8×10^{9}	7.6×10^{9}
L. lactis subsp. *lactis* 284	1.9×10^{10}	8.5×10^{9}	7.0×10^{9}
L. lactis subsp. *lactis* 729	3.4×10^{10}	1.6×10^{9}	1.0×10^{7}
L. lactis subsp. *lactis* 1605	1.1×10^{9}	9.0×10^{8}	7.2×10^{8}

Table 2. Effect of long-term storage in the lyophilized state on the viability and physiological activity of lactic acid bacteria

Strain	Storage period, years	Number of viable cells in ampule		Titratable acidity, °T
		immediately after lyophilization	after storage in the lyophilized state	
Genus *Lactobacillus*				
L. acidophilus 147	18	7.0×10^{7}	1.0×10^{4}	128
L. acidophilus 148	9	1.3×10^{10}	3.5×10^{8}	20
L. bulgaricus 152	9	2.7×10^{6}	1.2×10^{5}	76
L. casei 153	14	9.2×10^{9}	1.6×10^{8}	76
L. "caucasicus" 155	20	8.0×10^{7}	1.6×10^{6}	46
L. lindneri 158	13	5.0×10^{9}	1.0×10^{7}	120
L. plantarum 161	13	3.9×10^{8}	6.0×10^{5}	45
L. pentoaceticum 159	14	6.0×10^{9}	1.0×10^{7}	152
Lactococcus lactis subsp.				
lactis 167	15	6.0×10^{9}	1.0×10^{7}	42
lactis 169	15	2.0×10^{9}	1.7×10^{7}	60
lactis 283	10	9.8×10^{9}	4.6×10^{8}	106
lactis 284	9	8.5×10^{9}	2.4×10^{8}	100
lactis 729	14	1.6×10^{9}	6.0×10^{6}	120
lactis 1605	14	9.0×10^{8}	1.7×10^{7}	105
cremoris 164	13	6.0×10^{9}	4.0×10^{5}	76
cremoris 163	14	6.0×10^{9}	1.0×10^{4}	30
diacetilactis 165	10	1.5×10^{7}	2.4×10^{6}	38
diacetilactis 166	15	2.0×10^{9}	1.7×10^{7}	46
Leuconostoc mesenteroides 171	35	1.0×10^{9}	1.0×10^{7}	48

10 ml of the semiliquid MRS medium for 48–72 h. The culture broth was then neutralized with a 10% solution of $NaHCO_3$ and flooded with a 1.5-cm-thick layer of sterile mineral oil. Such stock cultures were stored for 12 months.

In control experiments, lactic acid bacteria were kept in the form of a milk clot in a refrigerator (4°C). Every 30 days, stock cultures were subcultured in skim milk and then in the aforementioned liquid medium. At certain time intervals, stock cultures were analyzed for survival, fermenting activity, and synthesis of bacteriocins, after which the cultures were reinoculated into skim milk and cultivated at the optimum temperature to produce a milk clot. The clot was then placed in a refrigerator for the next period of storage.

Viable cells were enumerated by the direct count of colonies grown on 2.5%-agar solidified MRS plates, which were inoculated with tenfold dilutions of revived

Table 3. Effect of storage under mineral oil on the viability and physiological activity of lactic acid bacteria

Strain	Number of viable cells in 1 ml		Titratable acidity, °T
	before storage	after 12-month storage	
Genus *Lactobacillus*			
L. acidophilus 147	4.8×10^9	1.0×10^7	248
L. casei 153	9.3×10^9	3.0×10^7	96
L. "caucasicus" 155	1.6×10^9	6.2×10^6	46
L. lindneri 158	6.1×10^9	1.0×10^7	38
L. plantarum 161	3.2×10^9	1.0×10^7	30
L. pentoaceticum 159	1.9×10^9	1.0×10^7	88
Lactococcus lactis subsp.			
lactis 167	1.5×10^9	1.0×10^7	54
lactis 169	5.2×10^9	6.2×10^5	50
cremoris 164	3.5×10^9	1.1×10^6	74
cremoris 163	1.4×10^9	1.0×10^7	168
diacetilactis 165	1.0×10^8	1.0×10^7	168
diacetilactis 166	1.5×10^8	6.2×10^5	50
Leuconostoc mesenteroides 171	6.3×10^8	1.0×10^5	30

cultures prepared with a physiological saline solution (0.8% NaCl).

