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Optimization of the storage method of *Lactococcus lactis* ssp. *lactis* strains to stabilize their probiotic potential

Abstract

The object of the study were strains of *Lactococcus lactis* ssp. *lactis* isolated from microbiota of fermented milk products brought from Buryatia (Russia), Lebanon, Iran, which have high probiotic potential. The purpose of this work was to optimize the storage method of *L. lactis* ssp. *lactis* strains to stabilize their probiotic potential. The various storage methods were used - in skim milk with frequent passages and lyophilization. The cultures were lyophilized on a 'Krist" installation of the "Betta A "(Germany). Lyophilized strains were restored and their physiological and biochemical properties were studied both immediately after recovery and in a number of passages after storage, their physiological activity was evaluated by the rate of clot formation in skim milk and antimicrobial activity using the microbiological method with the test-cultures and standard antibiotic solutions (Nisaplin, Chloramphenicol, Nystatin).

The highest survival rate was found during lyophilization with preliminary treatment. When cultures were restored, the survival rate in the first passage was over 70%, the strains had a high level of antimicrobial activity on both gram-positive and gram-negative bacteria and fungi, including pathogens. The results of the study showed that the most effective method of preserving bacteriocin-forming lactococci is lyophilization using a complex protective environment of the composition (g/l): sucrose-100, gelatin-10, monosodium glutamate-10, sodium citrate-5, which ensures long-term crop life. The introduction of lactococci into the intestinal microbiota leads to the replacement of pathogens and is useful for people with lactase deficiency. Strains enrich the dairy product with biologically active substances, which, in addition to the nutritional effects, have a beneficial effect on health. Lyophilization in combination with pre-treatment of cells contributes to the preservation of their probiotic potential.

Keywords: *Lactococcus lactis* ssp. *lacti,* antimicrobial activity, probiotic correctors, storage, lyophilization

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Daria Novoselova, Lidia Stoyanova
Department of Microbiology, Faculty of Biology, M.V. Lomonosov Moscow State University, Russia

Correspondence: Daria Novoselova, Department of Microbiology, Faculty of Biology, M.V. Lomonosov Moscow State University, 119234 Lenin's Gory, Moscow, Russia, Email novoselovadaria115@gmail.com

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Introduction

Among the probiotic correctors of normal microbiota the special interest present the lactic acid bacteria (LAB), isolated from national lactic acid products of fuctional nourishment. The history of this dairy product totals about 8 thousand years. Kurunga, yoghurt, matsun, Leben - are the names of the same product of lactic acid or mixed lactic acid and alcoholic fermentation. This products had used for treating the number of diseases of the gastrointestinal tract (GIT), cardiovascular system, to cure of tuberculosis for several thousands of years.[1] Lactic acid bacteria (LAB), isolated from fermented milk products, are of particular interest among the probiotic correctors of normal intestinal microbiota.

The main requirements for probiotics:[2]

a. Identification to the species by pheno- and genotypic characteristics;

b. Passportization of the strain (cultural, morphological, physiological, biochemical and genotypic properties of the microorganism);

c. Strains-producers of biologically active substances useful for humans. The presence of pronounced antagonistic properties in relation to pathogenic and conditionally pathogenic microorganisms;

d. Stability of signs: pathogenicity, immunological safety;[3,4]

e. The stability of their beneficial properties during storage.

Mesophilic *L. lactis* ssp. *lactis* have several advantages over other LABs due to their growth rate, do not have pathogenic forms ("GRAS" status), are part of the digestive tract microbiota, and are a starter culture for widely used lactic acid products. The fermented lactic acid products compared to natural milk due to the changed composition. As a result of fermentation, lactose is converted into lactic acid, so most people with lactase deficiency are able to tolerate normally fermented milk products. LAB consist more than 99% of the human intestine microbiota and suppress the growth of harmful microbes and help normalize the digestive tract.[4] The main requirements for probiotics are the stability of their beneficial properties during storage. Lyophilization is a common way of storing bacteria, since it helps to avoid infection of cultures during storage, ampoules with freeze-dried culture do not take up much space and their storage does not require special equipment.[5,6] Throughout the process, constraints (temperature, acid, osmotic, starvation and oxidative stresses) can affect the bacterial growth rate and survival, mainly causing permeabilization of the plasma membrane. In presence of oxygen, bacteria generate reactive oxygen species (ROS). However, if necessary, long-term storage should individually develop storage conditions for individual strains of microorganisms in order to ensure

