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INFLUENCE OF VARIOUS FACTORS ON THE *LACTOCOCCUS LACTIS* CELLS

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Abstract. *Bacteriocin-producing strains of Lactococcus lactis subsp. lactis of different origin were used in our experiments. UV-Irradiation of cells of bacteriocin-synthesizing lactoococci of different origin in a dose of 7.6–7600 erg/mm² was followed by their selective growth in an MRS medium, and their selection after UV-ray treatment. Some strains of lactococci had double UV treatment. The novel most active bacteriocin-producing strains were restored after lyophilization and storage. An exposure of 10 minutes should be considered an effective dose of treatment for wide strain 229. As a result, No.12 with an antibiotic activity of 3850 IU / mL was selected, which is 54 % higher than the initial one. The study of the physiological and biochemical properties of the variants, selected after UV-ray treatment, showed that they somewhat changed the rate of their growth and the accumulation of bacteriocin. The relative activity in the production of bacteriocin was calculated as the ratio of activity values and the number of produced cells. The inactivated homologous strain biomass and amino acids were applied as the factors of resuscitation. The control culture liquid was without supplements. VBNC cells of opportunistic strains formed within the first days of incubation. After 1 year of incubation, the VBNC values of Klebsiella pneumoniae 1954, Alcaligenes faecalis 415, Enterobacter aerogenes 418, Proteus vulgaris HX19222, Salmonella typhimurium were statistically equal (97.1–99.9%). After 1 day of stress, the strains of L. lactis did not form colonies up to 60–80%, after 5 days up to 82.1–99.6%, and after 1 year – 99.9%. Unwashed from the culture fluid, the inoculated cells proliferated and passed to the VBNC faster. With that inoculation, the nisin productive activity of cells was lower 10–78 times, depending on the strain of L. lactis. The study of resuscitation factors has shown that the supplement of homologous inactivated biomass of L. lactis (0.1%, 0.5%, 1%) was effective with 1% (a magnification of 2.65 at $p<0.05$) and 0.5% (magnification of 3.75 at $p<0.05$) only for the MSU strain. Strain F-116 was marked by a 4-fold increase in the ability for cultivation after the addition of 7 aminoacids: (glutamine, methionine, leucine, isoleucine, histidine, arginine, valine). After 1 year, the quantitative level of VBNC cells, which formed in the first days of incubation, was the same for opportunistic and probiotic cultures (97.1%–99.9%). All studied resuscitation factors were individually effective for bacterial strains.*

Key words**:** *Lactococcus lactis subsp. lactis, bacteriocin, nisin, opportunistic strains, factors, lactococci, resuscitation, UV-light, VBNC cells*

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1. INTRODUCTION

The most effective among the various factors whose impact can lead to a change in the properties of bacteria or a whole cell population are gamma or UV-irradiation [1]-[4]. It is known that sensitivity to UV is not the same for different bacterial species and strains. The radiation is enhanced by the formation of active free radicals, which are the cause of the non-genetic effects of radiation on organisms and capable to induce biochemical processes in cells. In the initial stage of the development of a culture, the enzymes of the intracellular fund are activated by "SOS" proteins, which protect the cell from unfavorable factors. It should be noted that natural peptides synthesized by microorganisms possess antimutagenic properties, which is important for creating probiotic preparations [5]. Genus *Lactococcus* has the "GRAS" status (absolutely harmless for human health and animals) according to the European Commission [6]. The physiological property of these bacteria is the synthesis of antimicrobial peptides – bacteriocins – which are the main component of probiotic preparations. Generally, probiotics may be defined as a safe and "natural" approach that helps to curb the population of bacteria that cause microbial infections. Lactococcci and their bacteriocins are widely used in various food fermentations and have a long history. Nisin is a unique, nontoxic antibiotic and it is the best studied compound in the bacteriocin group [7]. It is the only antibiotic substance given the status "GRAS", which is allowed for application as a food preservative under the code E234. Nisin is a low-molecular mass protein so it is easy to separate it into amino acids during digestion and it does not influence the microbiota of the gastrointestinal tract [8]. One of the very important properties of nisin is the activity against Gram-positive bacteria and bacterial spores of clostridia and bacilli, other non-spore forming bacteria, as well as many species of pathogenic *Streptococcus*, *Staphylococcus*, and *Listeria*, but it is not effective against Gram-

