

Nonculturable forms of bacteria in lyophilized probiotic preparations

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Submission date: August 3, 2013; Acceptance date: February 2, 2014; Publication date: February 9, 2014

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ABSTRACT

Background: Nonculturable cells are formed under stress. These viable but nonculturable (VBNC) cells retain the ability to revert to active growth and division when conditions become favorable, or after treatment with resuscitating factors. Information about the possible presence of VBNC in bacterial lyophilized probiotic preparations, foodstuffs, live vaccines, etc., indicates that human as well as animal intestines are a significant area for research.

Methods: Samples were stored for different periods of time (up to 30 years) according to the manufacturers’ manuals. Total counts were conducted using the Goryaev-Thoma counting chamber and actual viability was assessed by luminescence microscopy after staining with Live/Dead® (Baclight™). CFU/ml counts were made using solid or semisolid media. Viable cells that lacked the ability to form colonies were considered VBNC.

Results: We studied 11 batches of commercial probiotics (Russia) from different sources, containing lyophilized *E. coli*, lactobacilli, or bifidobacteria, in ampoules or vials. In *E. coli* preparations, depending on storage periods, the amounts of VBNC varied from 4.1% (3 years) to 99.7% (30 years) and showed different total viability (52.2 – 91.3%), as well as the percentage of VBNC cells. A different sample that had been expired for 11 years was 79.5% NC. It is also noteworthy that the 5-dose vials, 4 years past expiration, from yet another source, showed a higher amount of VBNC cells (85.5%). Two different batches that had been expired for three years contained 4.1 and 21.3% VBNC cells. 4 of the 5-dose vials of lyophilized lactobacilli were not expired and contained 58.8 – 80.4% VBNC cells. Total viability varied from 92.9 to 100%, and there was an unmistakable positive correlation between total viability and culturability. The last batch, which had expired 6 years earlier, has 23.7% viable cells and about 98% VBNC. Non-expired bifidobacterial samples contained 70.7 and 95.5% of viable cells and were 50 and 100% culturable.

Conclusion: We demonstrated the presence of VBNC cells in lyophilized probiotic preparations

that contained live bacteria. Probiotics stored past their expiration date may retain a high potential medical effect because they contained high numbers of viable cells. VBNC cells in studied preparations may have the potential to return to an actively growing state.

Keywords: nonculturable forms of bacteria, probiotics

INTRODUCTION:

In adverse conditions of natural or artificial habitats (exhaustion of nutrient supplies and energy, non-optimal levels of temperature influence, oxygenation, influence of chemicals, toxins of the organic nature, sharp changes in pH, exposure to white light, osmotic stress, etc.), non spore-forming microorganisms are exposed to stress that results in the formation of nonculturable forms of bacteria [1-3; 5-6, 8-12, 16-17, 19-20]. Thus, cells that have left the proliferative cycle retain the potential to return to the active growth and reproduction phase [14]. The term "viable, but nonculturable" was assigned to conditions where the adverse conditions were reversible (viable but nonculturable – VBNC) [8, 9]. VBNC cells have entered a “state of dormancy triggered by harsh environmental conditions” [16, 21]. Identification of the microorganisms in nonculturable states: in the environment, in humans, in animals, and in foodstuff, etc., is an important area of microbiological research. Apart from simply being viable and culturable or dead, cells can also be dormant (a state in which they are inactive but ultimately culturable) or active but nonculturable (as is the case with VBNC cells) [15].

Dormant conditions have different degrees of depths. In the presence of some stressful circumstances, the metabolism is slowed [1]; however, it can be measured and tested for [17]. At other conditions, the metabolism completely stops. For example, in a stationary phase, or in conditions of proliferative rest, cells keep metabolic activity [1, 2, 3, 10]. This metabolic activity exists in the form of respiration or fermentation [16-17, 22-23]. Furthermore, cells may incorporate radioactive substances and have active protein synthesis [17]. While in other types of dormancy, there is a gradual slowing of metabolism [3, 4, 6, 9, 12]. Ametabolism (anabiosis, cryptobiosis, abiosis) [1, 13] is a condition in which a cell's metabolic processes have completely stop. However, ametabolism can be reversed under favorable conditions. Characteristics of a VBNC state include maintaining apparent cell integrity, having low metabolic activity, having reduced nutrient transport [15-18], exhibiting dwarfing, possessing apparent capacity to regain culturability in vivo [15-17], and higher autolytic capability than exponentially growing cells [17],

Depending on the factors inducing the transition to a dormant condition, the dormancy can either be classified as endogenous or exogenous [1, 13]. Endogenous dormancy is part of the natural cycle of development and is caused by a cell's internal mechanisms in reaction to stressful influences from its habitat. Dormant cells of this kind include bacterial and fungal spores, etc. [12, 14].