not only their survival, but also the preservation of their physiological and biochemical properties, which are of particular value for probiotic cultures.[7] When stored in the form of a milk clot, bacteriocin-forming lactococci require reseeding at least 1 time per month, however, with frequent passages in the culture with relatively high survival rates, a decrease in their milk-clotting (C-activity) and inhibitory activity was observed, and low-activity dissociants accumulate in the population.[8] The aim of this stage of the work was to optimize the storage method of *L. lactis* ssp. *lactis* strains to stabilize their probiotic potential..

Methodology

The object of the study were strains of *L. lactis* ssp. *lactis* isolated from microbiota of milk products brought from Moscow region and Buryatia (Russia), Lebanon, Iran (table 1), which have high probiotic potential.[9] Various storage methods were used - in skim milk with frequent passages and lyophilization. The cultures were lyophilized on "Krist installation of the Betta A" (Germany). The strains recovered in skim sterile milk (reverse) after lyophilization were cultured in a fermentation medium and their physiological and biochemical properties were studied both immediately after recovery and in a number of passages. Bacteria of the genera *Lactococcus* were grown in the image and in a modified medium MPC. As a control, cultures of lactic acid bacteria were used, stored in test tubes in the form of a milk clot in a domestic refrigerator at 4°C, which were periodically sub-cultured once every 30 days in reverse and on liquid media of the above composition as applied to a specific strain.

Table 1 The origin of the strains of *Lactococcus lactis* ssp. *Lactis*

Strains	Origin	Antimicrobial activity (Nisin) IU/mL	GenBank
K-205	Isolated from the national lactic acid product "Kurunga"	2700	EF 11 4305
194	Isolated from cow milk (Buryatia)	3900	DQ 255954
729	Isolated from fresh milk that does not contain Preservatives (Moscow)	200	EF 102814
IR-4	Isolated from microbiota of the Iranian Doogh	3500	MF990372
TM-2	Obtained from self-fermented cottage cheese	3200	CIM-B-8354

The antagonistic properties of the selected strains with respect to gram-negative and gram-positive bacteria, as well as myxomycetes, were determined by the microbiological method by agar diffusion.[10] A study of the national Buryat therapeutic and preventive drink kurunga showed that the microbiota of the drink was lactic acid bacteria and yeast. Microscopic examination of the field of view reveals yeast and lactic acid bacteria represented by rods and cocci, single and assembled in chains of different lengths.

The microbiota of the Iranian Doogh drink consisted of yeast cells, lactobacilli, and lactococci, which were diverse in their segregation into chains - single, diplococci, and cocci, collected in short chains. Moreover, there is a disunity of microbiota: lactobacilli are adjacent to lactococci, and yeast cells seem to live in their own community. Involutional forms, multiple lysed cells are visible in the field of view of the microscope (Figure 1B). Traditional fermented dairy foods including cottage cheese have been major components of the diet for centuries. The bacterial communities of traditional craft cheeses vary according to geographic origin. In addition, we have allocated new and valuable laboratory resources to improve the production of cottage cheese. Microbiota from self-fermented cottage cheese contains lactobacilli, lactococci (Figure 1C). TM-2 strain obtained from self-fermented cottage cheese and proposed as a mono-ferment for the production of cottage cheese from Lebanon (table 1).

