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VIABILITY AND ANTAGONISM OF CRYOPRESERVED LACTIC ACID BACTERIA

Kaiyrtay Kh. Almagambetov, Zinigul S. Sarmurzina, Anzhela A. Chshelkina, Daniyal Sh. Zhakenov, Bakhytgul J. Sadykova, Gulmira N. Bisenova, Serik M. Shaikhin

LLC "Republican collection of microorganisms", Astana, Republic of Kazakhstan

Corresponding author: Kaiyrtay Kh. Almagambetov, e-mail: rcmkz.list.ru
Co-authors: Zinigul S. Sarmurzina, e-mail: sarmurzina@list.ru
Anzhela A. Chshelkina, e-mail: ashhelkina@mail.ru
Daniyal Sh. Zhakenov, e-mail: zhakenov.daniyal@mail.ru
Bakhytgul J. Sadykova, e-mail: bakhytgul.sadykova130384@mail.ru
Gulmira N. Bisenova, e-mail: bissenova84@mail.ru
Serik M. Shaikhin, e-mail: rkm_shaikhin@mail.ru

Abstract

Preserving and advancing bioresources involving industrial microorganisms is of paramount importance for every nation. However, long-term storage of these strains often leads to diminished viability and biological activity. Thus, it is crucial to investigate the properties of cryopreserved strains stored at -80°C in a 10% glycerol solution within low-temperature refrigerators. This study aimed to comparatively analyze the viability of 129 lactic acid bacteria strains, including *Lactobacillus sp*., *Lactococcus sp*., and *Pediococcus sp*., cryopreserved from 2006 to 2020. Among them, 93 *Lactobacillus sp*. strains were categorized into three groups based on storage dates (2006-2007, 2013-2014, and 2017-2020). Viability titers were determined using the standard serial dilution method, counting microorganisms in CFU/ml. Regardless of the storage duration or species affiliation, the study identified lactic acid bacteria strains exhibiting both high (107 - 109 CFU/ml) and low (104-106 CFU/ml) viability titers. Additionally, the antagonistic activity of 33 *Lactobacillus sp*. strains was investigated using the delayed antagonism method, subdividing them into 17 strains with sufficiently high viability titers and 16 strains with low viability titers. The results revealed that 20% of strains with high viability titers and 27% with low viability titers exhibited relatively high antagonistic activity (with a zone of inhibition ranging from 10 to 18 mm). In both groups, strains with low antagonistic activity (with a zone of inhibition measuring 5-9 mm), particularly against Gram-positive and Gram-negative bacterial test-cultures, predominated. Significantly, 51% of lactobacilli strains demonstrated pronounced antagonism against *Candida albicans* ATCC- 885-653 test-culture. These findings underscore the practical importance of the study, emphasizing the necessity to analyze and select optimal concentrations of intracellular or extracellular cryopreservatives and to determine the initial viability

titer when storing strains for cryopreservation. Tailoring cryopreservation solutions to each strain can enhance the preservation of their original properties, ultimately improving overall preservation quality.

Key words: Antagonism; cryopreservation; lactic acid bacteria; viability.

Introduction

The development and use of probiotics in young farm animals and poultry to increase resistance to intestinal infection, and immunomodulatory effect is actual in veterinary medicine [1]. Lactic acid bacteria, especially lactobacilli, are most often used as probiotic microorganisms.

Therefore, the preservation and replenishment of collections of lactic acid bacteria are necessary for the development of modern effective probiotic preparations based on them. More than 130 strains of lactobacilli of various species isolated from human organisms, fermented milk products, vegetation, etc. are deposited in the biobank of the Republican Collection of Microorganisms. The main task of the biobank of industrial microorganisms is to preserve the viability and original biological properties of deposited strains of lactobacilli. Long-term storage of lactobacilli strains in the biobank is carried out by cryopreservation at minus 80°C in 10% glycerol solution.

Cryopreservation is one of the most widely used methods for long-term storage of microorganisms [2]. Eukaryotic and prokaryotic microorganisms quite stably retain viability and original biological properties both under conditions of cold stress and vacuum drying.