The fermentative activity of cultures (i.e., their ability to accumulate organic acids) was assayed as the titratable acidity of culture liquids [11] and was expressed in Turner degrees (one Turner degree is defined as the volume (in ml) of 0.1 N NaOH necessary to neutralize acids formed in the process of bacterial growth; 1°T corresponds to 0.0075% lactic acid).

The antibiotic activity of lactic acid bacteria was studied with respect to a wide range of test cultures, since the bacteriocins produced by lactic acid bacteria are very diverse [1, 12–14]. As test cultures, we used six species of gram-negative bacteria (*Alcaligenes faecalis* 82, *Comamonas acidovorans* 45, *Escherichia coli* 52, *Pseudomonas fluorescens* 80, *Proteus vulgaris* 206, and *Serratia marcescens* 208), six species of gram-positive bacteria (*Bacillus coagulans* 429, *Bacillus mycoides* 32, *Bacillus subtilis* 2, *Micrococcus luteus* 128, *Mycobacterium smegmatis* 377, and *Staphylococcus aureus* 144), three species of yeasts (*Candida guilliermondii* 217, *Rhodotorula aurantiaca* 226, and *Saccharomyces cerevisiae* 230), and two species of fungi (*Aspergillus niger* 369 and *Fusarium oxysporum*).

Test cultures of bacilli and mycobacteria were grown on plates containing (g/l) glucose, 10.0; peptone, 5.0; NaCl, 5.0; agar, 25.0; and Hottinger broth in an amount corresponding to 28 mg% nitrogen (pH 7.0). Yeasts were grown on plates prepared with 6–8°B wort and 2.5% agar (pH 6.8); fungi were grown on plates prepared with 3–4°B wort and agar (pH 6.0). Test cultures of yeasts and fungi were grown at 28°C; bacilli, staphylococci, and micrococci, at 37°C; *E. coli*, at 42°C; and *B. coagulans*, at 55°C. Plates were inoculated with suspensions of 1-day-old bacteria or yeasts and 5-day-old fungi prepared with a physiological saline solution to give a concentration of 1 billion cells/ml by the bacterial turbidity standard. Upon submerged cultivation, plates were inoculated with 0.1-ml cell suspensions.

The antibiotic activity of lactic acid bacteria was assayed by the disk diffusion method; the diameter of growth inhibition zones was expressed in mm [15].

RESULTS AND DISCUSSION

Survival rate is one of the major parameters characterizing the ability of microorganisms to revive. Data on the effect of lyophilization on the survival of eight strains of lactic acid bacteria (Table 1) show that the survival rate of cells subjected to lyophilization changed insignificantly, ranging from 50 to 70%. Thus, shortly after lyophilization, the relative number of viable cells of the homofermentative bacterium *Lactobacillus casei* 153 was 65.7%, and the relative numbers of viable cells of *L. caucasicus* and *L. lactis* subsp. *lactis* 170, 283, 284, and 1605 were 50 and 54–57%, respectively. After five years of storage in the lyophilized state, the percentage of viable cells decreased to 7.3–23.3% (Table 1).

After 14 years of storage in the lyophilized state, the number of viable cells of *L. casei* 153 decreased by an order of magnitude (from 9.2×10^9 to 1.6×10^8 cells/ml) and that of *L. cremoris* 163, by five orders of magnitude. After 20 years of storage, the survival rate of *L. caucasicus* 155 fell by about 2 orders of magnitude,

Table 4. Antagonistic activity of bacteria of the genus *Lactobacillus*

Strain	Maintenance method	*Alcaligenes faecalis*	*Bac. coagulans*	*Bac. mycoides*	*Bac. subtilis*	*Comamonas acidovorans*	*E. coli*	*M. luteus*	*M. smegmatis*	*Proteus vulgaris*	*P. fluorescens*	*Serratia marcescens*	*Staph. aureus*
L. acidophilus 146	S	0	0	13	11	12	12	15	15	0	10	0	12
	L	8	0	14	11	13	13	15	17	0	12	0	12
L. acidophilus 147	S	12	11	18	12	11	12	18	20	12	14	10	13
	L	0	0	13	11	14	12	15	19	0	11	0	13
L. acidophilus 148	S	0	15	23	8	11	12	12	16	8	11	0	15
	L	0	0	9	7	11	10	18	23	0	12	0	10
L. bulgaricus 150	S	0	0	12	10	12	12	15	14	0	11	0	11
	L	0	0	14	12	13	12	15	15	0	12	0	12
L. bulgaricus 152	S	0	8	16	0	0	12	13	15	0	10	0	0
	O	11	10	13	0	10	8	12	15	0	11	0	12
L. casei 153	O	0	9	13	0	0	8	10	16	0	0	0	0
	L	0	0	13	8	0	10	15	18	0	12	0	0
L. "caucasicus" 155	O	0	12	0	7	0	0	9	15	0	0	0	0
	L	0	0	10	8	0	0	10	16	9	9	0	0
L. lindneri 158	O	10	0	12	14	0	15	15	15	10	14	0	13
	L	0	8	13	10	0	12	14	16	10	0	10	9
L. pentoaceticum 159	O	0	0	10	12	0	15	12	18	0	0	0	0
	L	10	0	12	12	0	12	15	18	10	0	10	0
L. plantarum 161	O	0	14	9	0	0	17	14	11	0	0	0	0
	L	0	0	15	0	0	17	14	18	0	0	0	0

