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Non-culturability and Nisin Production of *Lactococcus lactis*

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Abstract

We studied formation of non-culturable forms of three bacteriocin (nisin) producing strains of *Lactococcus lactis* sub sp. *lactis*: MSU, 729 and F-116 under carbohydrate starvation stress. Two different types of inoculum were applied: A) unwashed cells with culture liquid, B) cells washed twice with normal 0,9%saline. Resulting total numbers of cells were 0.6 1.0×10^8 cells/ml for both types of inoculum. Population obtained using type A inoculum demonstrated active growth phase within first 1-5 days of incubation (up to 2.4×10^9 cells/ml) while those obtained using type B inoculum did not grow within that period. Type B population of strain MSU showed phenotypic dissociation that resulted in appearance of micro colonies. After that, we observed active growth phase (up to 5.2×10^9 cells/ml). Type B cultures of strains 729 and F-116 did not grow during the whole experiment. It was shown that type A population shifted into non-culturability faster than type B. This is due to differences in metabolic strategies and stress sensitivity of these types of population. After 1 year of incubation (383 days) culturability decreased by 3 orders of magnitude for type B (5 orders for type B population of strain MSU) and by 6 orders of magnitude for type A population. We also observed considerable reduction of cell size for type A population of strains 729 and F-116. Studies of bacteriocin activity showed that in type B population cells were up to 78 times more productive compared to those of type A cultures. This phenomenon can be explained by differences in survival strategies of population that use antibacterial potential of bacteriocins for their benefit.

Keywords: *Lactococcus lactis*; Nonculturable; Nisin; Bacteriocin; Activity

Introduction

Bacterial cells in response to various stressful factors can reversibly lose ability to form colonies on traditional nu-trient media [1-11]. This state of a microorganism is called viable, but nonculturable (VBNC). Formation of noncul-turable cells is confirmed for a wide range of microorganisms among which there are pathogenic and opportunistic types for the people [4]. Some microorganisms shift to non-culturability only under the influence of a specific com-plex of stresses. Each of them or their consecutive introduction causes essentially different results. With culturability loss the microorganism undergoes a number of morphological and physiological changes. They include considerable dwarfing, slowing of metabolism, change of lipid and protein composition of a membrane.

When studying different types of microorganisms it is also necessary to consider that the possibility of formation of nonculturable forms and speed of this process are strains dependent. Such data are published for *Campylobacter jejuni, Escherichia coli, Lactococcus lactis* [2,6-9,12]. For example, for *E. coli* it was discovered that depending on isolation source and culture conditions, the microorganism can obtain or lose the ability to form nonculturable cells [4]. Occurrence of nonculturable bacteria in natural and artificial environments explains difficulties in search and isolation of causative agents during the outbreaks of infectious diseases in humans and animals using culture based techniques [3]. Also there are problems in assessment of microbial contamination of environment and food stuff. Besides studying conditions of nonculturable cells formation of industrial microorganisms will help better understand processes of maturing of the fermented foods [13,14]. For example, Ganesen et al. [13] with coauthors showed that studied *Lactococcus* cultures, isolated from cheese, formed nonculturable cells, under carbohydrate starvation conditions during long-term incubation in the minimal synthetic environment [13,14]. Production of nisin (most known bacteriocin) by nonculturable cells has not yet been studied. Objective of this research is detection of specific features of formation of nonculturable forms of *Lactococcus lactis* strains during a long-term starvation and their nisin producing activity.

Materials and Methods

In this study we used 3 strains of *L. lactis* subsp. *lactis*, producers of nisin A (included in Gene Bank): 2 from the department of microbiology collection, isolated from various fermented milk products - MSU (DQ255952) and 729 (EF102814) and one genetically engineered strain F-116 (EF100777), obtained by method of merging of protop-lasts[15,16]. Microorganisms were recultured twice in Elliker's broth (Sigma, Steinheim, Germany) for 24 hours. After that, in the same medium we prepared 18-hour cultures which were used in further studies.

To obtain nonculturable *Lactococcus* we used the minimal medium the following composition (mmol/l), using reagents by Sigma, Steinheim, Germany: glutamate - 21; histidine - 0.3; isoleucine - 0.8; leucine - 1.5; methione - 0.8; valine - 2.6; NH_4Cl - 9.5; K_2SO4 - 0.28; KH_2PO_4 - 1.3; Na acetate - 15; glucose - 50; lactose - 2.92; MOPS - 190; tricine - 4; CaCl2 - 0.0005; $MgCl_2$ - 0.52; $FeSO_4$ - 0.01; NaCl - 50; biotin - 0.0004; pyridoxal-HCl - 0.01; folic acid - 0.0023; riboflavin - 0.0026; niacinamide - 0.008; thiamine-HCl - 0.03; panthotenic acid - 0.02; $(NH_4)_6Mo_7O_{24}$ - 0.000003; H_3BO_3 - 0.0004; $CoCl_2$ - 0.00003; $CuSO_4$ - 0.00001; $MnCl_2$ - 0.00008; $ZnSO_4$ - 0.00001;(Sigma, Steinheim, Germany). This medium creates conditions of carbohydrate starvation during long-term cultivation [13,14]. pH was stabilized at

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the level of 6.8 made with 0.19M 2-(N-morfolino) ethansulphonic acid (MES). Bacteria were incubated in 0.5 ml flasks that contained 300 ml of experimental medium. Experiment was conducted in two variants. In the first variant flasks with minimal culture medium were inoculated with 5% (v/v) of native cultures (these population will be further referred to as *L. lactis* MSU, 729 and F-116 type A). In the second variant the cells washed twice with 0.9% NaCl solution and resuspended to the initial volume (these conditions were designated as *L. lactis* MSU, 729 and F-116 Type B). The volume of inoculum also was 5% (v/v). All cultures were incubated at 30°C without agitation for more than 1 year (383 days). The initial number of population for all cultures made 0.6 - 1×10^8 cells/ml, and initial values of optical density were 0.1 ± 0.01.