At the same time, it is known that during cryopreservation microbial cells are exposed not only to low temperatures but also to damaging physicochemical factors arising from water phase transitions, such as ice crystal formation, changes in the pH of the medium, and significantly changes intra- and extracellular osmotic and oncotic concentration gradients. Freezing and thawing with intracellular ice crystals and high concentrations of intra- and extracellular ingredients damage membrane structures. Free radicals and peroxide compounds formed as a result of oxidative reactions also disrupt the structure of polymers within cellular and intracellular membranes - lipids and proteins, and damage nucleic acids.

Analysis of literature data on the determination of viability of cryopreserved lactobacilli has shown the following. Some studies show stable preservation of viability and biological activity by lactobacilli under cryopreservation conditions [3, 4]. In other studies, it was revealed that some strains, regardless of the species affiliation of lactobacilli, lost their viability under conditions of low-temperature stress, their lgKOE/ml decreased by 1 - 2 orders of magnitude [5]. While some studies revealed better survival of bacterial cultures in case of slow cooling of cells [6], others showed that, on the contrary, at low cooling rates, most of the cells die at the freezing stage, possibly due to prolonged exposure to cold osmotic stress [7].

Preservation of the antagonistic activity of lactobacilli is a necessary condition for their use in the development of probiotic preparations [8]. It is conditioned by the production of organic acids (lactic, acetic), hydrogen peroxide, and lactocin production [9, 10].

It is known from the literature that the antagonistic activity of lactobacilli does not depend on their species affiliation and may differ between different strains within a species [11].

The antagonistic activity of lactobacilli to Gram-negative Enterobacteriaceae is well known and is the basis for the use of lactic acid bacteria in dysbiotic conditions as probiotics, both in medicine and veterinary medicine. At the same time, concerning yeasts of the genus Candida, several studies have revealed both high inhibitory activity and its absence in lactic acid bacteria. In vitro, co-cultivation showed a mutual inhibitory effect between *Candida albicans* and *Lactobacillus plantarum*. The authors of the study explain this by competition for nutrients and colonization of the epithelial mucosa of the oral cavity and vagina [12, 13]. Also, the co-cultivation of the production strain of lactobacilli and Candida on a solid nutrient medium revealed a significant decrease in the number of colonies for all *Candida albicans* strains taken in the experiment compared to the control [14].

Preservation of viability of cryopreserved lactobacilli is not a guarantee of preservation of their antagonistic activity. Therefore, this study aimed to analyze both the viability and antagonistic activity of lactobacilli strains cryopreserved in a biobank. Commonly used methods for determining viability by serial dilutions [15, 16] and antagonistic activity of lactic acid bacteria against test cultures [17, 18, 19] were used.

Materials and Methods

The study was conducted at the Republican Collection of Microorganisms (RCM) biobank between April 2022 and June 2023. Microbiological procedures were performed within controlled environments using nutrient media, reagents, and light microscopy to examine Gram-stained micro-specimens. The research materials comprised cryopreserved lactic acid bacteria strains sourced from fermented dairy products, human and animal intestines, and plant surfaces, all stored in low-temperature refrigerators at -80°C.

Lactobacilli, lactococci, and paediococci were used for the determination of viability titre. The viability titre of 93 strains of lactobacilli represented by 13 species (*L. casei*, *L. brevis*, *L. fermentum*, *L. plantarum*, etc.), 19 strains of lactococci (*L. lactis*, *L. diacetilactis*) and 17 strains of paediococci (*P. pentosaceus*) was studied.

Thirty-three cryopreserved strains of lactobacilli, including *L. casei* - 9, *L. acidophilus* - 6, *L. fermentum* - 6, *L. plantarum* - 5, *L. brevis* - 4, *L. cellobiosus* - 2 and *L. pentosus* - 1 strain were used to determine antagonism.

The following test cultures were used to evaluate the antagonistic activity: *Escherichia coli* 157,

Staphylococcus aureus 209 P, *Serratia marcescens* 221 F, *Salmonella typhimurium* TA 98 and *Candida albicans* ATCC- 885-653.

The following nutrient media were used for the cultivation of lactic acid bacteria and test cultures: MRS-1, MRS-4, Sabouraud, Czapek, Endo, MPA, MPB.

To determine the viability titre, the serial dilution method was used, which is a series of serial dilutions, each with the same dilution factor, with the diluted material from the previous step being used for the subsequent dilution. The number of bacteria present in the original sample was calculated by multiplying the number of colonies formed by the dilution factor, in CFU/ml.

