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EXPERIMENTAL ARTICLES

Adaptation of Lactic Acid Bacteria to Unfavorable Growth Conditions

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Abstract—The adaptation of lactic acid bacteria (LAB) to unfavorable growth conditions, e.g., depletion of nutrient sources, overthreshold cell density of a population, or antibiotic impact, was shown to include: (1) formation of cyst-like dormant cells (CDC) providing for survival and species preservation and (2) realization of intra-population phenotypic variability, which is demonstrated by development of non-dominant colonies on plates inoculated with CDC suspensions. In *Lactobacillus plantarum*, the dormant cells, which retained viability and heat resistance for a long time, were formed in 10- and 20-fold concentrated suspensions of the stationary phase cells. In 4-month cell suspensions, two types of cells were present, CDC and L-forms. The CDC of *Lactococcus lactis* were formed in (1) post-stationary cultures grown under glucose limitation and (2) in stationary phase cultures resuspended in starvation medium (without glucose). Populations of CDC stored for different periods of time varied in the ability for phase variation; as a result, both variants exhibited a shift of the population's CDC spectrum to the transition of the dominant S-colony type to the R-type up to complete substitution (by day 25). In *Lactobacillus acidophilus* AT-41, CDC appeared in (1) post-stationary cultures grown on a nitrogen-limited medium; (2) autolyzing cultures treated with ampicillin or erythromycin; and (3) concentrated (10- and 20-fold) suspensions of stationary-phase cells. At plating of *L. acidophilus* CDC, the substitution of the S-type for the dominant R-type in variants (1) (day 30), (2) (100 μg/ml ampicillin, day 10), and (3) (day 25) was 68.6%, 30.1%, and 61.2%, respectively. The S-variant of *L. acidophilus* was used for development of a novel lactofermented product based on vegetable (beet) juice fermentation, which sustained high titer of viable cells (2×10^6 cells/ml).

Key words: lactic acid bacteria, *Lactobacillus acidophilus, Lactobacillus plantarum, Lactococcus lactis*, adaptation, dormant forms, population variability, selective media.

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Permanent interest in the study of the adaptation mechanisms of lactic acid bacteria (LAB) is caused by deterioration of the environmental conditions with a consequent diminution of protective forces of the human organism and the outburst of diseases associated with bacterial imbalance in the gastrointestinal tract [1–4]. The LAB, symbiotic partners of animals and plants, are actively used for the manufacture of pro- and prebiotic products, natural food preservatives, and as therapeutic agents in medicine [5–9]. The attention of researchers to the LAB survival strategy is induced by scientific interest in the theory of stress and by the demand for lactic acid products containing viable LAB cells, which retain viability for a long time, adapt quickly to the conditions of the gastrointestinal tract, and resume active metabolism.

Microorganisms respond to such changes of environmental conditions as depletion of nutrients, energy sources, spatial resources, or under the impact of damaging factors (deteriorating physicochemical conditions, antimicrobial agents, etc.) by means of evolutionally developed, inherited adaptation mechanisms. Formation of the dormant forms (DF) for survival of a population under unfavorable growth conditions is the most important of such mechanisms [9–11].

The intrapopulation phenotypic variability makes its own contribution to the adaptation of microorganisms; it affects, inter alia, the effectiveness of symbioses and substitution for or maintenance of productive

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variants [12, 13]. Intrapopulation transitions are believed to be a result of reversible intragenomic rearrangements, a specific type of mutations resulting from the transposition of mobile genetic elements, "switching on/off" gene expression, phage or plasmid conversion, etc. [14–16]. According to some hypotheses, the phase variation is related to the resting state and to the action of signal molecules which control the expression of the stationary phase genes. Previously, we demonstrated the dependence between the phenotypic variability revealed on solid media and the type of dormant bacterial cells (*Bacillus cereus*, *B. licheniformis*) used as inoculum [17–19]. The highest genotypic instability was observed in cyst-like dormant cells (CDC), and their plating resulted in significant changes of the population spectrum due to development of the colonies of non-dominant variants [17, 18]. These results demonstrated that microorganisms possess flexible mechanisms of adaptation to the environmental changes which go beyond the tolerance limits for a given species.

The goal of our work was to study the adaptation responses of lactic acid bacteria to stressors, both programmed (depletion of nutrient sources) and nonprogrammed (antibiotic treatment) in their ontogenesis.

MATERIALS AND METHODS

The objects of our research were lactic acid bacteria *Lactobacillus acidophilus* AT-41 from the culture collection of the Department of Milk and Dairy Products, Moscow State University of Applied Biotechnology (MSUAB), *Lactococcus lactis* TB2 from the collection of microbial cultures of the State Research Institute of Genetics and Selection of Industrial Microorganisms, and *Lactobacillus plantarum* from the collection of microbial cultures of the Department of Microbiology, Biological Faculty, Moscow State University.