Samples were taken periodically for assessment of numbers of colony forming units (CFU/ml) by plating on solid Elliker medium. Samples were also counted in Goryaev or Thoma chamber. Ratio of viable to dead cells in popula-tion defined in a luminescent microscope OPTON (Carl Zeiss, Germany), magnification by 320 (8×40) using Live/Dead® (Baclight™, Life Technologies, Carlsbad, CA) staining kit. Dynamics of formation of nonculturable forms revealed as a difference between the number of colony forming units and total number of viable cells in population. Optical density was measured on the spectrophotometer type KFK-3 with wavelength of 450 nanometers and optical distance of 5 mm. Bacteriocin production (nisin) was determined by a method of diffusion of substance into agar [17]. Bacteriocin extraction from culture liquid was carried out in a mix of acetone, glacial acetic acid and water in the ratio 4:1:5 at 55°C for 90 min. Extracts were diluted in the phosphate buffer (pH 5.5) in the ratio 1:10 and injected into the prepared holes in the medium. Estimation of bacteriocin activity level was carried out by measuring growth inhibition zones of test culture - *Bacillus coagulans* 429 with the subsequent recalculation of actual quantity on a calibration curve. The phosphate buffer for bacteriocin titration contained (g/l): 6.6 KH_2PO_4, 0.142 $K_2HPO_4\times3\ H_2O$, sterilized at 121°C within 20 min. The 24-hours test culture of *B. coagulans* 429 wasa suspension with optical density value of 0.7 units (450 nanometers and optical distance 0.5 cm). As a standard commercial preparation of nisin was applied ("Nisaplin" with 2.5% pure bacteriocin nisin A, activity of 1000 IU/mg, firms Alpin&Barret, LTD, Great Britain).

Results and Discussion

Results of experiments are presented in Tables 1-3 and Figures 1-6. Weshowed that from first days of cultivation the difference between the type A and type B population was evident. The former within 1-5 days after inoculation underwent a phase of active growth and increased in numbers from 0.6 - 1×10^8 viable cells/ml to 1.5 - 1.6×10^9 viable cells/ml depending on the strain. For this variant of experimental conditions the maximum number of bacteria in 1 ml did not depend on quantity of the cells, used as inoculum. At the same time, growth rate differed depending on initial concentration of cells/ml and had a maximum during the first hours after inoculation. Thus, if initial cell concentration was 2.8 - 4.8×10^7 cells/ml, within the first six hours of incubation increased in total number of cells/ml by 1-1.2 orders of magnitude (that corresponds to about 3.5 division cycles) was observed. For type B population we didn't observe increase in number of cells, within the first 1-5 days (Fig. 4.1). Such difference is apparently associated with introduction together with unwashed cells some waste products of bacteria, including bacteriocins, which act as growth stimulating factors [18,19]. It is besides noted, that the population, which have passed a stage of active growth, by two weeks reduced pH value of the media by about 2.6 (from 6.8 to 4.2) units, while for parallel population decrease of this value was 0.3-0.5 units. This may have been associated with slower consumption of sugars by these cells and less decrease of acidity of the environment respectively. After more than 1 year of incubation the pH value of the media was 5.1-5.2 for all type A population, 6.2 for type B population of the strains 729 and F-116 and 5.5 for the type B population of strain MSU. For type B population of the strain MSU, when plating on Elliker's solid medium in 12 days we observed the phenotypic dissociation which manifested in emergence of microcolonies, with diameter less than 0.1 mm (typical colonies have diameter of 3 - 4 mm), seen only by means of a binocular microscope (magnification by 10 times). Thus, the culture splits in two sub-population, micro- and macrocolonial. The subpopulation, that formed micro colonies, by 12th day of cultivation, entered a phase of active growth and, by that time the number of micro colonies exceeded quantity of normal *Lactococcus* colonies by 5.5 times, total number of cells was 1.5 ± 0.2×10^8 (initial number was 9 ± 1×10^7). Later the number of cells in the population, defined in the counting chamber, increased to 5.2 ± 0.6×10^9/ml (a difference by 63.5 times). The growth was accompanied by synthesis high-adhesive, optically dense substance probably of proteinaceous and/or polysaccharide nature. The optical density of this culture was by 3.4 times higher (1.6), than in parallel culture (0.5 with a number of cells 2.6 ± 0.3×10^9/ml), adhesiveness was revealed as increased sticking of cells to microscope slides and the floor of the counting chamber, which could be observed under a microscope. Process of phenotypic dissociation of the strain MSU, type B population, probably is a spontaneous reorganization of the genetic material of a cell as an adaptation to environment conditions, which is described in literature [18,20].

