TOLERANCE TO FREEZE-DRYING CONDITIONS OF *Lactobacillus delbrueckii* subsp. *bulgaricus* STRAINS AFTER ACIDIC EXPOSE

Ivo Ganchev

Trakia University of Stara Zagora
Faculty of Technics and Technologies of Yambol
38 Graf Ignatiev str., Yambol 8602, Bulgaria
E-mail: ivotg@abv.bg

Received: 02 April 2023
Accepted: 02 May 2023
DOI: 10.59957/jctm.v59.i1.2024.3

ABSTRACT

The goal of this work was to investigate the effect of fermentation pH and time on the acid tolerance, glycolytic activity, and survival during freeze drying of L. delbrueckii subsp. bulgaricus WDCM 00102 strains during cultivation in the medium, based on pretreated biomass of Spirulina platensis. During the study, when the fermentation process is carried out at a constant pH, a higher value of glycolytic activity is established in the cells of the probiotic culture, which decreases when the pH is lowered. The survival rate of cells of L. delbrueckii subsp. bulgaricus WDCM 00102 after lyophilization shows similar values after cultivation for 25 and 37 h ($p < 0.05$), while the opposite dependence is established in experiments related to changes in the pH of the cultivation medium. pH and fermentation time significantly influence the glycolytic activity of L. delbrueckii subsp. bulgaricus WDCM 00102 and on the resistance of its cells to acid stress and lyophilization conditions.

Keywords: Lactobacillus delbrueckii subsp. bulgaricus WDCM 00102, acidic stress, freeze drying process, glycolytic activity.

INTRODUCTION

Lactic acid bacteria include a large range of microbial species that belong to probiotics, which, when taken optimally, have a beneficial physiological effect on the host organism. They are distinguished by high adaptability to the culture medium, in which they grow to a high cell density, survival to the unfavorable conditions of the technological process (cooling, etc.), they retain their viability for a long time even in the unfavorable conditions of the gastrointestinal tract [1]. The technology for obtaining probiotics is based on the cultivation of the microbial culture at the optimal temperature for their growth and development between 37 °C and 42°C, while the pH value lies in the area between 6.0 and 7.0 [2 - 4]. Lactic acid bacteria of *Lactobacillus* genus belong to the group of heterotrophic species, the growth and development of which is influenced by the content of fermentable carbohydrates, amino acids and their constituent proteins, water-soluble B group vitamins, nucleic acids, unsaturated free fatty acids and various minerals in the culture medium [5]. Preservation of their properties for a long period of time (up to 2 years) is achieved by applying vacuum-sublimation drying [6]. The lyophilization process takes place in an environment ensuring the viability of the cells of the probiotic culture. It includes in its composition cryoprotectants such as sucrose, lactose, trehalose, sorbitol, proteins and skimmed milk [7]. Other studies lead to the conclusion that the preservation of viability of *Lactobacillus* strains during vacuum freeze-drying is closely dependent on the conditions of the fermentation process, mainly pH and temperature [8]. Lowering the pH value of the culture medium to pH 5.0 - 6.0 creates conditions for increasing the number of surviving cells of the *L. reuteri* strain and increasing the cryotolerance

during lyophilization [9]. The acidification of the medium due to the high fermentation activity of the *L. acidophilus* RD758 strain also ensures the stability of its cells during vacuum freeze-drying. Another factor that stimulates the accumulation of lactic acid and ensures cryotolerance is the fermentation temperature, increasing the value from 30°C to 37°C and 42°C leads to an increase in the number of surviving cells of the *L. acidophilus* strain after lyophilization [10]. A similar pattern between fermentation process factors and freeze-drying survival was not found for *Bifidobacterium* strains [11].