L. acidophilus was grown on MRS medium containing the following (g/l): $Na_2HPO_4 \cdot 12H_2O$, 2; $CH_3COONa \cdot 3H_2O$, 5; ammonium citrate, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.05; peptone, 10; beef extract, 10; yeast extract (Difco), 5; glucose, 20.

L. lactis was grown on M12 medium containing the following (g/l): $Na_2HPO_4 \cdot 12H_2O$, 8.5; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.12; peptone, 5; beef extract, 2.5; yeast extract, 5; glucose, 5; ascorbic acid, 0.5.

L. plantarum was grown on a Bifidum medium containing the following (g/l): casein hydrolysate, 30; yeast extract, 5; glucose, 7.5; lactose, 2.5; cysteine, 0.5; NaCl, 2.5; $MgSO_4 \cdot 7H_2O$, 0.12; ascorbic acid, 0.5; $CH_3COONa \cdot 3H_2O$, 0.3. The inoculum (the stationary growth phase culture) was added to the initial optical density (OD) of cell suspensions 0.2 (λ = 650 nm, l = 10 mm, Specord, Germany). Cultivation was carried out in 250-ml flasks with 50 ml of the medium on a shaker (140–160 rpm) at 37°C (*L. acidophilus*), 28°C (*L. lactis*), or 30°C (*L. plantarum*).

To obtain the CDC, LAB were exposed to various stresses, i.e., directional modification of cultivation conditions and the application of antimicrobial agents. Variant (1): CDC were formed in autolyzed suspensions with a high cell density (space exhaustion stress): the cells from the stationary growth phase were precipitated by centrifugation (5000 *g*, 15 min); the pellet was resuspended in the supernatant volume 10, 20, or 30 times less than the initial volume before centrifugation. Variant (2) included either the cultivation in a medium with a 5-fold decreased content of nitrogen sources or glucose (the stress of depletion of nutrient sources), or the transfer of the stationary phase cells to the starvation medium with no carbon sources (starvation stress). Variant (3): the exponential phase cells were treated with amplicillin or erythromycin in the concentrations of 30 and 100 μg/l (the stress caused by an antimicrobial agent). The cell suspensions obtained by all the variants were incubated under static conditions at room temperature for 7 days to 12 months.

The variants of *L. acidophilus* and *L. lactis* were obtained by plating of the DF of these bacteria on agarized (1.5%) MRS and M21 media followed by incubation at 37 and 28°C, respectively. The colonies of certain morphotypes (5 clones each) were successively reinoculated in 5–6 transfers.

The viability of LAB was determined as the number of colony-forming units (CFU) obtained by plating on agarized media of cell suspensions from respective dilutions.

The heat resistance of LAB variants was determined from the CFU number after heating of the cell suspensions (0.2 ml) in an U-10 ultrathermostat at 60°C for 10 min.

Endogenous cell respiration was detected according to Scholtz–Ostrovsky on a LP7E polarograph in a 1-ml oxygen cell [20].

Antagonistic activity was determined by the method of developing mixed populations of LAB and tester strains, compared to the growth of test organisms in monocultures [21]. Daily LAB cultures (the second transfer) in liquid media were diluted with saline solution to the density of 10 units according to the turbidity standard of the State Institute of Standardization and Control (SISC) to obtain standard LAB suspensions. Cultures of the test organisms *Escherichia coli* K-12 and *Staphylococcus aureus* 209-P (the second transfer), freshly grown on agarized media, were washed off the slanted beef-extract agar by saline solution and diluted to the density of 10 according to the turbidity standard. In the control variants, 1 ml of the suspension of each test organism was added per test tube with 9 ml of liquid nutrient medium. In the experimental variants, 1 ml of the standard LAB suspension was also added into the test tubes. The cultures in the control and experimental variants were incubated at 37°C for 24 h, and then the number of viable cells (CFU) of the tester strain was determined by plating of aliquots on nutrient agar.