The total numbers of cells/ml in population of all strains lactococci after reaching maximum concentrations (1.5-5.2×10^9/ml for population, that underwent active growth phase and 0.7-1×10^8/ml for others) remained approximately at the same level, or decreased, but no more than 1 order of magnitude. Percentage of viable bacteria depended on a strain and experimental conditions (Figures 4-6; Table 1-3). Thus, for F-116 strain the percentage of viable cells in both populations was close to 100% during the whole experiment. Type B population of the strain 729, had viability close to 100% till 6.5 months of incubation. By 10th month of incubation this value decreased by 24%. After a year of observation the percentage of viable cells for type A population of strains of MSU and 729, became close to 100% again. However it occurred simultaneously with the decrease of total cell numbers in popula-tion, meaning that was due to lysis of dead cells. The least viability of bacteria was noted for type B population of the strain MSU. The number of viable cells in it was gradually decreasing and by 10 months the population was 38.1% viable at total number 3.3×10^9/ml, and by a year of incubation-54.2% at total number 2×10^9/ml. In addition, from Figures 1-3 it is evident, that those cultures which underwent a stage of active growth were characterized by greater decrease of viability in time.

It has been found experimentally that nonculturable cells start forming right after inoculation. In case of type A population their formation occurs parallel to growth. It, most likely, is a slowing factor for growth rate of population. By 24 hours of cultivation 60 - 80% of cells did not form colonies on a solid nutrient medium. And by fifth day of incubation under conditions of carbohydrate starvation 82.1 - 99.6% of cells in population (see Table 4.1), depending on a strain and the variant of experiment did not form colonies. As it was noted earlier further dynamics of culturability loss depended on a strain and experimental conditions (Table 1-3). So, for the type A population, we observed greater decrease in rates of a culturability compared to parallel cultures. It is probably due to the fact, that the cells, which

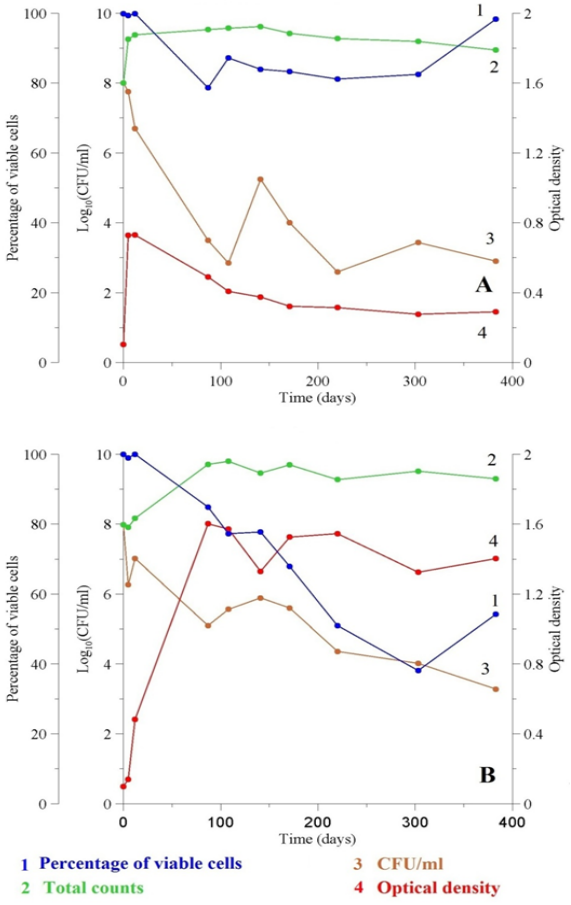

Figure 1: Characteristics of viability parameters for populations of *Lactococcus lactis* strain MSU. A – type A population of strain MSU, B – type B population of strain MSU.

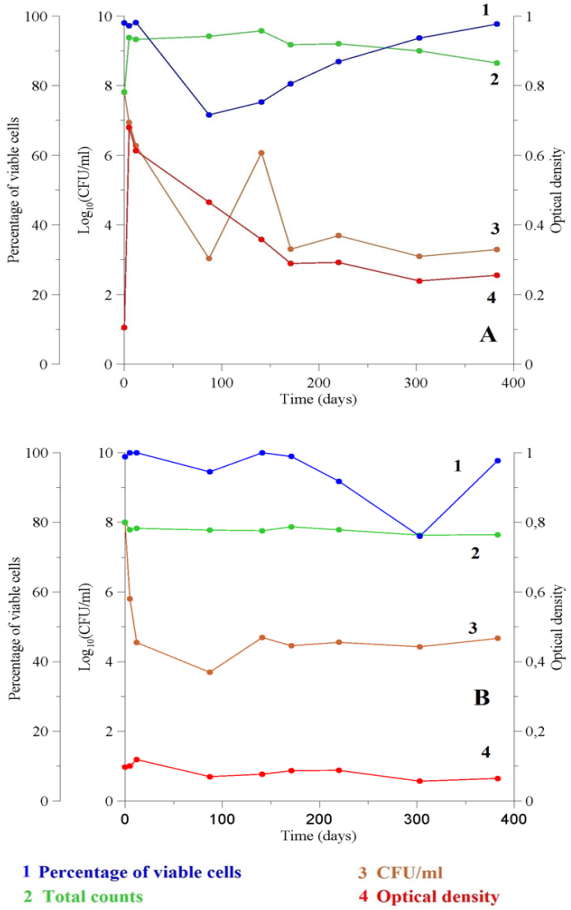

Figure 2: Characteristics of viability parameters for populations of *Lactococcus lactis* strain 729. A – type A population of strain 729, B – type B population of strain 729.

used resources of the medium during growth, became more sensitive to carbohydrate starvation stress, and possibly to a stress caused by decrease in pH values of these cultures. By three months of incubation these cultures contained 103 - 104/ml culturable cells. Difference between culturability (CFU/ml) and total number of viable cells was 5 - 6 orders of magnitude which, therefore, represented concentration of nonculturable microorganisms in population. By 10 months of incubation the type A population contained 1.2 - 2.7×10^3/ml culturable cells. Thus, the difference between total viability and culturability was 6 orders of magnitude. For the Type B cultures numbers of CFU/ml was 1 - 1.7×10^4/ml that was by 4 orders of magnitude less, than total number of viable cells. By a year of incubation, the largest percentage of nonculturable cells is noted for type A population of a strain MSU. Number of CFU/ml for this culture was 7.9 ± 0.9×10^2/ml. The difference between a culturability and total number of viable cells was 6 orders of magnitude.