Note: S is subculturing; L is lyophilization; O stands for "storage under mineral oil." Given are the diameters of growth inhibition zones expressed in mm.

and the survival rate of *L. lactis* 167 stored for 15 years, by 2 orders (Table 2). In general, after 9 to 20 years of storage in the lyophilized state (*Leuconostoc mesenteroides* was stored for as long as 35 years), all 17 lyophilized cultures of lactic acid bacteria retained a fairly large number of viable cells (from 10^4 to 10^8 cells/ml).

It is known that changes in the medium composition or long-term subculturing of homofermentative lactic acid bacteria can make them capable of producing, in addition to lactic acid, considerable amounts of volatile fatty acids and CO_2, as well as some amounts of glycerol, diacetyl, acetone, and ethanol. On the other hand, heterofermentative lactic acid bacteria may lose the ability to produce volatile fatty acids, carbon dioxide, and ethanol and thereby follow the homofermentative pathway of glucose assimilation, which will alter their values of titratable acidity [1, 16].

After 13 and 14 years of storage in the lyophilized state, the titratable acidity of *L. pentoaceticum* 159 and *L. lindneri* 158 suspensions containing equal numbers of viable cells (10^7 cells/ml) was higher (152.0 and 120.0°T, respectively) than after long-term storage under mineral oil (88.0 and 38.0°T, respectively).

The titratable acidity of the homofermentative bacteria of the genus *Lactococcus* (*L. lactis* subsp. *cremoris* 163 and 164 and *L. lactis* subsp. *diacetilactis* 165) stored in nutrient media under mineral oil for 1 to 6 years was considerably higher than in the case of storage in the lyophilized state. Correspondingly, the survival of these species kept in the lyophilized state showed a greater decrease (by four, five, and one order of magnitude, respectively) than in the case of storage under mineral oil (Tables 2 and 3). It should be noted that the concentration of viable cells of *Leuconostoc mesenteroides* 171 stored in an ampule in the lyophilized state for 35 years was the same (10^7 cells/ml) as in the case of one year of storage under mineral oil. The titratable acidity of the *Leuconostoc mesenteroides* culture revived after storage in the lyophilized state was 48°T, i.e., 1.6 times higher than in the case of storage under mineral oil.

The maximum concentration of organic acids corresponding to a titratable acidity of 248°T was observed for the homofermentative lactic acid bacterium *L. acidophilus* 147 stored for one year under mineral oil.

Table 5. Antagonistic activity of bacteria of the genus *Lactococcus*

Strain	Maintenance method	*Alcaligenes faecales*	*Bac. coagulans*	*Bac. mycoides*	*Bac. subtilis*	*Comamonas acidovorans*	*E. coli*	*M. luteus*	*M. smegmatis*	*Staph. autreus*	*Candida guilliermondii*	*Asp. niger*	*Phodotorula aurantica*
Lactococcus lactis subsp.													
lactis 167	O	0	0	14	0	0	0	0	13	0	0	0	0
	L	0	0	0	0	0	0	0	13	12	12	13	0
lactis 169	O	0	12	15	0	9	12	14	15	0	0	0	10
	L	0	0	0	8	0	10	0	16	12	14	0	8
lactis 170	O	0	0	10	7	0	13	11	15	0	0	0	0
	L	0	0	8	0	0	10	9	10	0	0	0	0
lactis 283	S	0	20	10	10	0	0	26	18	28	0	0	0
	L	0	22	16	12	0	0	20	18	28	0	0	0
lactis 284	S	18	32	15	18	18	14	34	32	28	18	20	0
	L	16	20	12	16	16	14	30	28	24	16	18	18
lactis 729	S	0	0	16	10	0	0	18	20	0	0	0	16
	L	0	0	14	10	0	0	15	17	0	0	0	0
lactis 1605	S	6	0	16	8	0	12	14	16	18	14	10	0
	L	0	0	16	7	0	8	10	15	14	10	8	0
cremoris 163	O	8	0	13	0	0	0	0	20	0	0	0	0
	L	0	0	0	0	0	0	0	0	13	12	0	0
diacetilactis 165	O	0	0	9	10	0	0	12	12	0	0	0	0
	L	0	0	0	0	0	17	0	0	0	0	0	0
Leuconoctoc mesenteroides 171	S	0	0	10	0	0	0	12	12	0	0	0	0
	L	0	0	8	0	0	17	0	0	0	0	0	0