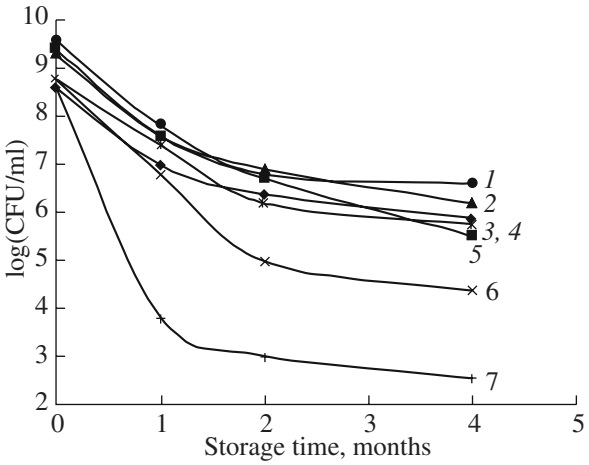

Fig. 1. Dynamics of viable dormant LAB cell numbers (CFU) in long-stored suspensions: *L. plantarum* in phosphate buffer with SiO_2, 30-fold concentration (*1*); *L. plantarum* in phosphate buffer with SiO_2, 20-fold concentration (*2*); *L. acidophilus* in growth medium, 20-fold concentration (*3*); *L. acidophilus* in growth medium, 10-fold concentration (*4*); *L. plantarum* in the native growth medium with $CaCl_2$, 10-fold concentration (*5*); *L. plantarum* in growth medium, without concentration (*6*); *L. acidophilus* in growth medium, without concentration (*7*).

The adhesive ability of LAB cells was assessed from cell counting in an Axio Imager D1 microscope (Carl Zeiss, Germany) before and after their adsorption on a hydrophilic glass surface; the cells were stained with the fluorescent dye Acrydine Orange.

The laboratory sample of the lactofermented vegetable beverage was prepared from beet juice. The *L. acidophilus* culture was used as a starter. Cultivation was carried out in 500-ml flasks with 250 ml of the medium on a shaker at 140–160 rpm. The organoleptic (color, fragrance, taste) and physicochemical characteristics of the ready beverage (active acidity, content of dry substances, titrated acidity) were determined by the standard procedures [22].

Statistical analysis was performed by standard mathematical methods (Student's *t*-test and calculation of the mean square root deviation) using the Microsoft Excel XP software package. The data set was considered as uniform, if the mean square root deviations σ did not exceed 10%. The differences between the data sets were considered reliable at a probability criterion $P < 0.05$.

RESULTS

The adaptation mechanisms of lactic acid bacteria were studied by exposing their cultures to the stressors which the bacteria most often encounter in their habitats. The adaptation response was assessed by maintenance of the viability of LAB cells for a long period of time (1–12 months) and formation of metabolically inactive dormant forms.

The first series of experiments simulated the stress conditions caused by depletion of nutrient sources and/or the lack of space (high cell density); under these conditions, spontaneous autolysis of some part of the population is induced. The extracellular concentration of anabiosis autoinducers increases due to their release from autolyzed cells, inducing the formation of DF from the cells remaining intact [17, 18]. This situation was simulated by artificial concentrating (10-, 20-, or 30-fold) of LAB cell suspensions followed by their storage for 4 months in the media of varied composition (in the spent medium with and without 0.02% $CaCl_2$; in phosphate buffer with 0.09% SiO_2). In autolyzed concentrated cell suspensions of *L. plantarum*, the number of viable cells (Fig. 1) decreased by three orders of magnitude during the first 2 months of storage and then stabilized at the level of 0.03–0.09% of the initial level; this value depended, inter alia, on the medium where the cells were stored. Microscopic observations showed that a part of the population was lysed, while some cells remained intact; after 1-month incubation, 50% of them acquired refractivity, which is a morphological feature of DF [23]. Electron microscopic analysis demonstrated that after 1–4 months storage, the population of surviving cells was represented by the two types of cells with the ultrastructural organization essentially different from that of the vegetative forms (Fig. 2a–c). In DF of the first type, the cell wall became denser and thicker during the storage; the periplasmic space, electron-transparent or filled with dense granules, was enlarged; the cytoplasm texture became heterogeneous and lumpy; and the nucleoid was compacted (Fig. 2b). This combination of ultrastructural characteristics enables classification of such cells as cyst-like dormant forms described previously for a number of non-spore-forming bacteria [10, 18, 23]. The second type of surviving cells was represented by L-forms without a cell wall, with an intact cytoplasmic membrane, lumpy cytoplasm, and a weakly compacted nucleoid (Fig. 2c). The surviving cells obtained in long-stored (4 months) autolyzed suspensions exhibited no metabolic activity (polarographic measurements showed the absence of endogenous respiration) and were highly resistant to elevated temperatures (Table 1). Heat treatment (60°C, 15 min) of vegetative cells from stationary growth phase (48 h) and post-stationary cells (4 mouths of storage) resulted in their death, whereas the surviving cells of experimental variants preserved viability at a level of 0.8–8.0% (of their number before the heat treatment).