The antagonistic activity of lactobacilli was studied by a modified delayed antagonism method - well diffusion method. The method is based on the diffusion of antibiotic substances formed by the tested strains of lactobacilli into the agar medium containing the test culture and inhibiting the growth of the latter. A 200 µl daily culture of lactobacilli grown on MRS-1 was placed in the volume of 200 µl into wells cut in the thickness of dense nutrient medium with the sowing of the test culture.

Results

Viability titres of cryopreserved lactic acid bacteria - lactobacilli, lactococci, and paediococci - were studied in a comparative aspect (Table 1). In 73.5% of the studied strains of lactobacilli, the viability titres were quite high ($4.1x10^7$ - $4.1x10^9$ CFU/ml), and low titres ($2.0x10^4$ - $4.8x10^6$ CFU/ml) were found in 26.5%. Among cryopreserved lactococci and paediococci, strains with viability titres of 10^7 - 10^9 CFU/ml were also predominant (in 79% and 94%, respectively). Low viability titres were detected in 21% of lactococci and 6% of paediococci.

Table 1 - Viability titre of lactic acid bacteria cryopreserved in 2006-2020.

Number of lactic acid bacteria (n, %)	Viability titre (CFU/ml, %)	
Lactobacillus sp. n - 93	$2,0×10^4$ - $4,8×10^6$ 26,5%	$4,1×10^7$ - $4,1×10^9$ 73,5%
Lactococcus sp n- 19	$1,0×10^4$ - $7,4×10^6$ 21%	3,5×107 - 1,2×109 79%
Pediococcus sp. n-17	$1,0×10^4$ - $6,2×10^6$ 6%	$2,9×10^7$ - $1,6×10^9$ 94%

The viability titres of 93 strains of lactobacilli were studied separately in a comparative aspect by years of cryopreservation (Table 2). The viability titres of 3 groups of lactobacilli cryopreserved in 2006-2007 (37 strains), 2013-2014 (32 strains), and 2017-2020 (22 strains) were studied.

Table 2 - Viability titre of lactobacilli cryopreserved 2006-2020

Number of lactobacilli (n, %)	Viability titre (Mcr, CFU/ml, %)	
2006 - 2007 гг. n-39	$1,0\times10^4$ - $3,9\times10^6$ 25,6 %	$4,9\times10^7$ - $4,2\times10^9$ 74,4%
2013 - 2014 гг. n-32	$2,0\times10^4$ - $4,6\times10^6$ 37,5%	$3,0\times10^7$ - $2,7\times10^8$ 62,5%
2017 - 2020 гг. n-22	$1,0\times10^4$ - $8,0\times10^6$ 22,7%	$3,9\times10^7$ - $2,1\times10^9$ 77,3%

Comparative analysis of the viability titre of 93 strains of lactobacilli represented by 13 species (*L. casei*, *L. brevis*, *L. fermentum*, *L. plantarum*, etc.) cryopreserved in different years showed the following. Out of 39 strains of lactobacilli cryopreserved in 2006, 29 (74.4%) showed sufficient viability titre (10^7 - 10^9 CFU/ml), and low titre (10^4 - 10^6) was detected in 10 strains (25.6%). The viability titer of lactobacilli cryopreserved in 2013 - 2014 in 20 cases (62.5%) was 10^7 - 10^8 CFU/ml, and in 12 (37.5%) - 10^4 - 10^6 CFU/ml. In the third group of lactobacilli cryopreserved in 2017 -2020, 16 (77.3%) of the 22 strains studied showed medium viability titre (10^7 - 10^9 CFU/ml). Low viability titre (10^4 - 10^5 CFU/ml) was detected in 5 strains (22.7%).

Figure 1 shows the result of determining the viability titre of L. plantarum 8RA-3pl+(15), it is equal to 6×10^8 CFU/ml. While in *Streptococcus lactis* AMS-23 (0048) and *Streptococcus cremoris* K-1 (0049) titres do not exceed 1×10^5 CFU/ml (Fig.2).