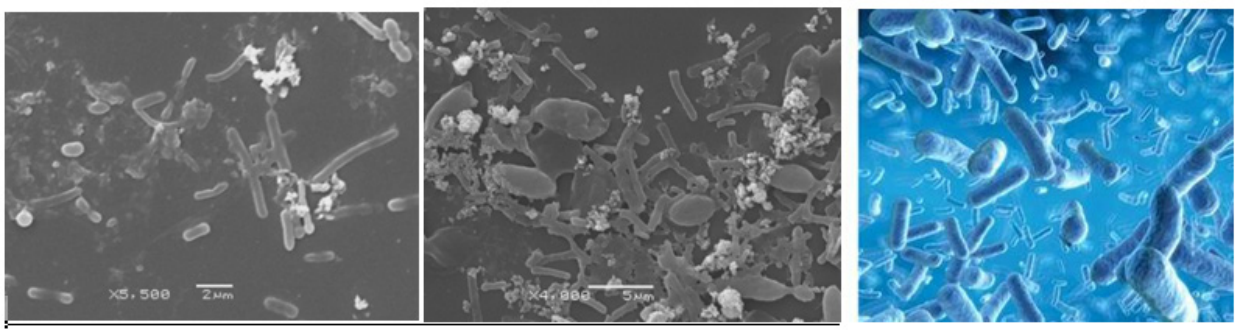

Figure 1 Microbiota of kurunga (A), Iranian Doogh (B) and self-fermented cottage cheese from Lebanon (C) (electron micrograph (x5000 and x7000).

The cultures were grown to a stationary phase, cell suspensions were washed using centrifugation (1380 g, 15min, 25°C), then mixed with cryoprotectants that reduce the strength of water activity and change the osmotic pressure in the medium: 20% sucrose solution, 10% sucrose +5% gelatin and a protective medium containing 1% gelatin + 10% sucrose + 1% sodium glutamate + 0.5% sodium citrate (Table 2). Next, cell lyophilization was performed on a" Krist Betta A" installation (Germany) under vacuum at -30°C. The entire production process of freeze-dried bacterial biomass generally includes microorganism culture and cell preparation, freeze-drying, packaging, storage and rehydration. Lyophilized strains were restored in the image, their physiological and biochemical properties were studied both immediately after recovery and in a number of transfers after storage. Their physiological activity was evaluated by the rate of clot formation in skim milk and antimicrobial activity using the microbiological method with the test- cultures and standard solutions of antibiotics (Nisaplin, Chloramphenicol, Nystatin).

All experiments were carried out in completely independent triplicates (n = 3). Statistical processing of research results was performed using computer programs Excel 2016 (Microsoft Inc., Statistical for Windows, v.5.0 StatSoft Inc), calculating the arithmetic mean, confidence intervals, and standard deviation. The significance of differences between the mean values was assessed using the Student's t-test ($p \leq 0.05$).

Table 2 The effect of the composition of the protective environment on the survival of *Lactococcus lactis* subsp.*lactis* strain 194 during lyophilization

The composition of the protective environment	The number of cells/mL	
	Before lyophilization	After lyophilization
Sucrose, 20%	$1{,}69\times10^{10}$	$7{,}9\times10^{9}$
Sucrose, 10% + Gelatin, 5%	$1{,}52\times10^{10}$	$1{,}06\times10^{10}$
Sucrose, 10% + Gelatin, 1% + monosodium glutamate, 1% + sodium citrate, 0.5	$1{,}46\times10^{10}$	$1{,}30\times10^{10}$
Skim milk (reverse)	$1{,}7\times10^{10}$	$8{,}2\times10^{9}$

Results

Removal of intracellular water from bacteria before reaching a low level of water activity can lead to stabilization of microbial biomass by slowing down or stopping the metabolic activity of cells. It has been established that, depending on the composition of the protective environment, the survival rate of a culture during sublimation, one of the main indicators reflecting the ability of lyophilized microorganisms to recover, ranges from 30 to 88%. The use of a sucrose solution as lyoprotectants with the addition of gelatin, glutamate and citrate is optimal for lyophilization; the survival rate when using this complex protective medium is up to 70% ($P<0.05$). A high survival rate is a necessary requirement for probiotic drugs. It is known that the health benefits of probiotics are generally achieved by ingesting at least 10^6-10^7 CFU of viable microorganisms per gram or ml of carrier product.[2]