A similar situation was observed in 10- and 20-fold concentrated cell suspensions (in the growth medium) of another LAB, *L. acidophilus*. After 1 month of storage, the CFU number decreased by 2 orders of magnitude and then stabilized; after 4 months of incubation, it was 1.0 and 1.5% of the initial value for 10- and 20-fold concentrated suspensions, respectively (Fig. 1). Electron microscopy of concentrated *L. acidophilus* suspensions stored for 4 months also revealed two types of cells (Fig. 2d, e). The cells of the first type had a dense,

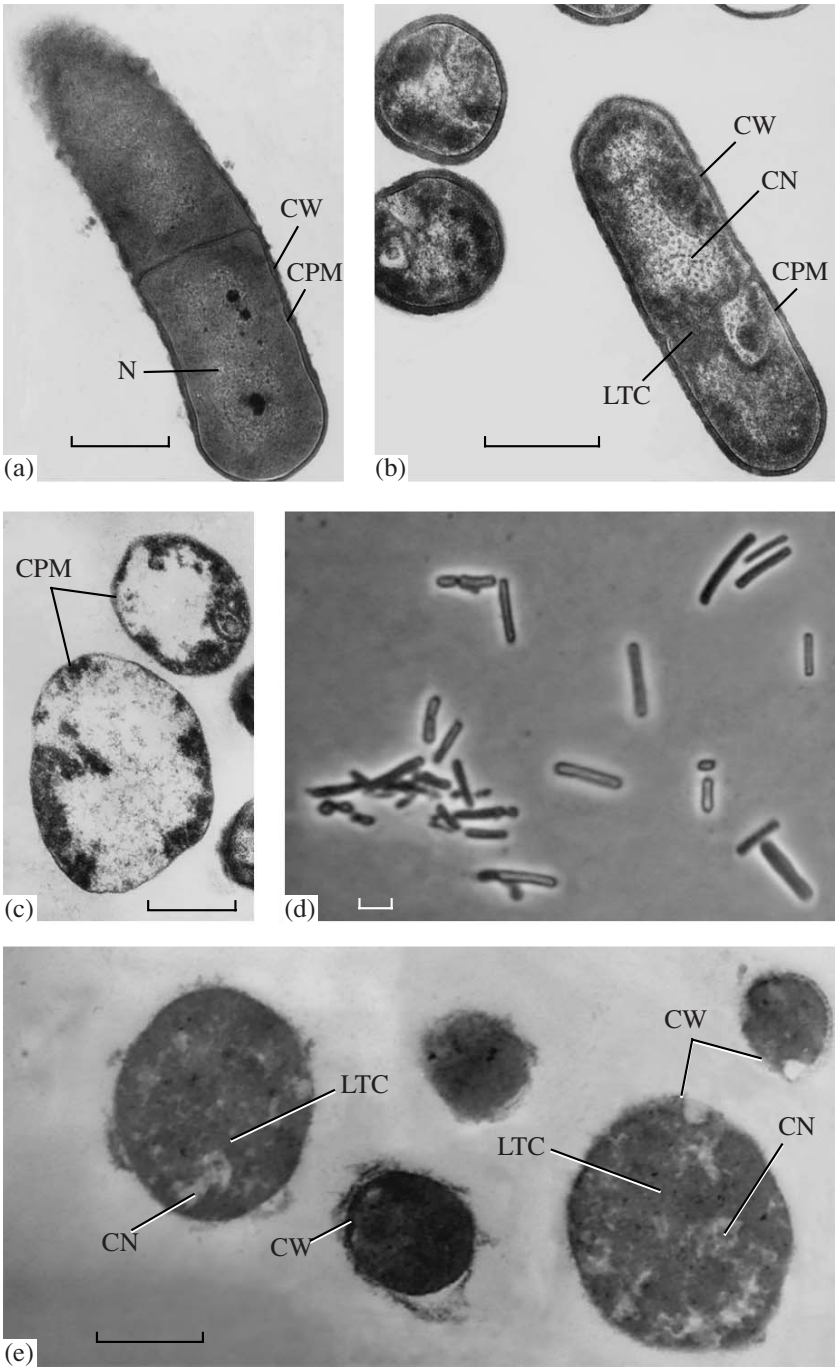

Fig. 2. Microphotographs of dormant LAB cells obtained in 20-fold concentrated cell suspensions. (a–c) Ultrathin sections of *L. plantarum* DF: CDC, storage time 3 weeks (a); CDC, storage time 4 months (b); L-forms without cell wall, storage time 4 months (c). (d, e) CDC of *L. acidophilus*, storage time 4 months: phase contrast (d); ultrathin sections (e). Designations on the photographs: CW, cell wall; CPM, cytoplasmic membrane; N, nucleoid; CN, compacted nucleoid; LTC, lumpy-textured cytoplasm. Scale bar: 1 μm (a–c, e); 2 μm (d).

thickened cell wall, heterogenic texture of the cytoplasm, and a more compacted nucleoid than the vegetative cells. The cells of the second type were of a much smaller size and had a more electron-dense cytoplasm. Similar to *L. plantarum*, the surviving cells of 4-month suspensions of *L. acidophilus* exhibited no endogenous respiration and possessed higher resistance to stress impacts (heat resistance).