Exogenous rest is connected to the influence of external factors, such as the conservation of osmotically active agents (osmobiosis) such as salts, sucrose, etc., freezing (cryobiosis), and dehydrating (anhydrobiosis) in solutions. In our opinion, it is necessary to consider the lyophilization process of combining cryobiosis and anhydrobiosis, as well as the influence of low and high temperatures in conjunction with this type of dormancy [2].

Dormant nonculturable cells possess an increased resistance to damaging factors. For example, endospores are often able to withstand environmental factors such as heat and extreme pH, exhibiting a “global resistance for prolonged periods of time” to such adverse conditions [16, 24-25]. However, upon the reversion from dormant forms to metabolic activity, the cells that are most adapted (a serotype, a clone, etc.) to changed environmental conditions can benefit from this advantage and dominate. Preservation of both viability and the possibility of returning to the active division can be estimated after millions of years [3]. However, there is an assumption that the formation of VBNC and the loss of the ability to form colonies is a stage of cell population decline [7]. It should be noted that cells incapable of forming colonies on standard nutrient media often have microscope-confirmed structural integrity. Furthermore, return from an endospore state to an active metabolic state in spore-forming bacteria and fungi can require multiple factors, sometimes interacting in a specific order. Some VBNC bacterial cultures have exhibited similar requirements [16, 18]. It is therefore possible that some VBNC cultures require a more complex media to be resuscitated [16-18]. Some studies have shown that the retained metabolic activity of VBNC cells includes the ability of pathogenic cells to remain virulent in a VBNC state [17, 26-27]. As it is known, microorganism colonies can be formed by more than one cell type, the biological activity of preparations connected with live bacteria (probiotics, vaccines, dietary supplements, etc.) is underestimated when only the CFU/ml is measured. Therefore, a question of studying the viability potential of lyophilized cells in long stored preparations not through CFU/ml, but directly through the calculation of living cells, is actually the purpose of the research: to find VBNC among lyophilized cells of industrial probiotics.

A popular method of determining viability of a culture is plate counting, which requires proliferation of a culture on a nutrient medium [15, 28]. However, studies have shown a “striking difference” between plate count results and L/D count results (which use bioactive compounds to designate live and dead cells), as well as the possibility that a more complex medium may be required to return VBNC cells to a culturable state [17-18]. One study has suggested that plate count methods only accurately count cells which are viable and culturable on agar [15], which can cause issue due to the fact that less than 1% of microorganisms in natural water and soil samples can be cultured in such a way [17, 29]. Although traditional laboratory culture conditions and methods often cannot meet the requirements of VBNC organisms to resume growth, being once again exposed to a favorable environment has shown the potential for VBNC cells to maintain significant activity and even resume proliferation [16-18]. Therefore, examining the true count of viable cells (both active and VBNC) could lead to much more accurate information about the functionality of certain foodstuffs.

METHODS:

The objects of research in this study were preparations of probiotics manufactured in vials and ampoules (preparations codes: Colibacterin - CB, Bificol - BFC, Bifidumbacterin - BB) produced by “Mechnikov BioMed” (the Moscow Region), “IMBIO” (Nizhny Novgorod), and “Mikrogen” (Stavropol) that were either non-expired or expired for different periods of time. These preparations were stored in a refrigerator according to the manufacturer’s instructions. Characteristics of probiotics can be seen in table 1.

The total number of bacterial cells was counted in a Goryaev-Thoma counting chamber under a microscope ("Mikmed-5" of LOMO) with the magnification set on 320 (8×40). For the visual viability assessment, cells were stained with a commercial kit of bioluminescent dye Live/Dead® (Baclight™) under a luminescent microscope (Zeiss, Germany). Viable microorganisms with intact membranes had green luminescence, while dead microorganisms with damaged cytoplasmic membranes were luminesced red.

Table 1 Characteristics of probiotic preparations used in this study.