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negative enterobacteria and fungi [7]. The receipt of highly active producers of bacteriocins of a wide spectrum of action is an actual task. Various methods are used for breeding. Due to the effect on cells, such factors as radiation and other stresses (starvation, temperature, oxygenization, osmotic pressure, lyophilization etc.) appear viable, but the properties of uncultivated cells (VBNC) were insufficiently studied [1]-[4], [9]-[14]. VBNC cells can be resuscitated using serum, amino acids, vitamin K, blood-substitute etc. [11], [15]-[16].After exposure, the bacteria may return to their proliferation and metabolism.

There is evidence that VBNC bacteria appear under the influence of gamma-radiation at a dose higher than 0.5–2 kGy and UV-radiation at a dose of about 300 mJ/sm² [1]-[4]. The cells in the VBNC state are small and do not form colonies (CFU/ml). Metabolic processes, including the synthesis of bacteriocins, reduce the speed. The presence of VBNC bacteria in food, water, soil, organisms etc. may significantly reduce the total number of registrable pathogens; therefore, there is a danger of infection. Thus, it is necessary to study the condition of the formation of the VBNC state and the factors of cell resuscitation.

The aim of our research was the induction of biochemical processes of lactococci under the influence of UV-radiation, and the studying of the formation of the VBNC state under starvation influence and the cell reversion into normal state.

2. Materials and methods

In our experiments we used 3 probiotic strains of lactococci: one wild strain 229 with a bacteriocin activity of 2500 IU/ml (as nisin) was isolated from the cow milk from the Moscow Region; fusant strain F-116 (4200 IU/ml) was obtained after a protoplast fusion of two related strains with low nisin-synthesizing activities; and a classic nisin producing strain MSU with the activity of 2000 IU/ml [17], [18]. All strains were identified as *L.lactis* ssp. *lactis* (GenBank accession no. DQ 255954, EF100777, DQ255952, respectively). The induction by ultraviolet light (UVL) was studied for the variability of the wild strain 229 on the synthesis of nisin. The source of UV-irradiation was an installation of two parallel-mounted lamps BUV-15 (30 W), the main emission of which falls on the area of 253.7 nm. Doses of UV-irradiation were measured using a UVD-4 dosimeter. The biomass was determined nephelometrically at a wavelength of 600 nm. In this work, the effect of UVL on intact cells of the actively growing culture of the logarithmic growth stage, most sensitive to the action of UVL, was studied. Prior to irradiation, the cells were separated from the culture liquid by centrifugation at 1380 g for 20 min, washed twice with stabilizing ammonium citrate buffer with disodium ethylenediaminetetraacetate dehydrate (EDTA-Na_2)with a pH of 6.4. The bacterial suspension was resuspended in buffer in an amount of 5 ml, and irradiated in an open Petri dish for 1, 5, 10, 15, 20 minutes, which corresponded to 7.6-76 thousand erg / mm². To determine the survival rate, tenfold dilutions of control and experimental samples exposed to irradiation were carried out. Irradiated cells from a series of dilutions in the buffer were grown in a rich full-fledged MRS medium with the following composition (g / l): casein hydrolyzate – 10, glucose – 20, K_2HPO_4 xH_2O – 2, yeast extract – 2, Na-5 acetate, $MgSO_4xH_2O$ – 0.2, $MnSO_4$ xH_20 – 0.05 and an addition of 25 ml of 0.7% cysteine solution, and 1 ml of tween-80 suspensions of bacteria with subsequent sowing on dense media. The grown colonies were chopped by a replicator into agar medium MRS supplemented with 1.5% agar and 200 μg / ml nisin in order to accelerate the selection process according to the specified characteristic. They were incubated for two days in a thermostat at 28°C. After each exposure, the samples were centrifuged and washed with buffer to stop the mutagen. Then a direct count of the colonies was carried out. The bacteriocin product was provided by a diffusion method on the agar plate with *Bacillus coagulans* 429 as an indicator culture [14]. The grown colonies were interchanged by the replicator on the selective medium of MRS, and then on the agar MRS and MRS medium with *B. coagulans*. The influence of nisin as a selective component of the medium on the segregation of clones and their antibiotic activity was studied. The spontaneous and UV-induced variability of the strains was determined on the basis of antimicrobial activities. Colonies with the greatest inhibitory zones were selected. Some lactococci had double UV treatment. Active variants of lactococci were lyophilized on a “Krist” system of the brand Betta A (Germany) under vacuum at a temperature of -30 ° C to a residual moisture content of 2.5–4.0% and stored. Changes in their antibiotic and enzymatic activity were studied afterwards. Sensitivity to antibiotics was determined by the discodiffusion method [18]. The relative activity in the production of bacteriocin was calculated as the ratio of activity values and the number of produced bacteriocin cells. Quantitative determination of the antimicrobial activity was performed by measuring the zones of growth suppression with subsequent calculation involving a calibration plot for standard nisin solutions. Solutions of the preparations served as the standards: “Nisaplin (activity 1 mln. IU/g, “Aplin & Barrett”, Ltd.,UK). The obtained active variants of lactococci strains were also compared according to their cultural, physiological, and biochemical properties with the classical nisin-producing strain MSU and the recombinant strain F-116. The spectrum of inhibitory effects of the strains was studied by culturing strains under steady-state conditions in the medium of the above composition. The microorganisms used in these experiments were from the CM-MSU (Table 2).