During incubation we observed increase in numbers of culturable cells in population (Figures 1-3). It can be explained either with the secondary growth of culturable part of the population, not visible in the counting chamber (but apparent as an increase in CFU/ml values), or by spontaneous return of part of a population into culturable state due to stimulation by endogenous factors [18,19]. In this process there probably is an influence of natural heterogeneity of the cells typical to any population of viable organisms, based, for example, on uneven distribution of macromolecules or ribosomes between daughter cells or a temporary difference in number of copies of genetic material [5].

Some cells, probably, are able to come back to a culturable state under the influence of weak stimulating agents, such as the substances, released from lyzed cells, or grow in conditions of severe nutrient limitation. It allows population to have a number of cells, which can quickly react to inflow of nutrients in their environment. Also interesting is the fact, that the population that have undergone a stage of active growth during incubation process, showed higher increase in number of culturable cells, than at the others. Quantitative increase culturable cells by fifth month of incubation for the population, which have passed through a stage of active growth, was 1 - 3 order. Thus, for type A population *L.lactis* of the strain 729, the culturability increased from 1.1×10^3/ml to 1.2×10^6/ml. Such increase can be explained by a difference in level of a trophic stress (the populations which have undergone a stage of active growth were under stronger trophic stress). It was reported, that stress intensity influences degree of population heterogeneity of microorganisms, for example, due to adaptive mutations [6,18,20].

It should also be noted, that subpopulation of type B culture of strain MSU, had different characteristics, and pH value of the medium was intermediate between values measured for other type B cultures and parallel population. After a year of incubation pH values for type

A population were 5.1 - 5.2; for type B population of the strains 729 and F-116 - 6.1 - 6.2; and for type B population of a strain MSU, - 5.5. Micro colonial part became completely non-culturable by 3 months of incubation and micro colonies were no longer observed. However, the subpopulation forming colonies of normal size was losing culturability at the slowest rate among all cultures studied in this experiment (after 3.5 months of incubation the number CFU/ml was $3.7 \pm 0.4 \times 10^5$). Thus, two subpopulation influence each other, increasing stress resistance of minor population, including, probably, at the expense of allocation of nutrients from the medium to synthesis of polysaccharides/peptides. Therefore, such splitting of homogeneous culture in two subpopulation allows to maintain proliferative potential of a microorganism for a longer period due to "altruistic" death of the most cells, giving a microbe an advantage over other bacteria in micro-ecological niches and when colonizing a new environment [5,6].

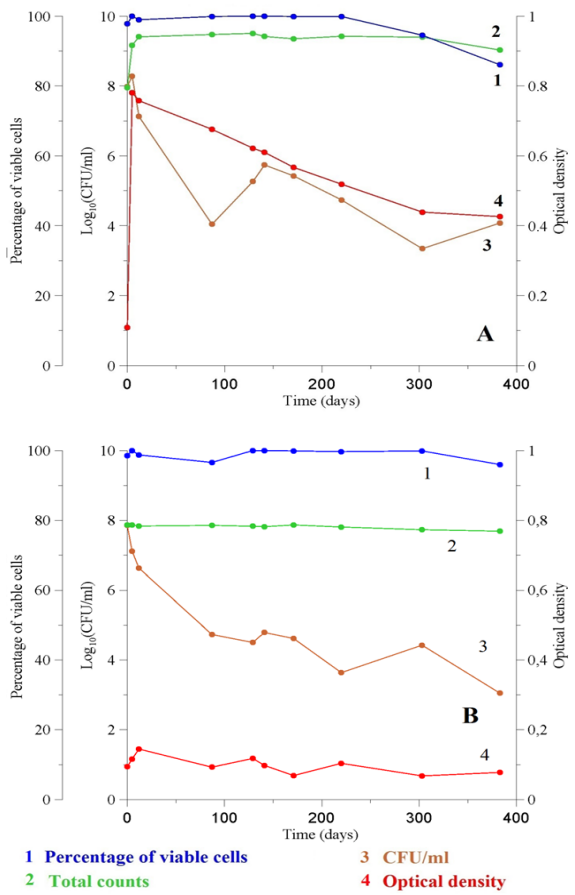

Figure 3: Characteristics of viability parameters for populations of *Lactococcus lactis* strain F-116. A - type A population of strain F-116, B - type B population of strain F-116.

The results obtained in this study, differ from literary data [13]. For example, two of the three strains of *L. lactis*, mentioned by Ganesan B. with colleagues, completely, but gradually, became nonculturable in 4 days to 4 weeks depending on a strain and experimental conditions. For our strains we didn't observe full transition to nonculturable state during 1 year of experiment. By that time culturability for type A population was $7.9 \pm 0.9 \times 10^2$ - $1.2 \pm 0.1 \times 10^4$ CFU/ml (the difference between the number CFU/ml and amount of viable cells was 5 - 7 orders. This fact means population, contained more than 99.9% of nonculturable cells), depending on a strain. For type B population $1.1 \pm 0.1 \times 10^3$ - $4.7 \pm 0.5 \times 10^4$ CFU/ml (3 - 4 difference by orders - more than 99.9% of cells did not form colonies) depending on a strain. Differences between results of this study and literary data once again indicate that rate and completeness of transition of population into nonculturable state depends on a strain and experimental conditions. In addition the sources of isolation of strains also influence stress resistance as it was shown by Asakura [21] with coauthors. In the single work we know, strains of *L. lactis* were isolated from cheese ferment [13], and in our expe-riments we used cultures isolated from milk [15,16].