The survival rate of *L. acidophilus* 148 maintained for 9 years by subculturing was two orders of magnitude lower than after 9 years of storage in the lyophilized state; in this case, the titratable acidity of the subcultured bacterium (128.0°T) was 6.4 times higher than that of the bacterial culture kept in the lyophilized state.

The lactic acid bacterium of the subgenus *Thermobacterium* (Orla-Jenson), *L. bulgaricus* 152, stored by subculturing, showed almost the same survival as in the case of storage in the lyophilized state, but its titratable acidity (41.0°T) was 1.8 times lower than in the latter case. The number of viable cells in lactococcal cultures maintained by subculturing was three orders of magnitude lower than in the case of storage in the lyophilized state. Storage by subculturing also led to a reduction in the ability of these homofermentative bacteria to produce lactic acid. It is assumed that long-term storage by subculturing affects the survival rate of cultures and their physiological activity due to the action of physicochemical factors of the medium, periodical culture dehydration, and the accumulation of inactive dissociants in bacterial populations [17].

The range of the antibiotic activity of 20 strains of lactic acid bacteria belonging to the genera *Lactobacillus* and *Lactococcus* subjected to storage under different conditions is shown in Tables 4 and 5. The antibiotic activity of lactic acid bacteria results from a combined action of bacteriocins, as well as of organic acids, alcohols, peroxides, and other metabolites. It is evident from Tables 4 and 5 that bacteria of the genus *Lactobacillus* exhibited a wider range of antibiotic activity than bacteria of the genus *Lactococcus*. The lactobacillar strains under study inhibited the growth of both gram-positive bacteria (the facultatively anaerobic cocci *Staphylococcus aureus* and *Micrococcus luteus*, aerobic coryneform *Mycobacterium smegmatis*, and the bacilli *Bacillus mycoides* and *Bac. subtilis*) and gram-negative bacteria (the facultatively aerobic *E. coli* and aerobic *Pseudomonas fluorescens*) but virtually did not suppress the growth of the acid- and heat-tolerant bacterium *Bac. coagulans*, gram-negative rods *Alcaligenes faecalis*, or the facultatively aerobic bacteria *Serratia marcescens* and *Proteus vulgaris*. The strains of the genus *Lactobacillus* failed to inhibit the growth of the yeasts *Candida guilliermondii, Rhodotorula aurantiaca,*

and *Saccharomyces cerevisiae* and the microscopic filamentous fungi *Aspergillus niger* and *Fusarium oxysporum*. The effective antimicrobial spectrum of lactococci was comparable with that of lactobacilli. Thus, *L. lactis* subsp. *lactis* 167 and 284 efficiently inhibited the growth of gram-positive and gram-negative bacteria, as well as of the yeast *C. guilliermondii* and the fungi *Asp. niger* and *Fusarium*. The antibiotic activity of lyophilized cultures tended to decrease, which was attributed to the damaging effect of lyophilization on cellular nucleic acids, proteins, and peptides [18] or to the accumulation of inactive dissociants in bacterial populations [17]. The subculturing and selection of active variants of lactic acid bacteria after storage in the lyophilized state led to the restoration of their physiological activity.

Thus, lyophilization provides the highest survival of lactic acid bacteria during long-term storage. Storage by immersion in mineral oil also ensures a high rate of survival of lactobacilli, but not of lactococci. The storage of lactic acid bacteria by subculturing requires that they are periodically grown in skim milk to restore their viability and antibiotic activity.

Studies along this line (the maintenance of pure cultures of lactic acid bacteria by subculturing, under mineral oil at 20°C, and in the lyophilized state at 4°C) are in progress in our laboratory. Attempts are now being made to elaborate individual conditions of storage of particular strains to ensure not only a high survival rate for cells, but also the preservation of their valuable physiological and biochemical properties.

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