Table 1. Heat resistance of *L. plantarum* dormant forms (4 months of storage)

Conditions of DF formation	Number of viable *L. plantarum* DF, CFU/ml (% of CFU before heat treatment)	
	Before heat treatment	After heat treatment, 60°C, 15 min
10-fold concentration, storage in growth medium with $CaCl_2$	3.2×10^5 (100)	2.7×10^3 (0.8)
30-fold concentration, storage in phosphate buffer with SiO_2	4.0×10^6 (100)	3.2×10^5 (8.0)
Poststationary culture without concentration	2.3×10^4 (100)	0
Control, stationary-phase cells (48 h)	3.9×10^8 (100)	0

Dormant LAB cells, an adaptive response to unfavorable conditions, were also obtained by cultivating the bacteria on modified media with an increased C : N ratio: 5-fold decreased nitrogen content for *L. acidophilus* and 5-fold decreased glucose content for *L. lactis*, as well as by the transfer of the stationary phase cells to a starvation medium without glucose. As in the case of C > N modification of the medium, the biosynthesis of autoregulators with the function of anabiosis autoinducers is stimulated. Their elevated level induces DF formation, as was previously demonstrated for some bacteria [10, 18, 23].

In our experiments, a rather high number of viable cells (CFU), about 1–10% of their quantity in the stationary phase, was maintained in 1–4 month post-stationary cultures of *L. acidophilus* grown under nitrogen limitation and of *L. lactis* grown under glucose limitation or incubated in the starvation medium (without glucose) (Fig. 3). At the same terms of incubation, the CFU number in *L. acidophilus* control cultures decreased by 5–6 orders of magnitude; in the cultures of *L. lactis* incubated for 15 days, it dropped to a single CFU/ml (Fig. 3). After 1 month of incubation, the surviving LAB cells of the experimental variants acquired refractivity; their ultrastructure became similar to that described previously for the DF obtained in concentrated cell suspensions. Their ultrastructural characteristics, heat resistance, and the absence of metabolic activity supported their classification as dormant forms.

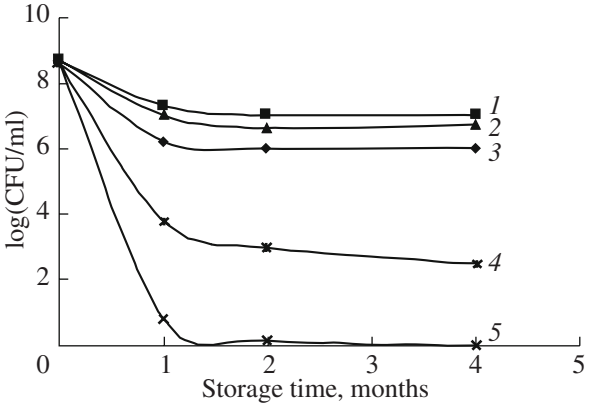

Fig. 3. Dynamics of viable dormant LAB cell numbers (CFU) in long-stored suspensions: *L. acidophilus* in nitrogen-limited medium (*1*); *L. lactis* in glucose-limited medium (*2*); *L. lactis* in starvation medium (*3*); *L. acidophilus* in MRS growth medium, without concentration (*4*); *L. lactis* in M21 growth medium, without concentration (*5*).

Thus, under stress conditions induced by high cell density or depletion of nutrient sources, LAB form cyst-like cells that possess all the features of dormant forms.

Plating of the obtained dormant LAB cells on solid media revealed changes in the spectrum of the phenotypic variants within the population evident as the morphological characteristics of the colonies. In autolyzing the high-density cell suspensions of *L. acidophilus*, a substantial increase of the minor S-type variant was detected already on the 10th day of incubation, especially in the variants with a 20-fold concentration (Fig. 4). S-type colonies were predominant in the experimental samples stored for 25 days and more. In the control (storage in the growth medium), the phase variation index was much lower: the content of the S-variant was 6 and 30% on the 10th and 25th day, respectively. Modification of the spectrum of phenotypic variants within the population is an adaptive mechanism that determines emergence of various phenotypes in order to enable selection of the variants stable under the given conditions and, hence, the species survival. It is provided by the differing properties of the variants. Our experiments demonstrated that the minor variant of *L. acidophilus* had a higher maximal specific growth rate and biomass accumulation level (1.5- and 1.2-fold, respectively) than the dominant R-variant (Fig. 5). The resistance of the S-variant cells to high temperature (60°C, 10 min) was 3.3 times higher than in the cells of the R-variant. The antimicrobial activity against tester strains *E. coli* K12 and *S. aureus* P-209 was also higher in the S-variant (Table 2). While the R-variant in the test system suppressed the development of *E. coli* and *S. aureus* by 2 and 5 orders of magnitude (by CFU values), respectively, joint cultivation