The lactococci and the opportunistic strains *Proteus vulgaris* HX19222, *Klebsiella pneumoniae* 1954, *Enterobacter aerogenes* GISK 418, *Alcaligenes faecalis* 415, and *Salmonella Typhimurium* 79 were used concerning the study of the VBNC state.

We used trophic starvation as a stress factor. For this purpose, opportunistic and lactococci strains were inoculated in special media. The monitoring of viability and nonculturability was performed in a period of up to 1 year. The total number of live and dead cells was counted under light and luminescent microscopes after staining with a Live/Dead® double staining kit. The colony numbers were determined (CFU/ml) on the plates with a nutrient medium. The value for the VBNC bacteria was determined by comparing the total

numbers of viable and CFU cells. Statistical data reliability was at the level of ≤ 0.05. The temperature inactivated the biomass of homologous strains and the 7 amino acids (glutamine, methionine, leucine, histidine, arginine, valine) were applied as a factor of resuscitation. The control culture liquid was without supplements. There is evidence of an effective influence of various factors for getting substrains with more bacteriocin activity in the VBNC state and the resuscitation of the cells.

3. Results

The results of studying the spontaneous variability of the natural strain *L. lactis* subsp. *lactis* 229 on the basis of nisin formation on an agar MRS medium showed that the culture was not homogeneous. The activity spread fluctuated from 500 to 2700 IU / ml, where 71% of the 52 clones selected for the study had an activity level in the range 2300-2700 IU / ml, while 29% had an activity below the level of the parent. UV irradiation for 1 min without a significant effect on the survival of lactococci still somewhat changed the distribution of clones according to their antibiotic activity: 11.8% of the 52 clones selected for the study lost activity, while 34.5% had a level of activity of the parent strain 229. However, clones with increased antibiotic activity were not detected. Only 11.1% "+" variants were isolated with treatment of the bacterial suspension for 10 minutes, with an activity level higher than the initial level (Table 1).