In the process of lyophilization, lactic acid bacteria are exposed to various types of stress (mechanical, osmotic, oxidative and thermal), which accompanies changes in their morphological characteristics, creates conditions for disrupting the structure of the cell membrane, reducing their metabolic activity and their viability [4]. Cell adaptation of *Lactobacillus* strains to cold stress conditions during lyophilization is associated with an increase in incubation temperature from 30°C - 37°C to 42°C [8]. Lowering the pH of the medium to a value of pH 5.0 ensured the resistance of a strain of *L. delbrueckii* subsp. *bulgaricus* at a lyophilization process temperature of – 20°C instead of pH 6.0 [12]. The impact of low acidity (pH 5.0) initiates the process of synthesis and accumulation of intracellular heat shock protein in *Lactobacillus acidophilus* strains, as a result of which the resistance of its cells increases at a vacuum-sublimation drying temperature of – 80°C. These regulatory proteins are able to take an interaction with RNA molecules and function as thier chaperones, thus allow to the translation process under low positive temperatures [10]. They also take place in the processes of desaturation of membrane fatty acids [13]. The overproduction of cold shock proteins such as CspA–CspE groups, that are characterised by a molecular mass of 7 kDa, resulted in an increased cell survival of *Lc. lactis*, *Lb. plantarum* after freezing. These physiological adaptations, which appear under conditions of cold stress, play a great role in the improvement of the cellular cryotolerance of *Lactobacillus* strains during frozen [14]. The end of the fermentation step led to low cell concentrations, which requirement deepening research in the direction of searching for new nutrient media based on microalgae, such as *Spirulina*, that is a photoautotrophic cyanobacterium, whose biomass includes mostlty proteins and carbohydrates in the concentration of 88 %, vitamins as growth factors for lactic acid bacteria during their cultivation, lipids about 4 % - 7 %, minerals and some natural pigments [15, 16].

The aim of this study includes investigation on the influence of the fermentation process conditions on the acid tolerance of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain during cultivation in the medium, based on pretreated biomass of *Spirulina platensis*., its survival rate during freeze drying and its glycolytic activity. The main factors, that includes this study and are part of technological process of the production of probiotic foods, were pH of the fermentation (under condition of constant pH value of fermentation medium and during fermentation, that takes place with changing of pH and the fermentation time (late exponential, stationary, late stationary phase of development of the probiotic culture).

EXPERIMENTAL

Bacterial strains and preculture conditions

L. delbrueckii subsp. *bulgaricus* WDCM 00102 of the collection of National Bank of Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria) was used in the present study. Preparation of the cultures involved inoculation and growth in De Man Rogosa and Sharpe (MRS) medium at 42°C for approximately 12 h, followed by introduction of glycerol at a concentration of 20 % and storage of the cultures at -80°C. 2 % of the stock culture was used for inoculation of 10 ml of MRS medium, and after culturing at 42°C for 24 hours, 2 % of the enriched culture was used to inoculate 100 ml of MRS medium, the culture was incubated under the same conditions. After incubation, the culture was subjected to centrifugation (3000 $min.^{-1}$, 10 min), the supernatant fluid was separated, and the cells were suspended in phosphate buffered saline. The cell suspension was inoculated into the experimental medium so that its count after plating on MRS agar and incubation at 37°C for 24 hours was of the order of 10^7 $cfu.mL^{-1}$.

Fermentation conditions

Cultivation of the strain was carried out in two *Spirulina platensis*-based media with a dry matter concentration of 9 %, one in which the pH was maintained constant throughout the millet, and the cultivation in the other was carried out with varying pH.

Fermentations were carried out in five-liter fermenters with a geometrical volume of 5 L and work volume of 4.75 L at 42°C and a stirrer speed of 400 rpm. Maintaining a constant pH value of the medium during the first cultivation of the strain was achieved by periodically adding 5 M NaOH to pH 6.8. During cultivation, samples were taken periodically (10 h during introduction of the strain into the late exponential phase), 25 h (during introduction of the strain into the mid-stationary phase) and 37 h (during introduction of the strain into the late stationary phase)) to determine colony-forming units of the strain by the Koch method and sugar concentration. Samples were subjected to centrifugation (3000 $min.^{-1}$, 10 min), the supernatant fluid was separated, and the cells were suspended in phosphate buffer solution (PBS), containing 10 % sucrose (w/v). The suspensions thus obtained were stored at -20°C.

Acid survival assay

A solution with NaCl content of 0.85 % was used for the analysis. To 0.3 mL of the cell suspension stored at −20°C, 2.7 mL of the saline solution tempered to 42°C was added. The solutions were incubated at 42°C for 3 h. Samples were taken at 0, 1.5 and 3 hours and assays were performed to determine the total number of colony forming units by the Koch method after inoculation and incubation medium on MRS agar. The assay was carried out three times for each fermentation point interval. Results are expressed as the mean and standard deviation of the log10 difference between the control (pH 7.0) and the sample after 1.5 and 3 hours.