№	Trademark	Contents	Manufacturer	Place of origin	Batch codes, expiration date (year)	Type of vessel, number of doses
1	Colibacterin	<i>Escherichia coli</i> M-17	NIIVS	Perm, Russia	CB 72-5 (1982)	Ampoules, 3 doses
2	Colibacterin	<i>Escherichia coli</i> M-17	IMBIO	Nizhny Novgorod, Russia	CB 70-3 (2001)	Ampoules, 3 doses
3	Colibacterin	<i>Escherichia coli</i> M-17	Mechnikov Biomed	Moscow region, Russia	CB 4-3 (2008)	Vials, 5 doses
4	Colibacterin	<i>Escherichia coli</i> M-17	Mechnikov Biomed	Moscow region, Russia	CB 24-3 (2009)	Vials, 5 doses
5	Colibacterin	<i>Escherichia coli</i> M-17	Mechnikov Biomed	Moscow region, Russia	CB 27-3 (2009)	Vials, 5 doses
6	Bificol	<i>Bifidobacterium bifidum</i> , <i>Escherichia coli</i> M-17	Mechnikov Biomed	Moscow region, Russia	BFC 20-1 2007	Vials, 5 doses
7	Bifidumbacterin	<i>Bifidobacterium bifidum</i> №1, 791	FGUP "NPO Mikrogen"	Stavropol, Russia	BB 44 (2012)	Vials, 5 doses
8	Bifidumbacterin	<i>Bifidobacterium bifidum</i> №1, 791	FGUP "NPO Mikrogen"	Stavropol, Russia	BB 56 (2013)	Vials, 5 doses
9	Lactobacterin	<i>Lactobacillus plantarum</i> or <i>L. fermentum</i>	Mechnikov Biomed	Perm, Russia	LB-55 (1994)	Ampoules, 5 doses
10	Lactobacterin	<i>Lactobacillus plantarum</i> 8R-A3 or 38 or <i>L. fermentum</i> 90T-S4 or 39	FGUP "NPO Mikrogen"	Perm, Russia	LB-80 (2010)	Vials, 5 doses
11	Lactobacterin	<i>Lactobacillus plantarum</i> 8R-A3 or 38 or <i>L. fermentum</i> 90T-S4 or 39	FGUP "NPO Mikrogen"	Perm, Russia	LB 93 (2013)	Vials, 5 doses

Determination of CFU/ml value for the cells of probiotic preparations was carried out by the standard plating of diluted suspensions of probiotics on a solid nutrient agar and Endo medium (*E. coli*) and on a semi-solid "Bifidum-medium" (for cultivation and allocation of Bifidumbacteria, produced by FGUN "State Scientific Center of Applied Microbiology" Obolensk). Nonculturable but viable forms of bacteria were revealed through the comparison of the total

number of cells, the number of viable cells, and the number of bacteria that did not form a colony, per 1 ml. Experimental data was statistically analyzed. Mean values and their standard deviations (I95) were calculated (p 0,05).

RESULTS:

Colibacterin

The initial stage of our research was to assess the levels of viability of lyophilized bacteria in commercial probiotic preparations. The data obtained in these experiments is presented in table 2.

Table 2. Studying of Colibacterin samples in various periods of storage

Characteristics of Colibacterin		Biological parameters			
Batch codes, expiration date (year)	Type of vessel, number of doses	Total number of cells in Goryaev-Thoma camera (cells/ml) (I ₉₅) p value	Live cells with "Live/Dead" (%)	CFU/ml (I ₉₅) p value	VBNC (%)
			Total number of live cells/ml with "Live/Dead" (I ₉₅) p value		
1	2	3	4	5	6
CB 72-5 1982	Ampoules, 3 doses	2,11±0,23×10 ¹⁰ (1,56÷2,67)×10 ¹⁰ 3 and 4<0,05 3 and 5<0,05	52,2	2,89±0,32×10 ⁷ (2,11÷3,65)×10 ⁷ 5 and 3<0,05 5 and 4<0,05	99,73
			1,1±0,12×10 ¹⁰ (0,81÷1,39) ×10 ¹⁰ 4 and 3<0,05 4 and 5<0,05		
CB 70-3 2001	Ampoules, 3 doses	3,11±0,34×10 ¹⁰ (2,29÷3,93)×10 ¹⁰ 3 and 4>0,05 3 and 5<0,05	86,7	5,55±0,61×10 ⁹ (4,09÷7,01)×10 ⁹ 5 and 3<0,05 5 and 4<0,05	79,5
			2,7±0,3×10 ¹⁰ (1,98÷3,42) ×10 ¹⁰ 4 and 3>0,05 4 and 5<0,05		
CB 4-3 2008	Vials, 5 doses	1,21±0,13×10 ¹⁰ (0,89÷1,52)×10 ¹⁰ 3 and 4>0,05 3 and 5<0,05	90,3	1,58±0,17×10 ⁹ (1,17÷1,99)×10 ⁹ 5 and 3<0,05 7 and 4<0,05	85,5
			1,091±0,12×10 ¹⁰ (0,80÷1,38)×10 ¹⁰ 4 and 3>0,05 4 and 5<0,05		
CB 24-3 2009	Vials, 5 doses	1,44±0,15×10 ¹⁰ (1,08÷1,8)×10 ¹⁰ 3 and 4>0,05 3 and 5>0,05	88,2	1±0,11×10 ¹⁰ (0,74÷1,26)×10 ¹⁰ 5 and 3>0,05 5 and 4>0,05	21,3
			1,27±0,14×10 ¹⁰ (0,90÷1,58)×10 ¹⁰ 4 and 3>0,05 4 and 5>0,05		
CB 27-3 2009	Vials, 5 doses	1,32±0,14×10 ¹⁰ (0,98÷1,66)×10 ¹⁰ 3 and 4>0,05 3 and 5>0,05	91,3	1,16±0,13×10 ¹⁰ (0,85÷1,47)×10 ¹⁰ 5 and 3>0,05 5 and 4>0,05	4,13
			1,2±0,13×10 ¹⁰ (0,89÷1,51)×10 ¹⁰ 4 and 3>0,05 4 and 5>0,05		