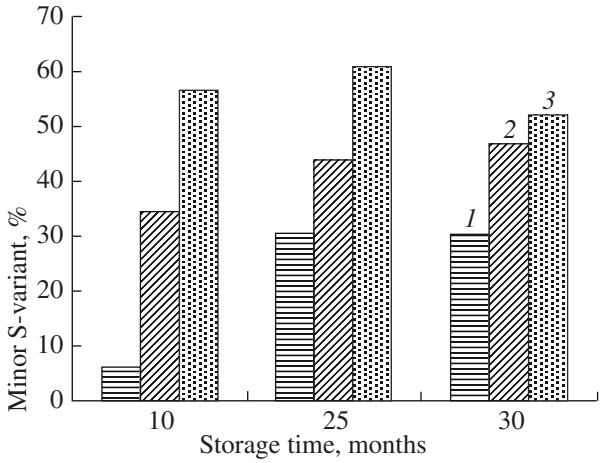

Fig. 4. Phase variation index (% of the minor S-variant) of *L acidophilus* at plating of the cells stored in the growth medium (*1*) or at 10- (*2*) and 20-fold (*3*) concentration.

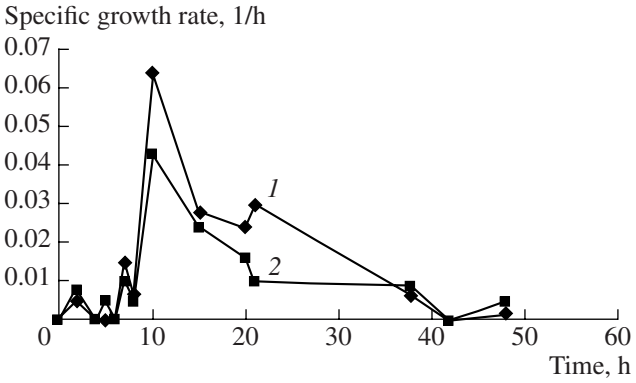

Fig. 5. Specific growth rates of the S-variant (*1*) and the R-variant (*2*) of *L. acidophilus.*

of *L. acidophilus* S-variant with the tester strains completely inhibited their growth. At the same time, the cell adhesive capacity was higher in the R-variant (Table 3), which is probably essential for its dominance at biotope colonization and suggests the preference of this variant in production of medicinal preparations. On the contrary, the S-variant of *L. acidophilus*, possessing better physiological and biochemical characteristics, will survive best under saprophytic conditions; thus, it would be reasonable to use it for industrial purposes. In our experiments, the S-variant was used as a starter for the new lactofermented dietetic beverage based on red beet juice. The beverage was obtained by adding the starter (stationary-phase culture of *L. acidophilus* S-variant) to the prescribed medium followed by incubation at 37°C under static conditions without stirring for 24 h. Organoleptic assessment of the laboratory sample of the lactofermented vegetable beverage showed its excellent properties (Table 4). During the storage of the finished product (20 days), the following physicochemical characteristics were monitored: pH, titrated acidity, and the content of dry substances (DS) (Table 5). After 24 h of fermentation, pH decreased to 4.2 and then stabilized. The indices of titrated acidity increased from 4.2 mol NaOH/l in the ready beverage (after 24 h) to 7.6 mol NaOH/l on the 20th day of storage. The content of DS during the fermentation (24 h) decreased by 1.6% and was 5.6% on the 20th day. An important consumer property of the beverage was the maintenance of a high titer of viable LAB cells (by CFU number): their content in the ready beverage (24 h) was 3.2×10^7 cells/ml and changed insignificantly after 20 days of storage (2.1×10^6 cells/ml), which indicated the high therapeutic and preventive properties of the product.

Plating of *L. lactis* DF suspensions obtained in glucose-limited cultures also showed a shift in the spectrum of phenotypic variants towards an increase of the minor R-variant (the S-variant is dominant in *L. lactis*). In this LAB species, the transition was much more pronounced than in *L. acidophilus* (Fig. 6a). After 25 days of storage, only the cells forming R-type colonies were observed in the suspensions of *L. lactis* DF from experimental variants (Fig. 6b). No emergence of the R-variant was observed in the control variants (*L. lactis* grown

Table 2. Antagonistic activity of the initial strain and the variants of *L. acidophilus* against tester strains *E. coli* K-12 and *S. aureus* 209-P