It was established that, depending on the composition of the protective environment, the survival rate of the culture, one of the main indicators reflecting the ability of microorganisms to recover, ranges from 30 to 88%. The use of sucrose solution with the addition of gelatin, glutamate and citrate as cryoprotectants is optimal for lyophilization, the survival rate using this complex protective medium is higher than the lyophilization of the strain in the fermented sample, where the survival rate was about 50%: the effect of concentration of the final metabolites for autolysis of cells during the drying of lactococcus.

Table 3 presents the results of a study of the effect of lyophilization carried out with the optimal protective medium (Table 2) on the survival of different bacteriocene-forming strains of *L. lactis* subsp. *lactis*. As can be seen from the results, in the process of lyophilization, the number of viable cells changed slightly. The highest level of cell survival was observed in strain 194, which was 88%, in strain K-205-67%, TM-2 and the lowest - in strain 729 - 55% ($P<0{,}05$).

With periodic reseeding of lactococci, the number of viable cells is 3 orders of magnitude lower than when these cultures were stored in a lyophilic state, the ability of lactic acid accumulation of this group of homoenzymatic lactococci and their antimicrobial activity was significantly reduced. When studying the spectra of the antimicrobial action of strains of *L. lactis* subsp. *lactis* before and after lyophilization (Table 4), there was a slight decrease in activity in lyophilized cultures, which may be due to damage to nucleic acids, intracellular proteins, peptides during lyophilization, or with accumulation in the population inactive dissociants,[10] which can occur during suspended culture (lyophilized storage), elimination of plasmids during prolonged storage of *L. lactis* during fasting.

Table 3 Change in the viability of strains of *L. lactis* subsp. *lactis* after lyophilization and recovery

Strains	Number of cells/ml	
	Before lyophilization	After lyophilization
194	$1{,}9\times10^{10}$	$1{,}3\times10^{10}$
K-205	$1{,}8\times10^{10}$	$8{,}6\times10^{9}$
TM-2	$1{,}7\times10^{10}$	$0{,}9\times10^{10}$
729	$1{,}6\ x10^{9}$	$0{,}8\ x10^{9}$
IR-4	$9{,}8\ x10^{9}$	$8{,}5\ x10^{9}$

Table 4 Antimicrobial spectrum of action of *Lactococcus lactis* subsp. *lactis* strains of before and after the lyophilization process

Test culture	TM-2		IR-4		194		K-205		729		Nisaplin
	before	after	before	after	before	after	before	after	before	after	
	The diameter of the zones of growth suppression, mm										
Bacillus mycoides	15,0	13,0	16,0	14,0	19,5	18,0	19,5	14,0	12	12	17,0
Bacillus subtilis	12,0	9,0	19,0	16,5	22,0	19,0	22,0	18,0	120	11	19,0
Bacillus coagulans	16,0	13,0	17,0	16,0	23,0	21,5	22,0	18,5	9	8	21,0
Bacillus cereus	14,5	13,0	18,0	16,0	21,0	18,0	21,0	18,0	11	10	18,0
Micrococcus luteus	19,0	17,0	20,0	19,0	22,5	20,0	19,5	16,0	11	11	21,5
Staphylococcus aureus	16,0	13,5	16,0	15,0	20,0	19,0	20,0	17,5	0	0	15,0
Alcaligenes faecalis	0	0	12	10	14,5	14,0	12,5	10,0	0	0	0
Escherichia coli	16	9,0	16	16	15,0	12,0	14,0	12,0	10	10	0
Proteus vulgaris	14	15	0	0	16,5	13,0	16,0	14,0	0	0	0
Pseudomonas aeruginosa	10	10	0	0	16,5	12,0	15,5	12,0	0	0	0
P. fluorescens	10	10	10	10	18,0	12,5	16,0	12,0	0	0	0
Candida albicans	10	10	10	10	16,5	18,0	10,0	12,0	0	0	0
Penicillum chryzogenum	10	10	10	10	17,5	19,0	10,5	12,0	0	0	0
Aspergillus niger	18	18	14	13	21,0	16,0	10,0	11,0	9	0	0