It is noted, that after undergoing active growth by *Lactococcus lactis* population the sizes of microorganisms appeared smaller, than initial cells used as inoculum, and, cells of parallel cultures, for which we did not observe increase in number (Figures 4-6).

During further incubation values of optical density for all cultures gradually decreased while total number of cells/ml remained fairly constant. It is apparently due to further reduction of the cell size in population in the course of transition to nonculturable state. Reduction of cell size for type A population of strain of *L. lactis* strain 729 is

Strain of *Lactococcus lactis*	General quantity of cells in Goryaev (Thoma) chamber CFU/ml		% viable cells in population General quantity of viable cells		Optical density		% viable cells, but not forming colonies	
	0 h	5 days	0 h	5 days	0 h	5 days	0 days	5 days
MSU type A	$1 \pm 0.11 \times 10^8$ --- $1 \pm 0.11 \times 10^8$	$1.8 \pm 0.2 \times 10^9$ --- $5.7 \pm 0.6 \times 10^7$	99.9 --- $9.9 \pm 1.1 \times 10^7$	99.2 --- $1.8 \pm 0.2 \times 10^9$	0.2	0.7	-	96.8
MSU type B	$9.0 \pm 1 \times 10^7$ --- $9.0 \pm 1 \times 10^7$	$8.1 \pm 0.9 \times 10^7$ --- $1.8 \pm 0.2 \times 10^6$	99.9 --- $9.9 \pm 1 \times 10^7$	99 --- $8.0 \pm 0.9 \times 10^7$	0.1	0.1	-	97.7
729 type A	$6.5 \pm 0.7 \times 10^7$ --- $6.4 \pm 0.7 \times 10^7$	$2.4 \pm 0.3 \times 10^9$ --- $8.7 \pm 1 \times 10^6$	98 0 $6.4 \pm 0.7 \times 10^7$	97.2 --- $2.3 \pm 0.3 \times 10^9$	0.1	0.7	-	99.6
729 type B	$1 \pm 0.1 \times 10^8$ --- $9.8 \pm 1.1 \times 10^7$	$6.1 \pm 0.7 \times 10^7$ --- $6.5 \pm 0.7 \times 10^5$	98 --- $9.7 \pm 1.1 \times 10^7$	100 --- $6.1 \pm 0.7 \times 10^7$	0.1	0,1	-	98.9
F-116 type A	$9.0 \pm 1 \times 10^7$ --- $9.7 \pm 1.1 \times 10^7$	$1.4 \pm 0.2 \times 10^9$ --- $1.9 \pm 0.2 \times 10^8$	97.9 --- $8.8 \pm 1 \times 10^7$	100 --- $1.5 \pm 0.2 \times 10^9$	0.1	0.8	-	86.8
F-116 type B	$7.3 \pm 0.8 \times 10^7$ --- $7.2 \pm 08 \times 10^7$	$7.3 \pm 0.8 \times 10^7$ --- $1.3 \pm 0.2 \times 10^7$	98.5 --- $7.0 \pm 0.8 \times 10^7$	100 --- $7.4 \pm 0.8 \times 10^7$	0.1	0.1	-	82.1

Table 1: Characteristics of viability parameters of *L. lactis* populations on 0 and 5 days of incubation.

most evident. Cells of this culture by 4-5 months of incubation were poorly distinguishable in Thoma counting chamber and in luminescent microscope. Cells of type A *Lactococcus lactis* population of strain F-116 also became smaller, but to lesser extent. It is known, that the tendency of cell dwarfing, is a general characteristic for process of transition of microorganisms into nonculturable state [4].

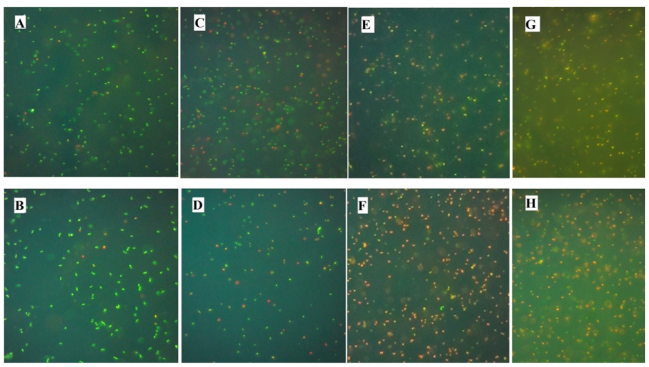

Figure 4: Viable and dead cells of *Lactococcus lactis*, MSU strain. A, C, E, G – type A population. B, D, F, H – type B population. Incubation times (from left to right): 5 days, 3 months, 7 months and 10 months. Live/Dead staining, magnification by 320 times.

At the same time with considerable reduction in cell size of the mentioned type A population *Lactocccus lactis* of strain 729 became weakly stainable with DNA-binding dyes from Live\Dead® (Baclight™) kit (very weak green or weak red luminescence). This fact can be explained by considerable condensation of cells' nucleoid, which makes DNA restrictedly available for dyes. It can also be concluded, that cell metabolism was considerably slowed in the population as nucleoid in such cells becomes restrictedly available for RNA polymerases as well. In type B population, despite essential decrease in optical density, no visual reduction of the cell sizes was observed (Figures 4-6).