Table 1. Effect of ultraviolet irradiation on the nisin-synthesizing activity of *Lactococcus lactis* subsp. *lactis* strain 229

Time of irradiation, min	Distribution of options for nisin-synthesizing activity, %			
	Lost activity	Below control level	Control	"+" variants
0	0	24.0±1.1	66.0±2.2	0
1	11.8±0.7	34.5±1.2	54.7±1.8	0
5	29.3±0.2	36.3±1.5	34.2±1.3	0
10	53.1±2.1	12.0±0.5	23.8±0.7	11.1±0.3

In a low-lying environment, UV exposure to the strain for 1 min caused a different distribution of the clones – 32.5% lost activity, 56% had activity from 2500 to 2900 IU / ml, i.e., induction of nisin formation was not observed. During treatment for 10 minutes, 14.2% of the colonies already had antibiotic activity in the range from 3000 to 3350 IU / ml. The distribution of lactococci by their nisin-synthesizing activity was as follows: 54% of surviving cells lost activity, 21.8% had lower activity, and 24.2% of cells had activity at a baseline level; therefore, an effective dose of treatment for this strain should be a dose of 76,000 erg / mm^2 (corresponds to an exposure of 10 min). As a result, strain No. 6 with antibiotic activity 25% higher than the original one was selected. Its colonies on the agar medium were larger, from 1.0 to 2.1 mm in diameter, and less transparent, possibly due to thickening of the peptidoglycan layer of the cell wall. There is also a dissociation of the culture under the UV-ray treatment, which is manifested in the change of the strain according to the morphological and biochemical features, as a consequence of which inactive populations accumulate inactive variants of antibiotic formation or induction of the desired trait. In the cultivation of variant No. 6 in the MRS medium, biomass and nisin synthesis reached their maximum by 9 hours, i.e. two hours ahead of the growth rate of the original strain 229. However, strain No. 6 lost the ability to utilize ribose, worse than the original fermented disaccharide maltose and lactose, but more actively fermented sucrose, and hydrolyzed dextrin. This variant became less sensitive to the antibiotic that inhibits the synthesis of the cell wall of Gram-positive bacteria – penicillins (benzylpenicillin, carbenicillin, ampicillin, methicillin, oxacillin) – but not to cephalosporins (cephalothin and cephalexin). It acquired resistance to broad-spectrum antibiotics inhibiting RNA synthesis aminoglycosides (sisomycin and kanamycin), and retained resistance to neomycin. Colonies that gave high activity were re-treated with UVL in the optimal dose (10 min exposure). At that, the survival rate of variant No. 6 was 0.3%, and the number of "+" variants was 17%. The following segregation of clones for nisinogenesis was observed: 22% had antibiotic activity from 1500–2000 IU / ml, 0% from 3000–3500 IU / ml, and 17% of positive bacterial clones ("+" variants) from 3500–3700 IU / ml, i.e., an increase in activity by 48%. It should be noted that in the population there were no clones sensitive to the nisin. Perhaps, along with the induction of nisinogenesis, the strain acquired resistance to nisin.

As a result of double, stepwise treatment of "+" strains, No. 12 variants were obtained with a nisin-synthesizing activity of 3850 IU / ml. A study of the dynamics of growth and development of variant No. 12 showed that double UV treatment extended the exponential phase of growth to 15 hours, and by the same time, the level of accumulation of nisin reached its maximum of 3850 IU / ml. With regard to fermentation of carbohydrates, this strain showed similarity to the initial strain regarding the consumption of pentoses, but became more resistant to a number of antibiotics: lincomycin, doxycycline, levomycetin, oleandomycin, streptomycin and tetracycline.

The results of our study of the spectra of antimicrobial action of culture liquids of the studied strains are summarized in Table 2. The wild strain 229 as strain MSU suppressed the growth of Gram-positive bacilli and cocci, including *Bacillus coagulans*, *Listeria monocytogenes,* and *Staphylococcus aureus* only in a manner similar to that of nisin. But nisin is not effective against enterobacteria. The study of the antimicrobial spectrum of action showed that fusant strain F-116 also suppressed the growth of Gram-positive bacteria as well as some Gram-negative cultures, including opportunistics and fungi, which is a

rare biological property for the natural strains of this species.

The fusant strain F-116 has a broad spectrum of antimicrobial action. It has been revealed, that the strain F-116 produced an antibiotic complex which differed from nisin [19]. UVL "+" variants No. 6 and 12 suppressed the growth of Gram-positive bacteria, but No. 12 had antifungal activity.