However, after the third passage of cultures in MRS or inoculum, the physiological and biochemical activity of the studied strains was completely restored: a dense milk clot formed after 10 hours of incubation, titratable acidity increased, antimicrobial activity reached its initial level, as before lyophilization. The results of the study showed that the most effective method of preserving bacteriocin-forming lactococci is lyophilization using a complex protective environment of the composition (g/l): sucrose-100, gelatin-10, monosodium glutamate-10, sodium citrate-5, which ensures long-term crop life. The sucrose was used as lyoprotectant since this sugar is currently used as standard in bacterial freeze drying.[11]

For the full restoration of the physiological and biochemical properties of lyophilized cultures, a stage of growing them in an elective environment specific for each microorganism is necessary. After lyophilization, the reconstituted strain TM-2 isolated from cottage cheese had a minimum fungicidal activity of 171.20 u/mL (by Nystatin), and the remaining strains were more active (262.81 u/mL). However, strain TM-2 was most active on *Aspergillus niger* (518.01 u/mL) after 12 hours of cultivation; strain 194 was the most active on *Candida albicans* yeast.

When lactococci recovered in MRS medium after lyophilization using a protective medium containing sucrose-100, gelatin-10, monosodium glutamate-10, sodium citrate-5, antimicrobial activity was revealed for Gram-positive bacteria (on the example of *M. luteus*), gram-negative (according to *E. coli*), the unique ability of lactococci to synthesize fungicidal metabolites was also confirmed (Figure 2).

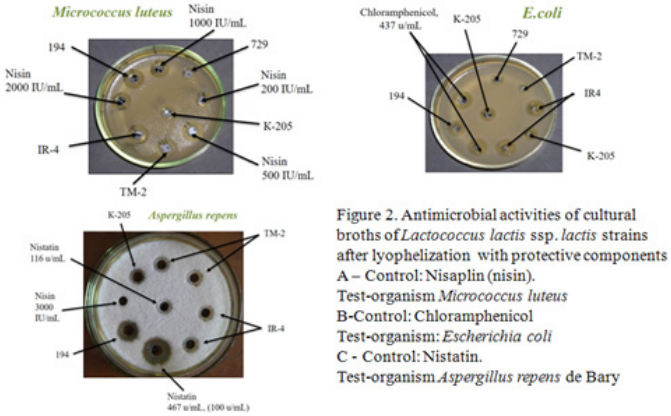

Figure 2. Antimicrobial activities of cultural broths of *Lactococcus lactis* ssp. *lactis* strains after lyophelization with protective components
A – Control: Nisaplin (nisin).
Test-organism *Micrococcus luteus*
B-Control: Chloramphenicol
Test-organism: *Escherichia coli*
C - Control: Nistatin.
Test-organism *Aspergillus repens* de Bary

Conclusion

So, the introduction of *L. lactis* ssp. *lactis* into the intestinal microbiota leads to the replacement of pathogens and is useful for people with lactase deficiency. Strains enrich the dairy product with biologically active substances, which, in addition to the nutritional effects, have a beneficial effect on health. Lyophilization in combination with pre-treatment of cells contributes to the preservation of their probiotic potential. The results of the study showed that the most effective method of preserving bacteriocin-forming lactococci is lyophilization using a complex protective environment of the composition (g/l): sucrose-100, gelatin-10, monosodium glutamate-10, sodium citrate-5, which ensures long-term crop life. For the full restoration of the probiotic potential of lyophilized cultures, a stage of growing them in an elective environment specific for each microorganism is necessary.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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