As it appears in table 2, lyophilized Colibacterin (5 batches), from different manufacturers that had been expired for different periods of time (from 1982 to 2009), were studied. In the Colibacterin preparation, the sample was manufactured in 3-dose ampoules (CB 72-5, production of the Perm NIIVS) with an excess storage period of almost 30 years, 99.73% of the living cells did not form colonies. As it was established under a microscope with a Live/Dead kit, the sample contained 52.2% ($1.1 \pm 0.12 \times 10^{10}$ cells/ml) of viable cells. The total number of cells was $2.11 \pm 0.23 \times 10^{10}$ cells/ml. The culturability assessment of this preparation resulted with $2.89 \pm 0.32 \times 10^7$ CFU/ml.

The Colibacterin utilized was manufactured in 3 ml ampoules by Nizhny Novgorod "IM-BIO," 11 years past expiration. 79.5% of its cells were incapable of colony formation. The CFU/ml of this sample equaled $5.55 \pm 0.61 \times 10^9$ /ml, the difference between CFU/ml and total number of cells is $3.11 \pm 0.34 \times 10^{10}$ /ml in the Goryaev-Thoma camera.

The fact that a higher number of viable E. coli bacteria was not able to form colonies (85.5%) in a preparation produced by Mechnikov Biomed that was stored for 4 years past expiration is interesting ($p < 0.05$), i.e. values were statistically reliable. It should be noted that it was manufactured in 5 dose vials. The percent of the living cells revealed using a Live/Dead kit was 90.3%. This value shows on the statistical equality to total number of cells in Goryaev-Thoma camera and value of live cells with "Live/Dead", i.e. values were statistically doubtful ($p > 0.05$).

Colibacterin from two batches (code CB 24-3 and CB 27-3) produced by Mechnikov Biomed, 3 years past their expiration, had viability rates of 88.2% and 91.3%, respectively. The indicator of the inability to form colonies was 21.3% and 4.13% for each of the two batches. All these parameters were statistically doubtful ($p > 0.05$), which shows a high level of cell viability and a small number of VBNC.

Bifidobacterin

As it is presented in table 3, for the preparations containing bifidobacteria (from three producers) which were stored for 2 and 5 years past expiration or still non-expired, we observed (according to luminescent microscopy) a high level of the preservation of viability in a mix of cells (70.3% for "Bificol", BFC-20-1).

The "Bifidumbacterin" preparations, produced in 5 dose vials by the Federal State Unitary Enterprise "NPO Mikrogen," Stavropol, without excess of periods of storage, reveal a level of cell viability from 70.7% to 95.45%. In the bifidobacteria reculturing of some batches (code BB-56 and BB-44), in the environment for their cultivation, there was a delay in the emergence of visual colony growth up to the 72nd hour (microscopic colonies were formed after 4 and 7 days and would later develop into normal colonies). In the first days of incubation in these preparations, no more than 0.01% could be revealed from the total number of the bifidobacteria that were able to form colonies.

For the "Bifidumbacterin" batch with an expiration date of 01/2008, we made an attempt to increase the number of colonies by the addition of 1% and 10% Amino-peptide in the final concentration. In the test tubes with additives, during the later days of incubation, a 2-fold increase in the numbers of culturable cells in comparison with the control (medium without Amino-peptide addition) was noted. These results allow us to assume that there was a transition of non culturable forms to a vegetative condition.