Obtained after treatment with UVL "+" variants after lyophilization, after 1-1.5 months, storage strains were restored and changes in their antibiotic and enzymatic activity in a number of passages were studied. It was established that, after 10 passages, strain No. 6 was noticed to resemble the original wild type both in terms of the level of antibiotic and enzymatic activity. Variant No. 12, obtained by stepwise, double UV light treatment, retained the stability of the changed properties, including the spectrum of antimicrobial activity (Table 2). The main component of the cell, responsible for sensitivity to ultraviolet rays, is its DNA. In response to DNA damage in the cell, the processes of restoring the original DNA structure and a mutagenesis mechanism are activated, ensuring the viability of cells with potentially lethal genome damage [5].

Table 2. Antimicrobial action of strains of *Lactococcus lactis* ssp. *lactis* in comparison with "Nisaplin"

Test-microorganisms	Strains					
	229	12	6	F-116	MSU	Nisaplin 3000 IU/ml
	Diameter of inhibition zone, mm					
Bacillus coagulans	17.0	23.0	20.0	26.0	15.0	17.0
Bacillus subtilis	17.0	21.0	19.0	24.0	16.0	18.0
Micrococcus luteus	19.0	21.0	9.0	27.0	18.0	21.0
Bacillus cereus	16.0	18.5	16.0	18.0	16.0	18.0
Staphylococcus aureus	20.0	21.5	19.0	19.0	19.0	25.0
Listeria monocytogenes	15.0	17.0	14.0	16.0	12.0	15.0
Salmonella Typhimurium	0	0	0	17.0	0	0
Escherichia coli	0	10.0	0	18.0	0	0
Proteus vulgaris	0	9.0	0	16.0	0	0
Klebsiella pneumoniae	0	0	0	12.0	0	0
Enterobacter aerogenes	0	0	0	14.0	0	0
Alcaligenes faecalis	0	0	0	16.0	0	0
Aspergillus niger	0	10	0	17.0	0	0
Rhodotorula aurantiaca	0	10.0	0	14.0	0	0
Candida guellermondii	0	9.0	0	16.0	0	0

The VBNC cells of the opportunistic cultures *Klebsiella pneumoniae* 1954, *Alcaligenes faecalis* 415, *Enterobacter aeruginosa* 418, *Proteus vulgaris* HX19222, and *Salmonella Typhimurium* 79 were formed within the first days of incubation. The VBNC value of these strains moved to statistically equal (97–99%). The study of the date of the transition of opportunistic bacteria in the VBNC state [Table 3] showed that after 1 year of incubation, 99.99% of *P. vulgaris* HX19222 did not form colonies, while with *K. pneumoniae* 1954 – 97.1% of the cells, i.e., bacterial cells, moved into the VBNC state. The values observed for *E. aerogenes* and *S. Typhimurium* GISK418 79 (about 99.1%) and *A. faecalis* 415 (99.33%) were close to VBNC values of aforementioned cultures. The most viable cultures were *E. aerogenes* and *P. vulgaris* GISK418 HX19222 (99.6% and 99.49% were alive, respectively). At the same time, *A. faecalis* 415 and *K. pneumoniae* 1954 had 68.6% and 66.4% of living cells, respectively.

Table 3. Viability and nonculturability of opportunistic bacteria

Bacteria	Maximum value CFU/ml (incubation period)	Values in a year incubation				
		CFU/ml	total cell number per ml	% live cell	% cells forming colonies	% VBNC cells
Proteus vulgaris HX19222	$4.77 \pm 0.52 \times 10^8$ (2 days)	$2.10 \pm 0.23 \times 10^3$	$5.80 \pm 0.64 \times 10^7$	94.4	0.0036	99.99
Klebsiella pneumoniae 1954	$3.89 \pm 0.26 \times 10^8$ (2 days)	$5.42 \pm 0.6 \times 10^6$	$2.76 \pm 0.3 \times 10^8$	66.4	2.9	97.1
Enterobacter aerogenes GISK418	$4.9 \pm 0.54 \times 10^8$ (7 days)	$2.15 \pm 0.24 \times 10^6$	$2.58 \pm 0.25 \times 10^8$	99.6	0.84	99.16
Alcaligenes faecalis 415	$7.1 \pm 0.78 \times 10^7$ (1 month)	$5.09 \pm 0.56 \times 10^5$	$1.10 \pm 0.12 \times 10^8$	68.6	0.67	99.33