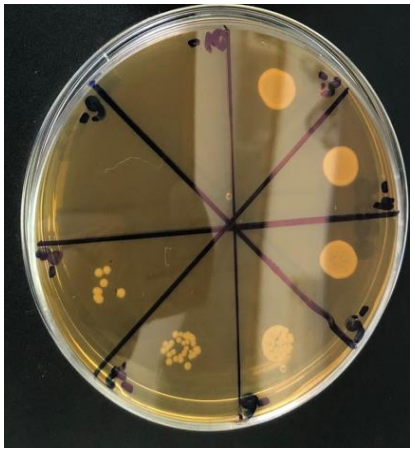


Figure 1 - Viability titre
L. plantarum 8RA-3pl+ (15) (0048)

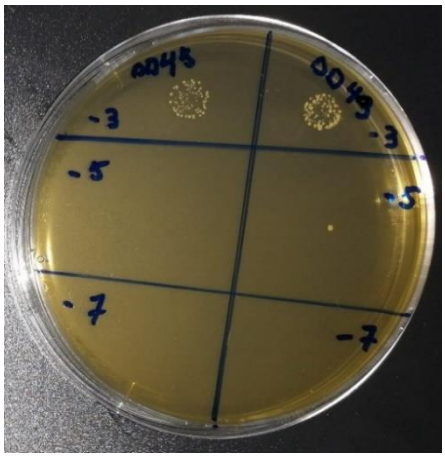


Figure 2 - Viability titres of *Str. lactis* and *Str. cremoris* (0049)

Along with the preservation of viability (10^7 - 10^9 CFU/ml), it is important to preserve the antagonistic activity of cryopreserved lactobacilli strains. Therefore, the antagonistic activity of 33 lactobacilli strains was investigated, including *L. casei* - 9, *L. acidophilus* - 6, *L. fermentum* - 6, *L. plantarum* - 5, *L. brevis* - 4, *L. cellobiosus* - 2

and *L. pentosus* - 1 strain. The above strains cryopreserved in the RKM biobank in 2002 - 2020 were divided into 2 groups according to the value of viability titre: 16 strains with low (10^4 - 10^6 CFU/ml) and 17 with relatively high (10^7 - 10^9 CFU/ml) number of viable microbial cells. The results of the study of the antagonistic activity of cryopreserved strains of lactobacilli with high and low viability titre are shown in Table 3.

Table 3 - Antagonistic activity of cryopreserved lactobacilli

Groups studied, number of strains (n)	Growth suppression zone size (mm)		
	10-18 mm	5-9 mm	1-4 mm
High viability titre, n - 17	20,0%	42,3%	47,7%
Low viability titre, n - 16	27,5%	30,0%	42,5%.

In our studies, the highest antagonistic activity towards test cultures (10 - 18 mm) was found in 20.0% of lactobacilli strains with relatively high viability titre and 27% with low titre. Low antagonistic activity (1 - 9 mm) was shown by 80% of strains with relatively high viability titre and 72.5% with low titre.

The antagonistic activity of the above 33 cryopreserved lactobacilli strains to each of the test cultures was also analyzed separately. The results of determining the antagonistic activity of the studied strains of lactobacilli to each of the test cultures are given below (Table 4).

Table 4 - Antagonistic activity of cryopreserved Lactobacillus strains to test cultures

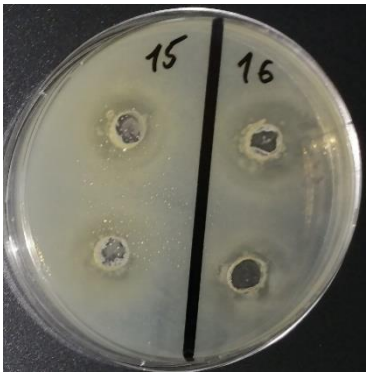
Test cultures	Number of lactobacilli strains (n) by zone of inhibition		
Zone of inhibition	10 - 18 mm	5 - 9 mm	1 - 4 mm
S. aureus 209P	4	15	14
S. marcescens 221 F	3	13	16
S. typhimurium TA98	1	15	17
E. coli 157	6	12	15
C. albicans ATCC-885	17	4	12

There is a significant difference in the frequency of antagonistic activity of cryopreserved lactobacilli strains to gram-negative and gram-positive bacterial test cultures on the one hand and to fungi of the genus Candida on the other. Expressed antagonism (10 - 18 mm) to *Escherichia coli* 157 was detected in 6 strains, to *Staphylococcus aureus* 209 P in 4 strains, to *Salmonella typhimurium* TA 98 in 2 strains, to *Serratia marcescens* 221 F in 3 strains, and to *Candida albicans* ATCC- 885-653 in 17 strains. If to bacterial test-

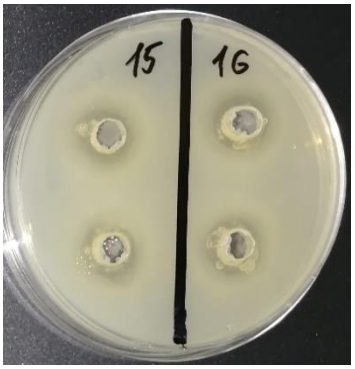
cultures the expressed antagonism (more than 10 mm) was revealed only in 6 - 18%, then to yeasts of Candida genus in 51% of cryopreserved strains of lactobacilli.