Experiments on bacteriocinogenic activity of lactococci population in conditions of carbohydrate starvation stress showed, that bacteriocin was synthesized in the first 24 hours after inoculation. However, no strain and experiment type dependent difference in final bacteriocin concentration in 1 ml of cultural liquid (2450 - 3000 IU/ml) was observed. During further 1 year incubation its concentration didn't change significantly. The only exception was strain 729 in both types of experiment. For this strain by 3 - 4.5 months we observed decrease in concentration of the nisin. However, by 3 months of incubation it was observed only in type A population, and in 4.5 months - for both cultures.

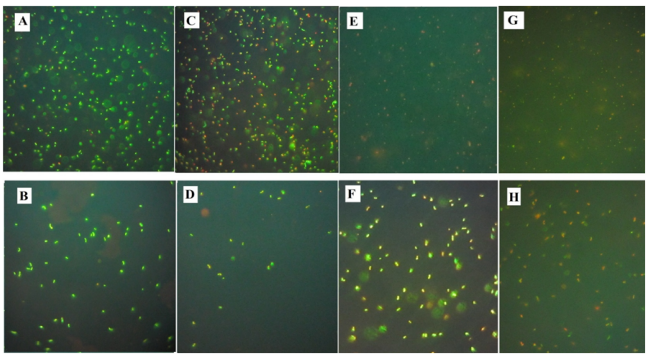

Figure 5: Viable and dead cells of *Lactococcus lactis*, 729 strain. A, C, E, G – type A population. B, D, F, H – type B population. Incubation times (from left to right): 5 days, 3 months, 7 months and 10 months. Live/Dead staining, magnification by 320 times.

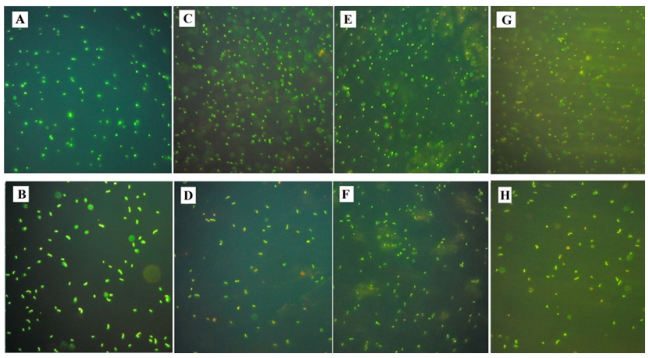

Figure 6: Viable and dead cells of *Lactococcus lactis,* F-116 strain. A, C, E, G – type A population. B, D, F, H – type B population. Incubation times (from left to right): 5 days, 3 months, 7 months and 10 months. Live/Dead staining, magnification by 320 times.

Strain of *Lactococcus lactis*	General quantity of cells in Goryaev (Thoma) chamber CFU/ml		% viable cells in population General quantity of viable cells		Optical density		% viable cells, but not forming colonies	
	12 days	12 weeks	12 days	12 weeks	12 days	12 weeks	12 days	12 weeks
MSU type A	$\frac{2.4 \pm 0.3\times10^{9}}{5 \pm 0.6\times10^{6}}$	$\frac{3.4 \pm 0.4\times10^{9}}{3.1 \pm 0.3\times10^{3}}$	$\frac{99.9}{2.4 \pm 0.3\times10^{9}}$	$\frac{78.6}{2.7 \pm 0.6\times10^{9}}$	0.7	0.5	99.8	99.9
MSU type B	$\frac{1.5 \pm 0.2\times10^{8}}{1.1 \pm 0.1\times10^{7}}$	$\frac{5.2 \pm 0.6\times10^{9}}{1.3 \pm 0.1\times10^{5}}$	$\frac{100}{1.5 \pm 0.1\times10^{8}}$	$\frac{84.9}{4.4 \pm 0.5\times10^{9}}$	0.5	1.6	92.9	99.9
729 type A	$\frac{2.1 \pm 0.2\times10^{9}}{1.9 \pm 0.2\times10^{6}}$	$\frac{2.6 \pm 0.3\times10^{9}}{1.1 \pm 0.1\times10^{4}}$	$\frac{98.2}{2.1 \pm 0.2\times10^{9}}$	$\frac{71.6}{2.0 \pm 2.2\times10^{9}}$	0.6	0.5	99.9	99.9
729 type B	$\frac{6.8 \pm 7.3\times10^{7}}{3.6 \pm 0.4\times10^{4}}$	$\frac{6.0 \pm 0.7\times10^{7}}{4.5 \pm 0.6\times10^{3}}$	$\frac{100}{6.8 \pm 0.7\times10^{7}}$	$\frac{94.5}{5.7 \pm 0.1\times10^{7}}$	0.2	0.1	98.9	99.9
F-116 type A	$\frac{2.5\pm0.3\times10^{9}}{1.3 \pm 0.2\times10^{7}}$	$\frac{3.0\pm0,3\times10^{9}}{1.1 \pm 0.1\times10^{4}}$	$\frac{98.9}{2.5 \pm 0.3\times10^{9}}$	$\frac{99.9}{3.0 \pm 0.3\times10^{9}}$	0.8	0.7	99.5	99.9
F-116 type B	$\frac{6.9 \pm 0.8\times10^{7}}{4.3 \pm 0.5\times10^{6}}$	$\frac{7.2 \pm 0.8\times10^{7}}{5.4 \pm 0.6\times10^{4}}$	$\frac{97.9}{6.7 \pm 0.7\times10^{7}}$	$\frac{95.6}{6.7 \pm 0.8\times10^{7}}$	0.1	0.1	93.6	99.9

Table 2: Characteristics of viability parameters of *L. lactis* populations in 12 days and 12 weeks of incubation.