Freeze drying

Cell suspensions stored at -20°C in PBS with 10 % sucrose were enumerated after inoculation on the medium on MRS agar and incubation at 37°C for 24 h; colony counts were in all cases around 10^7 cfu mL^{-1}. The suspensions were then frozen at -80°C for 12 hours and subjected to vacuum-sublimation drying for 40 hours in a Hochvakuum-TG –16.50 system with contact plates heating. The lyophilized culture was rehydrated in 2 % skim milk medium, and the cell concentration was determined by plating on MRS agar and incubating at 42°C for 24 h. Results are presented as the mean value with value of standard deviation of difference of cell concentration as log10 between the sample before freezing and the rehydrated sample after vacuum freeze-drying.

Glycolysis assay

50 mL of the frozen cell suspension was subjected to centrifugation at 3000x g for 10 min. The pellet was resuspended in 10 ml of 2.5 mM potassium phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$, and the suspension was centrifuged at 3000 $min.^{-1}$ for 10 min. The cells are washed once more with the buffer. After the second washing step, the cells were resuspended in 10 mL of 2.5 mM potassium phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$ pH and 20 g L^{-1} glucose. The suspension was incubated at 42°C for 5 h and the pH was measured every 30 min.

Cell enumeration

The concentration of viable bacterial cell counts was established before and after freeze-drying or desiccation and after storage. Dried microbial strain were resuspended in 1 mL medium of sodium chloride 0,85 %. Growth in skim milk after drying and rehydration during 15 min were carried out and then 0.5 mL of the rehydrated strains were used to inoculate on MRS agar and incubating at 42°C for 24 h.

Reducing sugar analysis

The concentration of reducing sugars was measured by using of dintrosalicilic acid with volume of 100 mL per sample in a capped test tube. The mixture was heated at 100°C for 10 min and then cooled to room temperature. The absorbance of samples was measured at 570 nm. The standard curve was maded by using of glucose solutions at various concentrations.

Statistical analysis

The statistical significance of the differences between the various results of different fermentation experiments was analyzed by two-tailed t-test at 95 % significance level according to Student´s test. p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The basis of the development of microbial preparations with a potential probiotic effect is the resistance of the cells of strains of lactic acid bacteria to the effects of freezing and the conditions of vacuum-sublimation drying and the preservation of their functional properties. Part of the approaches

to improve the cryotolerance of *Lactobacillus* strains during lyophilization and their storage under anabiosis conditions is based on their prolonged cultivation until reaching a stationary phase of development in a fermentation medium, which is accompanied by lowering pH to 5.0 and ensured the resistance of cells to temperature stress [17 - 19].

Fig. 1 represents the course of the fermentation process during the cultivation of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain in a medium, based on the biomass of *Spirulina* ssp. at concentration of 9 %. The exponential phase lasted from 11 hours during pH controlled fermentation to 13 hours for pH uncontrolled fermentation. The maximum number of the cell population reaches a value of 10^9 cfu.mL^{-1} at a biomass content of *Spirulina* ssp. of 9 % during pH uncontrolled fermentation, which changes to 10^{10} cfu. mL^{-1} at carrying out the fermentation process at constant pH. In the experiments conducted, the concentration of reducing sugars decreased rapidly during the first 12 hours of fermentation to levels below 1.25 g.L^{-1}; this amount was maintained until the end point (37 hours). In pH uncontrolled fermentation, the sugar concentration decreases more slowly and reaches 7.5 g.L^{-1} after 10 h of fermentation and 3.75 g.L^{-1} after 37 h. In the case of an uncontrolled pH fermentation, the pH drops to 4.5 after approximately 17 hours and remains there until the end of the fermentation. This is most likely due to the inhibitory effect of pH and the reduced activity of glycolytic enzymes at low pH values. The sugar was almost completely decreased during the fermentation under constant pH-value at the introducing into the exponential phase [20]. In contrast, in the case of uncontrolled pH fermentation,