Table 3. Studying of samples probiotic preparations, containing bifidobacteria with various periods of storage

Characteristics of preparation			Biological parameters				
Preparations	Codes of batches, expiration date (year)	Type of vessel, number of doses	Total number of cells in Goryaev-Thoma camera (cells/ml) (I_{95}) p value	live cells with "Live/Dead" (%)	CFU/ml (incubation time)		
				total number of live cells/ml with "Live/Dead" (I_{95}) p value	24 h.	72 h.	maximum CFU/ml (I_{95}) p value
1	2	3	4	5	6	7	8
Bificol	BFC 20-1 2007	Vials, 5 doses	5,96±0,66×10 ⁸ (4,38÷7,54)×10 ⁸ 4 and 5>0,05 4 and 8≥0,05	70,3	No growth	No growth	1±0,11×10 ⁹ B. bifidum (0,74÷1,26)×10 ⁹ 8 and 4≥0,05 8 and 5>0,05
				4,19±0,46×10 ⁸ (3,09÷5,29)×10 ⁸ 5 and 4>0,05 5 and 8>0,05			1,25±0,14×10 ⁷ E. coli
Bifdumbacterin	BB 44 2012	Vials, 5 doses	2,92±0,32×10 ⁷ (2,15÷3,69)×10 ⁷ 4 and 5>0,05 4 and 8<0,05	70,7	No growth	3,6±0,4×10 ⁴	1±0,11×10 ⁷ (0,74÷1,26)×10 ⁷ 8 and 4<0,05 8 and 5>0,05
				2,06±0,23×10 ⁷ (1,51÷2,61)×10 ⁷ 5 and 4>0,05 5 and 8>0,05			
Bifdumbacterin	BB 56 2013	Vials, 5 doses	6,76±0,74×10 ⁷ (4,98÷8,54)×10 ⁷ 4 and 5>0,05 4 and 8>0,05	95,45	6±0,66×10 ⁷	2±0,22×10 ⁸	2±0,22×10 ⁸ (1,47÷2,53) 8 and 4>0,05 8 and 5>0,05
				6,45±0,71×10 ⁷ (4,75÷8,15)×10 ⁷ 5 and 4>0,05 5 and 8>0,05			

Lactobacterin.

The results relating to the viability of lyophilized lactobacillus are presented in table 4. The quantity of cells not forming colonies (VBNC) varied from 54.4% to 99.9%. The greatest percent of cells that passed to VBNC is noted for LB-55 (1994). 92% of the living cells in Lactobacterin (exp. 2010) were not forming colonies. In another sample (exp. 2013) the quantity of VBNC was equal to or less than 54.4% (p<0,05). Therefore, the percent of the living cells that had lost the ability to form colonies depended on the date that the probiotic preparation was produced.

Therefore, in each of the studied preparations we found a certain percent of viable, but nonculturable bacteria, which are potentially capable of proliferation in favorable conditions.

Table 4. Studying of samples probiotic preparations, containing lactobacteria with various periods of storage

Characteristics of preparation			Biological parameters			
Preparations	Codes of batches, expiration date (year)	Type of vessel, number of doses	Total number of cells in Goryaev-Thoma camera (cells/ml), I ₉₅	live cells with "Live/Dead" (%)	CFU/ml (Incubation time=72h), (I ₉₅)	VBNC (%)
			p value	total number of live cells/ml with "Live/Dead", I ₉₅		
1	2	3	4	5	6	7
Lactobacterin	LB-55 1994	Ampoules, 5 doses	3,4±0,37×10 ⁹ (2,5÷4,3)×10 ⁹	66,5 2,3±0,25×10 ⁹ (1,7÷2,9)×10 ⁹	1,0±0,11×10 ⁷ (0,74÷1,26)×10 ⁷	99,9
			4 and 5 >0,05 4 and 6 < 0,05	5 and 4 >0,05 5 and 6 < 0,05	6 and 4 <0,05 6 and 5 < 0,05	
Lactobacterin	LB-80 2010	Vials, 5 doses	3,3±0,3×10 ⁹ (2,6÷4,02)×10 ⁹	92,04 3,06±0,23×10 ⁹ (2,5÷3,61)×10 ⁹	2,1±0,22×10 ⁹ (1,57÷2,63) ×10 ⁹	92,0
			4 and 5 >0,05 4 and 6 ≥ 0,05	5 and 4 >0,05 5 and 6 > 0,05	6 and 4 ≥0,05 6 and 5 >0,05	
Lactobacterin	LB 93 2013	Vials, 5 doses	2,9±0,3×10 ⁹ (2,18÷3,62)×10 ⁹	99,9 2,9±0,3×10 ⁹ (2,18÷3,62)×10 ⁹	1,35±0,15×10 ⁹ (0,99÷1,71)×10 ⁹	54,4
			4 and 5 >0,05 4 and 6 <0,05	5 and 4 >0,05 5 and 6 > 0,05	6 and 4 <0,05 6 and 5 >0,05	