Strain of *Lactococcus lactis*	General quantity of cells in Goryaev (Thoma) chamber CFU/ml		% viable cells in population General quantity of viable cells		Optical density		% viable cells, but not forming colonies	
	7 months	10 months	7 months	10 months	7 months	10 months	7 months	10 months
MSU type A	$1.9 \pm 0.2 \times 10^9$ / $3.9 \pm 0.4 \times 10^2$	$1.5 \pm 0.2 \times 10^9$ / $2.7 \pm 0.3 \times 10^3$	81.2 / $1.5 \pm 0.2 \times 10^9$	82.5 / $1.3 \pm 0.1 \times 10^9$	0.3	0.3	99.9	99.9
MSU type B	$1.9 \pm 0.2 \times 10^9$ / $2.3 \pm 0.3 \times 10^4$	$1.9 \pm 0.2 \times 10^9$ / $1.0 \pm 0.1 \times 10^4$	50.1 / $9.6 \pm 1.1 \times 10^9$	38.1 / $1.3 \pm 0.1 \times 109$	1.5	1.3	99.9	99.9
729 type A	$1.6 \pm 0.2 \times 10^9$ / $4.9 \pm 0.5 \times 10^3$	$1.0 \pm 0.1 \times 10^9$ / $1.2 \pm 0.1 \times 10^3$	86.9 / $1.4 \pm 0.2 \times 10^9$	93.7 / $9.4 \pm 1.0 \times 10^8$	0.3	0.2	99.9	99.9
729 type B	$6.1 \pm 0.7 \times 10^7$ / $3.6 \pm 0.4 \times 10^4$	$4.3 \pm 0.5 \times 10^9$ / $2.7 \pm 0.3 \times 10^4$	91.8 / $5.6 \pm 0.6 \times 10^8$	76 / $3.3 \pm 0.4 \times 10^9$	0.1	0.1	99.9	99.9
F-116 type A	$2.5 \pm 0.3 \times 10^9$ / $5.5 \pm 0.6 \times 10^4$	$1.9 \pm 0.2 \times 10^9$ / $2.2 \pm 0.2 \times 10^3$	99.9 / $2.7 \pm 0.3 \times 10^9$	94.6 / $2.4 \pm 0.3 \times 10^9$	0.5	0.4	99.9	99.9
F-116 type B	$6.5 \pm 0.7 \times 10^7$ / $4.4 \pm 0.5 \times 10^3$	$5.4 \pm 0.6 \times 10^7$ / $2.7 \pm 0.3 \times 10^4$	99.7 / $6.4 \pm 0.7 \times 10^8$	99.9 / $5.4 \pm 0.6 \times 10^9$	0.1	0.1	99.9	99.9

Table 3: Characteristics of viability parameters of *L. lactis* populations in 7 and 10 months of incubation.

Incubation period	Total cell counts cells/ml	Nisin activityIU/ml	Specific nisin activity IU/10^9 cells/ml	Difference rate of specific activity
	Lactococcus lactis strain MSU, type A			
	Lactococcus lactis strain MSU, type B			
1day (24 hours)	$1.5 \pm 0.2 \times 10^9$	3000	2069	25.1
	$4.7 \pm 0.5 \times 10^7$	2450	51906	
2 days	$1.6 \pm 0.2 \times 10^9$	3250	2083	35.3
	$3.4 \pm 0.4 \times 10^7$	2500	73529	
3 days	$1.4 \pm 0.2 \times 10^9$	3000	2097	27.1
	$4.4 \pm 0.5 \times 10^7$	2500	56818	
7 days	$1.8 \pm 0,2 \times 10^9$	2700	1525	41.7
	$4.4 \pm 0.5 \times 10^7$	2800	63636	
10 days	$2.1 \pm 0.2 \times 10^9$	2750	1322	35.1
	$5.3 \pm 0.6 \times 10^7$	2450	46402	
3 months	$3.4 \pm 0.4 \times 10^9$	2700	794	1/1.5
	$5.2 \pm 0.6 \times 10^9$	2700	524	
4,5 months	$4.2 \pm 0.5 \times 10^9$	2000	480	1.6
	$2.9 \pm 0.3 \times 10^9$	2270	783	
1 year	$8.8 \pm 1 \times 10^8$	2750	3111	1/2.6
	$2 \pm 0.2 \times 10^9$	2750	1375	

Table 4: Dynamics of nisin activity in populations of the strain of *Lactococcus lactis* MSU under the conditions of a trophic stress.

On the basis of data on activity in IU/ml, we standardized specific activity to one producing bacterial unit of population - 10^9 cells/ml (ratio of number of nisin activity units to one producing bacterial unit - 10^9 cells/ml). The calculation of IU/ml was made with calibration curve for nisin A, mentioned in materials and methods. The difference in specific productivity of nisin between variants of experiment is noticeable for all strains from first days of cultivation (Tables 4 - 6). However unlike growth processes here we observed inverse correlation. Results testify that type B cultures appeared more productive, than parallel population. Thus, after 24 hours of incubation specific activity differed by 20 - 40 times. It is possible to assume, that in our experiments the difference in survival strategy of the two types of population was observed. Type A population directed resources of the medium on growth and type B population were more sensitive to a stress and shifted to a certain state for survival of adverse conditions. In this case we considered intensified nisin production as a protective reaction for preservation of competitive advantage of the weakened population [22,23].