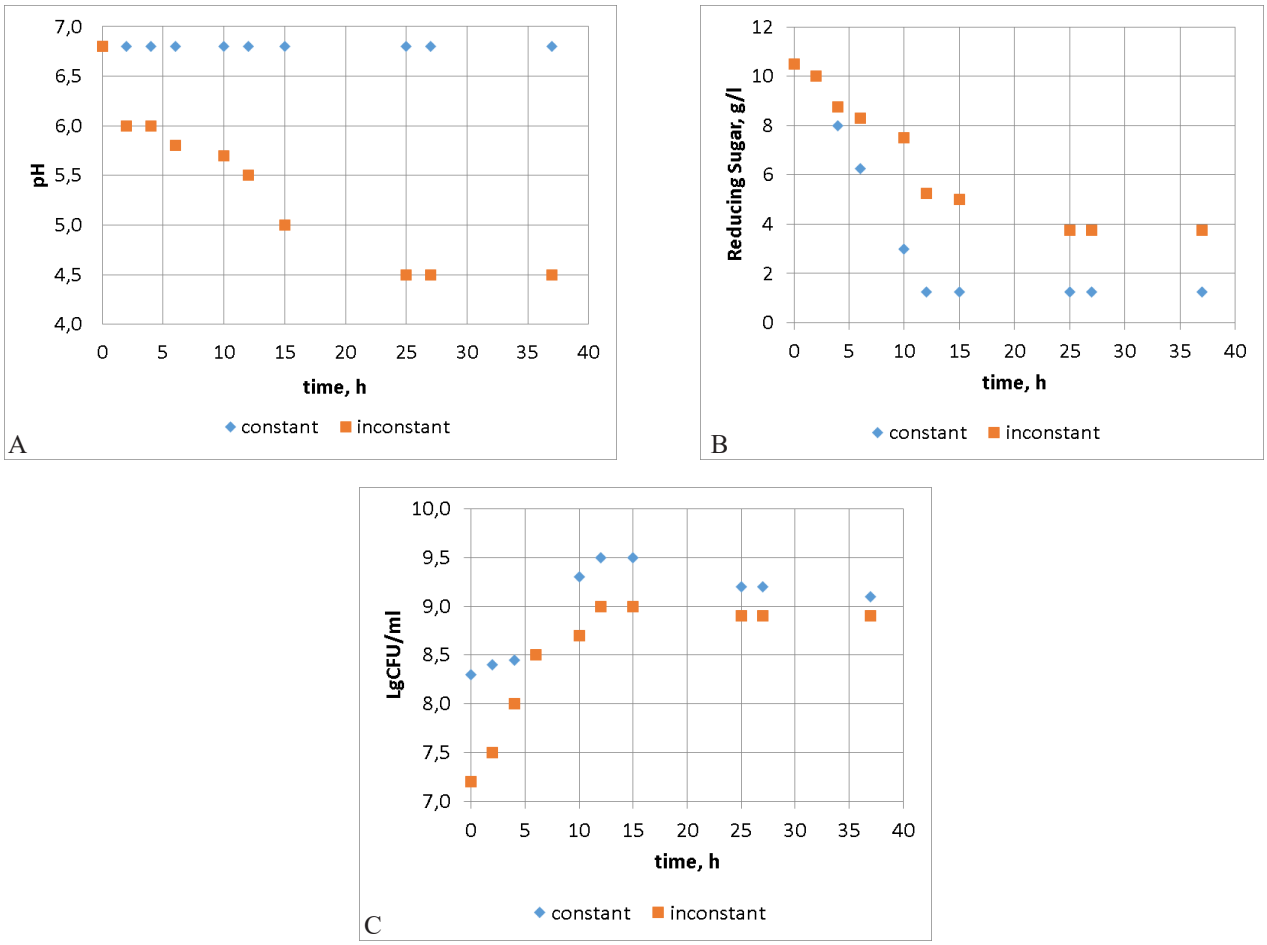


Fig. 1. Evolution of cell concentration (expressed as log CFU/ml) (C), pH (A) and reducing sugar concentration (B) during growth of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain at pH controlled fermentation (constant) and pH uncontrolled fermentation (inconstant). The data presented are mean values (from two independent fermentation) ± standard deviation.

sugar is consumed more slowly during the exponential phase but continues to decrease during the stationary phase, most likely due to increased ATP requirements to maintain pH homeostasis. Interestingly, however, cell viability did not decrease during the stationary phase, suggesting that *L. rhamnosus* GG cells are healthy and have sufficient energy reserves to survive. In contrast, in the case of uncontrolled pH fermentation, sugar is consumed more slowly during the exponential phase but continues to decrease during the stationary phase, most likely due to increased ATP requirements to maintain pH homeostasis [21].