DISCUSSION:

In our opinion, the obtained data indicates the presence of VBNC forms in lyophilized probiotic bacteria preparations. In such conditions of artificial anabiosis, these nonculturable forms can submit to the same laws of preservation and restoration that they do in nature. So, for the cells that have undergone lyophilization, Belgian scientists [4] studying a nonculturable condition of methane oxidizing bacteria have been led to a similar conclusion. Authors demonstrate that preservation (lyophilization, cryopreservation) induced VBNC state in significant part of cells.

It should be noted that suspensions of *E. coli* M17, which was incubated at room temperature in tubes with normal saline (an environment deprived of nutrients) and initially contained only singular CFU/ml for 72 hours, that were 30 years past expiration from Colibacterin, yielded an essential increase in culturability (by 3 orders of magnitude) that was clearly impossible to achieve by the division of the initial CFUs. A comparison of the results from plating the suspensions after the cultivations, which have shown a single quantity of CFU/ml after an initial resuspending of a probiotic, indicates that, in a nutritious agar, a statistically significant increase in concentration of bacteria (3 orders of magnitude) for a single cell in a hungry environment is impossible. Apparently, there was a restoration of damaged cells during the lyophilization process

or a storage of cells (a stressful factor), where the cells lose their ability to quickly transition to a condition of growth and reproduction.

The obtained data on nonculturable forms is important for controlling the true viability of cells in commercial lyophilized preparations based on microbial cells. The expiration date of such preparations, in our opinion, can be increased because of their containment of viable, but not readily culturable, cells. Aminopeptide (1% and 10%) is one of the factors that can transform nonculturable bacteria into a vegetative culturable state of bifidobacteria. For example, once in the intestines of a person or animal, these unaccounted nonculturable forms of probiotic microorganisms can start developing, providing for probiotics a long and effective influence. The approved method for assessing viability is also an express method for identifying viable cells in comparison with a method for defining CFU/ml.

Although some studies have been done on the ability of pathogenic VBNC cells to resuscitate in a complex environment, little consideration has been given thus far to the similar potential of probiotic substances. While the ultimate culturability of VBNC cells may currently be unclear [15], a variety of studies have shown that bacteria such as *E. coli* can remain inactive in foods and then resuscitate in the complex environment of the human gut [16-18]. More research into the potential of probiotics and other VBNC-containing functional foods may well shed light on further functionality as a result of complex resuscitation.

Although there does appear to be a direct correlation between the amount of cells that cannot form colonies and the amount of time that has passed since the preparation of cells had been produced, a significant amount of these non-reproductive cells remained viable. Furthermore, their viability was such that these non-reproductive cultures had the potential to resume proliferation when reintroduced to favorable conditions. This ability to remain alive in a dormant state, and even resume reproduction under the correct circumstances, presents an interesting potential for revival of “expired” cultures. Since so few similar studies have been done thus far, it would be beneficial for more research to be done on which factors most influence a specific culture’s going in to, and coming out of, a dormant state, and how easy it is to manipulate those factors.

Conclusion: We demonstrated the presence of VBNC cells in lyophilized probiotic preparations that contained live bacteria. Probiotics stored past their expiration date may retain a high potential medical effect because they contained high numbers of viable cells. VBNC cells in studied preparations may have the potential to return to an actively growing state.

Competing interests: The authors declare that they have no competing interests.

Authors’ contributions: Dr. Blinkova offered idea of experiment, a technique of its carrying out, is the principal investigator for the study, interpreted results of experiment and writing this article. Dr. Martirosyan provided discussion of the results, and assisted in writing this article. Pakhomov provided experiments, participated in discussion and registration of results and in writing this article. Dmitrieva O. provided experiments, participated in analyze, discussion and registration of results. Rachel Vaughan assisted in writing and editing this article. Altshuler M. participated in discussion of results.

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