Viability of the cells of opportunistic pathogenic strains, CFU × 10^9/ml (%)				
Tester strain	Experimental variants			
	Control (tester strain in monoculture)	Joint cultivation with *L. acidophilus*		
		Initial strain of lactobacilli	R-variant	S-variant
E. coli	1.3 (100)	0.07 (4.4)	0.04 (3)	Complete inhibition of growth
S. aureus	0.38 (100)	0.03 (1.68)	Growth inhibition by 5 orders of magnitude	Complete inhibition of growth

Table 3. Dynamics of adsorption of the cells of *L. acidophilus* variants on a hydrophilic surface

Variant	Portion of adsorbed cells, %		
	Time of adsorption, h		
	0.5	1.0	3.0
R	18.3	69.0	34.6
S	15.1	44.7	28.3

on complete M21 medium). The experiments with *L. lactis* showed that selective conditions were necessary for the predominant development of either S- or R-variants. Preferential growth of the dominant S-variant of *L. lactis* was observed on M21 medium both at 28°C and at 37°C, whereas 5-fold diluted M21 medium and 28°C provided selective conditions for the development of the minor R-variant.

The next series of experiments was carried out to investigate the possibilities of LAB adaptation to the stress induced by the antibacterial agents, in this case, antibiotics; this experiment simulated the situation that is frequent at antibiotic therapy and results in the death of LAB and development of dysbacteriosis. The treatment of the exponential-phase *L. acidophilus* cells with erythromycin (30 and 100 μg/ml) resulted in rapid lysis of most of the cell population. The cells that remained intact decreased in size and their ultrastructure became similar to the one described previously and typical of the DF of these bacteria (Fig. 2e). According to the CFU values, the number of such cells was very low (in the range of 50–100 CFU/ml); after 14 days they were not detected at all. When another antibiotic, ampicillin (30 or 100 μg/ml), was introduced into the *L. acidophilus* culture, the number of viable cells (CFU) after 10 days remained at the level of 0.8–1.0% of the initial value. A dissociative transition was observed in the surviving cells in the autolyzed suspensions, i.e., the minor S-variant substituted the dominant R-variant (Table 6). The portion of the S-variant practically did not depend on the antibiotic concentration but increased significantly during the storage of cell suspensions. Importantly, the S-variant has a higher heat resistance, which is evidence of its higher resistance to the damaging factors of a different nature.

Thus, the effect of antibiotics induced a quick response of the LAB culture, manifesting itself in autolysis of most cells of the population with the formation of a small number of surviving cells; the germination of the latter resulted in a change in the spectrum of the variants.

DISCUSSION

The results of the present work demonstrate a flexible response of LAB to environmental changes. Transition of the LAB cells into the resting state accompanied by CDC formation and emergence of intraspecies variability, i.e., transition of the dominant phenotype to the minor one, are the major mechanisms of LAB adaptation to stresses. These interrelated adaptation processes facilitate the survival of a population (species) under unfavorable growth conditions and selective development of the variant most adaptive to the new conditions at germination of surviving dormant cells.

The present work demonstrated for the first time that, during their development cycle or under unfavorable growth conditions, LAB, similar to other nonspore-forming bacteria, produce cells that possess all, the characteristics of dormant forms: prolonged maintenance of viability under conditions favoring cell autolysis (storage in liquid media at room temperature); absence of metabolic activity (endogenous respiration); resistance to stress impacts (heat treatment); and changes in the ultrastructural organization indicating essential intracellular structural rearrangements. The number of produced LAB DF depended on conditions of growth (modification of the medium) and post-stationary incubation of the cultures. For example, nutrient limitation, which shifted the metabolic equilibrium towards lipid metabolism and stimulated the biosynthesis of anabiosis autoregulators [10, 18, 23], contributed to a substantial increase of the DF number. In experimental variants with concentrated cell suspensions, the

Table 4. Organoleptic properties of the lactofermented beverage based on red beet

Property	Characterization	Grade
Color	Saturated vinous, corresponding to red beet color	7 (Excellent)
Fragrance	Corresponding to beet fragrance, profound, pronounced, without extraneous smell	12 (Excellent)
Taste	Pronounced beet taste, no extraneous taste, refreshing due to acidic flavor of the products of lactic acid fermentation	12 (Excellent)
Total grade		Excellent

DF were less numerous but more resistant to heat treatment than those formed in the development cycles. Importantly, the intraspecies polymorphism of dormant LAB cells was demonstrated on the *L. plantarum* model, where the L-type cells were present along with cyst-like forms. The methods of obtaining suspensions of persistent, stress-resistant dormant forms of LAB can be recommended for production of lactic acid milk foods and probiotic preparations in the food industry and medicine.