Fig. 2 presents the results of the sensitivity of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain to the acidity of the medium. Up to 10 h from the beginning of the fermentation process, any significant differences in the values of viable cells were established, while the increase in the duration of cultivation was accompanied by their significant decrease ($P < 0.05$), which was most pronounced after exposure to low pH of the medium of 4.2 for a time over 1.5 h. Carrying out the fermentation process at a constant pH value of 6.8 determines the higher resistance of the cells of the studied culture to the low pH of the medium when reaching the late exponential phase of development (11 - 13 h), as a result of which no statistical difference was observed significant decrease in the number of viable colonies ($P > 0.05$). An increase in the duration of cultivation was accompanied by a significant decrease in the number of viable cells, with a 4.27 log reduction observed in the study when stationary phase was reached (37 hours), while the change in pH value did not determine significant differences between the late exponential phase and mid-stationary phase cells ($P > 0.05$); but the reduction in both cases is great, viz. approximately 4.7 log. Cells from the late stationary phase were distinguished by less pronounced acid tolerance, as the cell concentration decreased by approximately 5.89 log. When a strain reached early and mid-stationary phase, a greater survival rate was observed when maintaining a constant pH ($P < 0.05$), while at stationary phase an inverse relationship was reported ($P < 0.05$). Increasing the pH of the medium before lyophilization had a negative effect on the cryotolerance of *L. bulgaricus* CFL1 [22]. The adaptation of *L. plantarum* L67, *Bifidobacterium longum* ssp. *longum* Reuter 1963, *L. acidophilus* NCFM strains to the conditions of

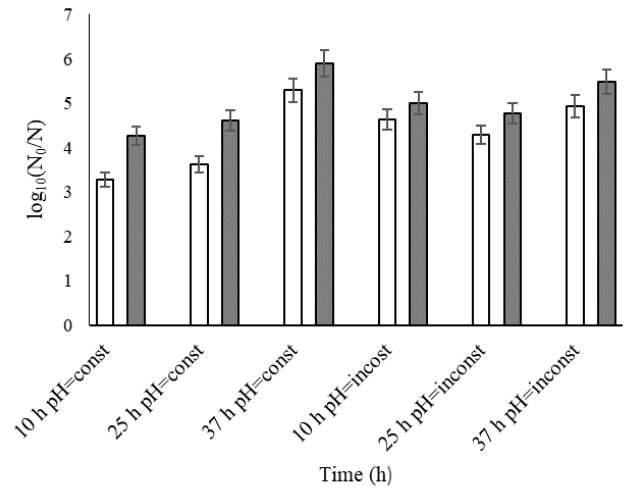


Fig. 2. Log_{10} decrease in the cell concentration (expressed as CFU/ml) of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain, collected after 10, 25 and 37 h of fermentation, after exposure to pH 2.5 for (□) 1.5 h and (■) 3 h. The cell concentration before freeze drying was approximately 10^7 CFU/ml in all cases. The data presented are mean values (from three independent experiments) ± standard deviation. pH controlled fermentation (pH=const), pH uncontrolled fermentation (pH=inconst).

the freezing and vacuum-freeze drying process is associated with an increase in the content of unsaturated fatty acids from the composition of the cytoplasmic membrane, as well as an increase in the expression level of *cspP* and *cspL* genes, encoding the formation of cold shock proteins after reaching the stationary phase of development [23 - 25], the increasing of the activity of the intracellular ATPase, which catalyzes the processes of removal of protons from the cytoplasm to the external environment [25]. The high content of residual reducing sugars in the cryoprotective medium, which act as endocellular cryoprotectants, also serves as a mechanism that ensures their protection against the processes of ice crystal formation, which occurs when the probiotic culture reaches a late exponential phase, when it accumulates a large reserve of ATP required for ATPase function [26]. Reduced glycolytic activity of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 in the present study strain could thus be due to the inactivation of the glycolysis enzymes, as a result of the low pH (in the case of fermentation, that takes place with decreasing of pH-value) or possibly the depletion of the carbon/energy source (under condition of constant pH value of fermentation medium).