Characteristic of gradually slowing nisin biosynthesis under conditions of trophic stress for all type B population was existence of three quantitative maxima of specific activity. For strain *L. lactis* MSU it was 2nd and the 7th days and 1 year, for strains 729 F-116 - 2nd, 10thdays and 1 year. The maximum level of specific activity was found for all strains on the second day. For the first time we revealed characteristics of fluctuation of the quantitative contents of nisin under stressful conditions when more than 99% of population of all *Lactococcus* cells shifted to nonculturable state. This can indicate preservation of bacteriocinogenic activity of microorganisms. Probably,

Incubation period	Total cell counts cells/ml	Nisin activityIU/ml	Specific nisin activity IU/109 cells/ml	Difference rate of specific activity
	Lactococcus lactis strain 729, type A			
	Lactococcus lactis strain 729, type B			
1day (24 hours)	$1.6 \pm 0.2 \times 10^9$	2800	1806	21.2
	$6.6 \pm 0.7 \times 10^7$	2450	38281	
2 days	$1.7 \pm 0.2 \times 10^9$	3250	1626	41.3
	$3.7 \pm 0.4 \times 10^7$	2500	67204	
3 days	$1.6 \pm 0.4 \times 10^9$	2450	1531	26.6
	$6.6 \pm 0.7 \times 10^7$	2700	40909	
7 days	$1.6 \pm 0.2 \times 10^9$	2750	1676	22.9
	$7.0 \pm 0.8 \times 10^7$	2700	38352	
10 days	$1.8 \pm 0.2 \times 10^9$	3000	1523	35.1
	$4.9 \pm 0.5 \times 10^7$	2800	57377	
3 months	$2.6 \pm 0.3 \times 10^9$	1332	510	78.2
	$6 \pm 0.7 \times 10^7$	2400	39867	
4,5 months	$3.8 \pm 0.4 \times 10^9$	985	262	77.1
	$5.8 \pm 0.6 \times 10^7$	1166	20207	
1 year	$4.4 \pm 0.5 \times 10^8$	2450	5518	10
	$4.4 \pm 0.5 \times 10^7$	2450	55180	

Table 5: Dynamics of nisin activity in populations of the strain of *Lactococcus lactis* 729 under the conditions of a trophic stress.

Incubation period	Total cell counts cells/ml	Nisin activityIU/ml	Specific nisin activity IU/109 cells/ml	Difference rate of specific activity
	Lactococcus lactis strain F-116, type A			
	Lactococcus lactis strain F-116, type B			
1day (24 hours)	$1.5 \pm 0.2 \times 10^9$	2450	1633	41.8
	$4.3 \pm 0.5 \times 10^7$	2950	68287	
2 days	$1.4 \pm 0.2 \times 10^9$	2450	1713	55.4
	$3.2 \pm 0.4 \times 10^7$	3000	94936	
3 days	$1.1 \pm 0.1 \times 10^9$	3250	3037	28.1
	$3.3 \pm 0.4 \times 10^7$	2800	85365	
7 days	$1.2 \pm 0.1 \times 10^9$	2950	2379	26.7
	$4.7 \pm 0.5 \times 10^7$	3000	63559	
10 days	$1.3 \pm 0.1 \times 10^9$	2450	1870	36.8
	$3.6 \pm 0.4 \times 10^7$	2450	68820	
3 months	$3 \pm 0.3 \times 10^9$	2250	755	40,5
	$7.2 \pm 0.8 \times 10^7$	2200	30556	
4,5 months	$2.6 \pm 0.3 \times 10^9$	2080	784	46.6
	$6.6 \pm 0.7 \times 10^7$	2320	35152	
1 year	$1.1 \pm 0.1 \times 10^9$	2800	2641	18.9
	$4.9 \pm 0.5 \times 10^7$	2450	49796	

Table 6: Dynamics of nisin activity in populations of the strain of *Lactococcus lactis* F-116 under the conditions of a trophic stress.

some amount of nisin in cultural liquid within a year can deteriorate or be consumed by cells. Decrease in nisin activity of strain 729 correlated with increase in CFU/ml numbers confirms this observation. However due to continuing process of nisin biosynthesis the accumulation level of nisin in cultural liquid for all lactococci after 1 year was close to initial values.

It is evident from Tables 4 - 6, that stressful conditions cause accumulation of a certain bacteriocin concentration in a nutrient medium. The nisin activity in suspensions was between 2000 to 3000 IU/ml during the whole period of incubation. The exception was strain 729. For it we observed decrease in activity in the period of 3 - 4.5 months. The samples taken at 3 months of incubation revealed 2.1-fold decrease in activity for type A population. Decrease in nisin activity level continued by 4.5 months and by that moment it was nearly 3 times lower than initial value. For parallel culture 2-fold decrease in bacteriocin activity was observed at 4.5 months of incubation. It is noteworthy, that the maximum of total number of cells in type A

population, and increase in CFU/ml for both cultures also occurred in this period. For type A population, the increase was by 3 orders of magnitude (from 1.1×10^3 to 1.2×10^6), and for parallel culture - 1 order of magnitude (from 4.9×10^3 to 4.9×10^4). Thus, it is quite probable, that cells used bacteriocin as a nutritious substrate. By 12 months concentrations of nisin were restored to almost initial values, probably, due to utilization of nutrients from lyzing cells and activation of its biosynthesis as after 4.5 months decrease in total number of cells in population of strain 729 was observed.

To summarize the experimental data we can assume, that as a stress response *Lactococcus lactis* subsp. *Lactis* strains activate survival mechanism that apparently includes synthesis of a certain concentration of bacteriocin. At the same time stress nisin productivity differs from productivity in optimal conditions.

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