As for the VBNC *L. lactis* ssp. *lactis* (strains MSU and F-116), which was formed from two different types of inoculum, washed (W) and unwashed (UW), after 24 hours of incubation, 60–80% did not form colonies on the synthetic medium. After 5–7 days of incubation, there was about 82–99.6% of them and, in a year, 99.9% of the cells did not form colonies depending on the conditions of the experiment. The lactococci population, which was obtained with unwashed inoculum, entered the state of VBNC faster and in a greater amount, but the total number of cells was opposite (Table 4). Perhaps, these cells were significantly more sensitive to trophic stress.

Table 4. Dynamics of bacteriocin-producing activity in *Lactococcus lactis* ssp. *lactis* MSU and F-116 strains populations during carbohydrate starvation

Time of incubation	Type of inoculum	Total number of cells per ml		Relative bacteriocin productivity IU/10^9 cells/ml		Multiplicity of differences in relative bacteriocin activity between UW and W variants	
		MSU	F-116	MSU	F-116	MSU	F-116
1 day	UW	$(1.5\pm0.2)\times10^9$	$(1.5\pm0.2)\times10^9$	2069	1633	25.1	41.8
	W	$(4.7\pm0.5)\times10^7$	$(4.3\pm0.5)\times10^7$	51906	68287		
2 days	UW	$(1.6\pm0.2)\times10^9$	$(1.4\pm0.2)\times10^9$	2083	1713	35.3	55.4
	W	$(3.4\pm0.4)\times10^7$	$(3.2\pm0.4)\times10^7$	73529	94936		
7 days	UW	$(1.8\pm0.2)\times10^9$	$(1.2\pm0.1)\times10^9$	1525	2379	41.7	26.7
	W	$(4.4\pm0.5)\times10^7$	$(4.7\pm0.5)\times10^7$	63636	63559		
10 days	UW	$(2.1\pm0.2)\times10^9$	$(1.3\pm0.1)\times10^9$	1322	1870	35.1	36.8
	W	$(5.3\pm1.6)\times10^7$	$(3.6\pm0.4)\times10^7$	46402	68820		
1 year	UW	$(8.8\pm1)\times10^8$	$(1.1\pm0.1)\times10^9$	3111	2641	0.44	18.9
	W	$(2.0\pm0.2)\times10^7$	$(4.9\pm0.5)\times10^7$	1375	49796		

Table 4 shows that the relative activity of bacteriocin-producing VBNC cells with W-inoculum was always greater than with UW-inoculum for the MSU and F-116 strains. Maximum differences were 41.7 (MSU) and 54.4 (F-116). In separate experiments the relative activity differed 78 times.

4. Conclusion

Thus, under the action of UV-rays and other stress factors, including trophic starvation, the mechanism of change of the cells properties was launched; they were allowed to obtain the *L. lactis* substrain with a higher bacterial productivity or created conditions for the emergence of VBNC bacteria. Furthermore, using a homologous biomass of lactococci and aminoacids, VBNC cells were resuscitated to a normal state. The studied processes are of practical importance. For example, the new substrains of lactococci are more promising for the production of bacteriocin. Bacteriocins are used for treatment or to ensure the safety of food products. The experimental detection of reversion shows the danger of a lack of appropriate monitoring of the presence of dormant microbes. It is necessary to discover the VBNC bacteria in clinical material, food, environmental objects, etc.

The study of resuscitation factors showed that the supplement of a homologous inactivated biomass of *L. lactis* (0.1%, 0.5%, 1%) was effective with 1% (a magnification of 2.65 at $p<0.05$) and 0.5% (magnification of 3.75 at $p<0.05$) only for the MSU strain. For the fusant strain, L. *lactis* F-116 showed a 4-fold increase in the ability of cultivation after the addition of a 7-aminoacid mixture.

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