The lowering of the pH to 4.5 during the fermentation of the medium by *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 was not accompanied by statistically significant differences in acid tolerance at 10 h, 25 h and 37 h from the start of inoculation at the recent study (Fig. 3). In all experiments, the decrease in cell viability was in the range of 1.25 to 1.94 log. When the fermentation process was carried out at constant pH, the decrease in the number of viable cells during freeze-drying studies increased as function of the fermentation time. An opposite trend was observed when the pH of the medium decreased to 4.5, and the number of colonies reached a maximum value at a later stage of fermentation ($P < 0.05$). Cells from the late exponential phase of under fermentation conditions of constant pH-value survived significantly better than those of fermentation, accomplishing with decrease of pH-value ($p < 0.05$), i.e. there were no significant differences in the degree of decrease in the number of viable colonies when the studied culture reached the middle stationary phase ($P > 0.05$), and entering the late stationary phase when changing the pH of the culture medium led to an increase in the number of viable cells compared to cells under conditions of the fermentation, that was carried out at constant pH-value ($p < 0.05$). The high resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophiles, Lact. rhamnosus* E800, *Lactococcus lactis*, *Listeria monocytogenes*, *Enterococcus faecalis* and *Streptococcus mutans* strains, that carry out the process of lactic acid fermentation to pH 2.0 - 3.6, when cultured in milk, is consequence of the biosynthesis of the chaperones such as GroES, GroEL, HrcA, GrpE, DnaK, DnaJ, ClpE, ClpP and ClpL, the repression of regulatory protein ClpC, induction of genes, involved in the biosynthesis of fatty acids (*fabH*, *accC*, *fabI*), and suppression of genes, involved in the biochemical pathway of isoprenoid synthesis (*mvaC*, *mvaS*) [21, 23, 28, 29].

The higher cell survival of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain on lowering the pH of the medium to reach the exponential phase or in the early stationary phase during fermentation is related to the physiological adaptation of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain to the changing environmental conditions. Fig. 4 presents the results of the analysis, related to the glycolytic activity, for the individual stages of rehydration (10, 25 and 37 h).

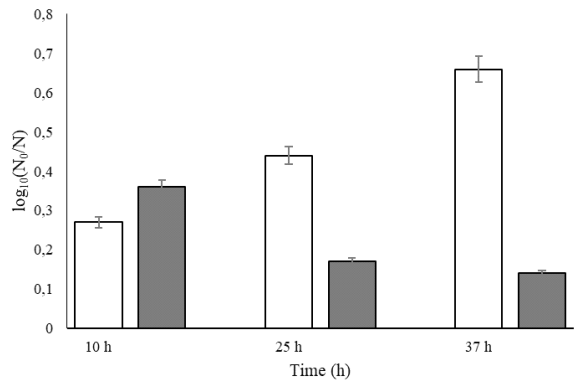
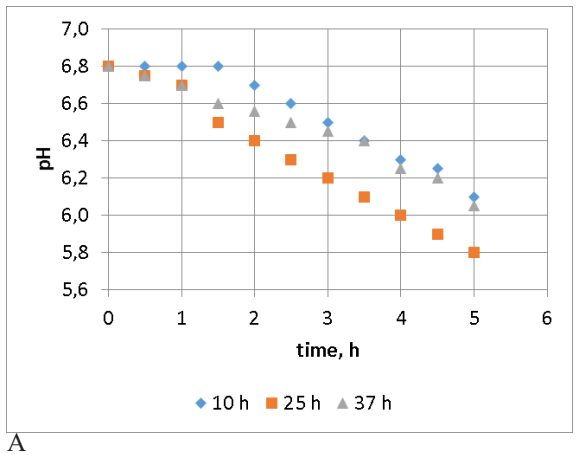


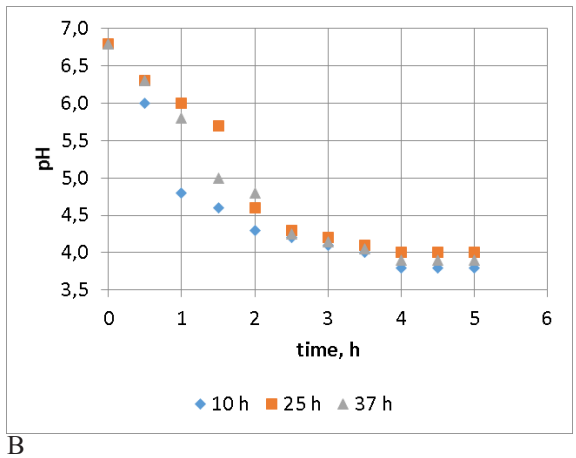
Fig. 3. Log10 decrease in the cell concentration (expressed as CFU/ml) after freeze drying of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain, harvested after 10, 25 and 37 h of fermentation. Symbols: (□) pH controlled fermentation (pH = const), (■) pH uncontrolled (pH =I nconst) fermentation. The starting cell concentration was approximately 10^7 CFU/ml, in all cases. The data are presented mean values (from three independent experiments) ± standard deviation.