One more significant result of this work is the detection of the mechanisms of regulation of the LAB intrapopulation variability. The frequency of transition of the dominant variants into the minor ones was found to depend on a stress impact. For example, in *L. acidophilus* grown under standard conditions, the portion of the minor variant after 11 days of incubation was 6% in the ageing culture; 56% in the 10-fold concentrated cell suspension; and 30% after treatment with ampicillin.

These results are in agreement with the literature data on the emergence of minor isolates (phenotypes) of *L. lactis* under nonoptimal or unfavorable growth conditions [2–4, 9]. Plating of *L. lactis* cell suspensions stored for a long time (1 year) under starvation (glucose-free medium) was shown to result in emergence of the variants different from the dominant type in both the morphological (colony size) and biochemical characteristics. These minor variants had higher resistance, to heating, oxidative stress, and to high concentrations of bile. The differences revealed at the level of chromosomal DNA demonstrated the changes in expression regulation of the genes responsible for utilization of lactose, maltose, and ribose, as well as of some stressor genes [9]. For *Enterococcus feacalis*, *Lactococcus lactis* and *Lactobacillus delbrueckii* subsp. *lactis*, the data were obtained on the phenotypic and genotypic differences between LAB intrapopulation variants (clones). The dominant phenotypes differed from other forms both in plasmid profiles and a number of biochemical characteristics, including the antagonistic and proteolytic activities [24–27].

Table 5. Physicochemical indices of the lactofermented beverage based on red beet

Property	Time of storage of the ready beverage, days		
	0	1	20
pH	6.7	4.2	4.2
Dry substances (DS), %	7.2	6.8	5.6
Titrated acidity, mol NaOH/l	4.0	6.1	7.6

The patterns of regulation of the LAB population variability revealed in this work can be of practical importance. The method for production of CDC with unstable genotypes, followed by their plating on solid nutrient media for development of the colonies with different phenotypes, may be recommended for extension of the dissociative spectrum and rapid isolation of the variants with required characteristics. For stabilization of the development of dominant or minor phenotypes, selective cultivation conditions (medium composition and growth temperature) should be used, as was demonstrated in experiments on the obtaining of *L. acidophilus* and *L. lactis* variants. Since these variants differ in the growth, physiological, and biochemical characteristics, the variants recommended for biotechnological or medical applications should possess the

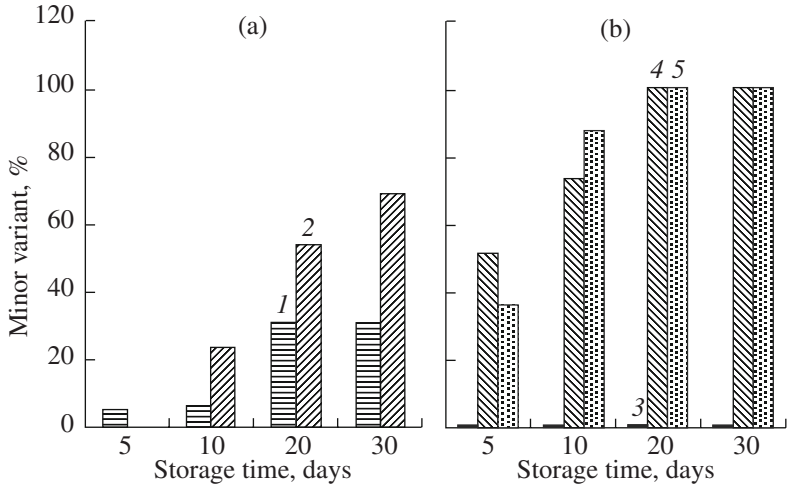

Fig. 6. Phase variation index (% of the minor variant): *L. acidophilus* (a) at plating of DF stored in the standard growth medium (*1*) and in nitrogen-limited medium (*2*); *L. lactis* (b) at plating of DF stored in the standard growth medium (*3*), in glucose-limited medium (*4*), and in starvation medium (without glucose) (*5*).

Table 6. Phase variation of *L. acidophilus* population after ampicillin treatment of the growing culture

Duration of ampicillin treatment, days	Emergence frequency, % of total CFU number					
	Antibiotic concentration, μg/ml				Control (no antibiotic)	
	30		100			
	$R_{dominant}$	S	$R_{dominant}$	S	$R_{dominant}$	S
0*	86.3	13.7	85.1	14.9	100	0
1	86.2	13.8	84.2	15.8	95.0	5.0
3	84.2	15.8	81.3	18.7	95.0	5.0
10	72.6	27.4	69.9	30.1	95.0	5.0
14	80.7	19.3	70.6	29.4	94.0	6.0

* Immediately after the ampicillin introduction into the culture.

following advantages: good growth and physiological–biochemical characteristics or pronounced adhesive properties. This conclusion was confirmed by the present study in the course of development of a novel lactofermented product based on the vegetable juice and possessing a high probiotic potency.

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