Figure 3 shows the results of the antagonistic activity of cryopreserved *L. plantarum* 8RA-3pl+ and *L. plantarum* 8RA-3pl- strains to test cultures. The antagonism to gram (+) and gram (-) bacterial test cultures is low but quite pronounced against fungi of the genus Candida.

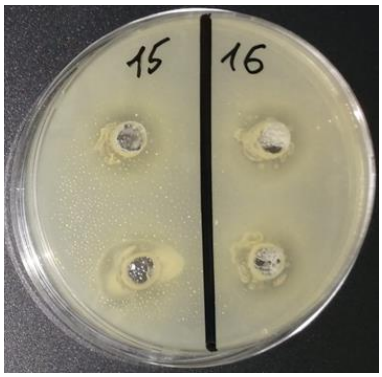
C. albicans ATCC- 885-653



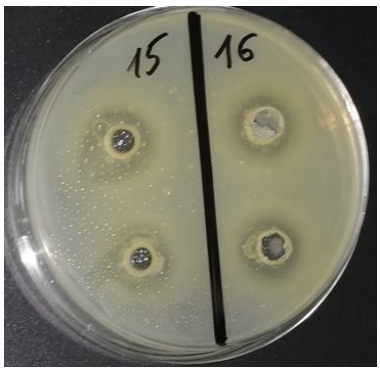
S. aureus 209 P



E. coli 157



S. marcescens 221 F



S. typhimurium TA 98

Figure 3 - Antagonistic activity of *L. plantarum*8RA-3pl+ (15) and *L. plantarum* 8RA-3pl-(16) to test cultures

Discussion

A study of the viability of 129 strains of lactic acid bacteria (*Lactobacillus sp*., *Lactococcus sp*., and *Pediococcus sp*.) cryopreserved between 2006 and 2020 revealed that microorganisms with high and low viability titres were equally found regardless of the storage dates. A separate study of the viability of 93 strains of *Lactobacillus sp*. divided into 3 groups according to storage dates (2006 - 2007, 2013 - 2014, and 2017 - 2020) also showed that the titre value did not depend on the duration of their storage in low-temperature conditions. Comparative analysis of the viability titre of cryopreserved lactobacilli of different species (*L. casei*, *L. fermentum*, *L. plantarum*, etc.) did not show any differences in titre depending on their species affiliation. This pattern was not only observed between different species of lactobacilli but also among strains within species. Thus, this study shows that there is no significant difference in the titres of viability and antagonistic activity between different taxonomic groups of lactic acid bacteria. At the same time, a difference in these indicators was found between strains within species. Such dynamics of viability and antagonism indicators may be due to different resistance to cold stress among strains of lactic acid bacteria.

The results obtained are consistent with other studies on the identification of cryo-resistant strains, on the determination of cryopreservation modes and concentrations of intra- and extracellular cryoprotectants necessary for optimal preservation of biological properties of lactic acid bacteria [11, 14, 20].

It is of practical interest that the study of antagonism of 33 strains of cryopreserved lactobacilli to test cultures revealed relatively high activity against fungi of the genus Candida, to a lesser extent against Gram (+) and Gram (-) bacteria. Thus, strains of lactobacilli with both low and relatively high titre of viability only in 20 -27.5% of cases showed pronounced antagonism to test cultures. However, in relation to *C. albicans*, more than half of the studied strains (51%) showed pronounced antagonism.

Conclusions

Viability titre values of lactic acid bacteria strains cryopreserved in different years are not related to storage duration.

No regular relationship between the antagonistic activity of the strains studied and the value of their viability titre was revealed. Cryopreserved strains of lactic acid bacteria can have rather high antagonistic activity at low viability titer and vice versa.

The antagonistic activity of the studied strains of lactobacilli is high in relation to *C. albicans* ATCC- 885.

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