Any significant changes of pH values were observed ($p > 0.05$) until reaching 5 h after incubation of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain in all points of fermentation experiments, that were carried out with the decrease of pH-value. Under conditions of the fermentation at constant pH, pH of inside of cells changed two times significantly for all three samples ($P < 0.05$). The significantly decrease was observed for the sample at 10 h of the cultivation, which also related to the lower pH value (approximately pH 3.9).

The glycolytic activity of the cells of *Lactobacillus casei* K17, *L. acidophilus* CLA, *L. rhamnosus* and *Bifidobacterium* BB-12, *Lactobacillus acidophilus* RD758 strains is very low, but the cryotolerance of the culture could be due to the increase in the number of unsaturated fatty acids in the composition of the membranes and the presence of residual reducing sugars such as glucose, which serve as endocellular cryoprotectants [21, 30 - 32], and due to lower lipid phase transition temperature (Ts) of the cytoplasmic membranes during freezing (Ts = -8°C) occurring at the same temperature range such as ice nucleation than freezing -sensitive cells (Ts = +22°C) [32]. The comparative studies on the effect of fermentation time on the resistance of lactic acid bacteria to freeze-drying conditions are scarce, further studies should be concentrated on the composition and structure of membranes during fermentation and acidification and



A



B

Fig. 4. Glycolytic activity assay of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain, harvested after 10, 25 and 37h of fermentation. pH controlled fermentation (A) and pH uncontrolled fermentation (B). The data are presented mean values (from three independent experiments).

the relationship to cryoresistance. of probiotic cultures. When a stationary phase of development was reached at 20 h after inoculation, the highest percentage of surviving cells was observed in *B. lactis* 10140 strain after vacuum-sublimation drying due to structural and physiological changes in the expression levels of stress-related proteins, the composition of the membrane and the structure of the cell wall, which leads to the acquisition of increased resistance of the cell to adverse conditions [33]. Reducing the pH value of the medium to a value of pH 5.1 determined a number of viable cells of *Lactobacillus bulgaricus* ND02 strain of $1{,}05.10^{11}$ cfu. mL^{-1} and the survival rate after lyophilization process was 68.3 %, while at pH 5.7 the survival was only 51.2 %. Long rod-shaped cells, which are observed when cultured at pH above pH 5.7, are characterized by a greater sensitivity to lyophilization conditions, while short-celled cells, found when cultured up to pH 5.1, are characterized by higher resistance of the conditions of vacuum-freeze drying process [33]. Other result was observed for the cells of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 strain in pH-controlled fermentation, is determined by the exposure of the stationary cells to conditions of decreasing amount of nutrient compounds for a long time or the higher residual amount of glucose, or the presence of *Spirulina platensis* biomass, the composition of which includes starch and proteins acting as cryoprotectants during lyophilization.

CONCLUSIONS

The main factors - pH and duration of fermentation, had a significant effect on the acid tolerance of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 and its survival during vacuum freeze-drying. Under constant pH culture conditions, the acid tolerance of the cells decreased as the probiotic culture reached the late exponential, mid-stationary and late-stationary phases. The statistically insignificant differences in glycolytic activity when cultured in medium with constant pH is due to the high content of residual reducing sugars and the high buffer capacity of the medium. As a result of the conducted study, a higher resistance to the acidity of the medium of the cells of the culture that reached the late exponential phase was found, compared to the stationary phase of development. The glycolytic activity of the cells after vacuum-sublimation drying keeps relatively constant values when the strains are cultivated in a medium with a constant pH value and significantly decreases when the pH value of the medium is reduced during fermentation. Fermentation conditions have a negligible effect on the chewability of the strain during vacuum freeze-drying, as the degree of reduction in the number of colony-forming units is statistically significant as a result of culturing the strain in a constant pH environment increases its resistance to lyophilization conditions. while lowering the pH of the medium results in less favorable outcomes in terms of survival rates. Its value increases statistically insignificantly when the culture reaches the middle and late stationary phase. In conclusion, the viability of the cells of the probiotic culture is stimulated by the impact of the low pH of the medium and its prolonged cultivation until reaching a stationary phase of development.

Acknowledgements

This research is supported by the Bulgarian Ministry of Education and Science under the National Program „ Young Scientists and Postdoctoral Students-2".

REFERENCES

1. L.K. Sarao, M. Arora, Probiotics, prebiotics, and microencapsulation: A review, Critical Reviews in Food Science and Nutrition, 57, 2, 2017, 344-371.
2. R.A. de Oliveira, A. Komesu, C.V. Rossell, R.M. Filho, Challenges and opportunities in lactic acid bioprocess design-From economic to production aspects, Biochemical Engineering Journal, 133, 2018, 219-239.
3. M. Ali Abdel-Rahman, Y. Tashiro, K. Sonomoto, Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: Overview and limits, Journal of Biotechnology, 156, 2011, 286-301.
4. E. Gerbino, P. Carasi, P. Mobili, M.A. Serradell, A. Gomez-Zavaglia, Role of S-layer proteins in bacteria, World J Microbiol Biotechnol, 31, 2015,1877-1887.
5. M.K. Tripathi, S.K. Giri, Probiotic functional foods: Survival of probiotics during processing and storage, Journal of functional foods , 9, 2014, 225-241.
6. A. Terpou, A. Papadak, I.K. Lappa, V. Kachrimanidou, L.A. Bosnea, N. Kopsahelis, Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value, Nutrients, 11, 2019, 1591, doi:10.3390/nu11071591
7. C. Champagne, A. Gomes da Cruz, M. Daga, Strategies to improve the functionality of probiotics in supplements and foods, Current Opinion in Food Science, 22, 2018, 160-166.
8. K. Fenster , B. Freeburg, C. Hollard, C. Wong, R.R. Laursen , A.C. Ouwehand, The Production and Delivery of Probiotics: A Review of a Practical Approach, Microorganisms, 7, 2019, 83; doi:10.3390/microorganisms7030083
9. O. Prakash, Y. Nimonkar, Y.S. Shouche, Practice and prospects of microbial preservation, FEMS Microbiol Lett., 339, 2013, 1-9.
10. Y. Wang, G. Corrieu, C. Beal, Fermentation pH and Temperature Influence the Cryotolerance of *Lactobacillus acidophilus* RD758, J. Dairy Sci., 88, 1, 2005, 21-29.
11. D. Dianawati, V. Mishra, N. P. Shah, Survival of Bifidobacterium longum 1941 microencapsulated with proteins and sugars after freezing and freeze drying, Food Res. Internat., 51, 2013, 503-509.
12. Z. Ai, X. Lv, S. Huang, G. Liu, X. Sun, H. Chen, J. Sun, Z. Feng, The effect of controlled and uncontrolled pH cultures on the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*, LWT - Food Sci. Technol., 77, 2017, 269-275.

13. S. Louesdon, S. Charlot-Rougé, R. Tourdot-Maréchal, M. Bouix, C. Béal, Membrane fatty acid composition and fluidity are involved in the resistance to freezing of *Lactobacillus buchneri* R1102 and *Bifidobacterium longum* R0175, Microbial Biotech., 8, 2, 2015, 311-318.
14. A. Rault, M. Bouix, C. Béal, Cryotolerance of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 is influenced by the physiological state during fermentation, Internat. Dairy J., 20, 2010, 792-799.
15. P. Gourbeyre, S. Denery, M. Bodinier, Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions, J. Leukoc. Biol., 89, 5, 2011, 685-695.
16. E. Barroso, A. Montilla, N. Corzo, C. Peláez, M. C. Martínez-Cuesta, T. Requena, Effect of lactulose-derived oligosaccharides on intestinal microbiota during the shift between media with different energy contents, Food Res. Internat., 89, 1, 2016, 302-308.
17. M. Chen, H. Tang, M. Chiang, Effects of heat, cold, acid and bile salt adaptations on the stress tolerance and protein expression of kefir-isolated probiotic *Lactobacillus kefiranofaciens* M1, Food Microbiology, 66, 2017, 20-27.
18. B. Montoro, N. Benomar, N.C. Gomez , S. Ennahar, P. Horvatovich, C.W. Knapp, A. Galvez, H. Abriouel, Proteomic analysis of *Lactobacillus pentosus* for the identification of potential markers involved in acid resistance and their influence on other probiotic features, Food Microbiology, 72, 2018, 31-38.
19. Q. Zhang, L. Ma, E. Jingjing, Z. He, C. Yao, Z. Chen, R. Ma , R. Wang, R. Sun, J.Cao , J. Wang, Effects of initial pH on heat resistance of *Lactobacillus plantarum* LIP-1, LWT - Food Sci. Technol., 138, 2021, 1-9.
20. V. Ferrando, A. Quiberoni, J. Reinhemer, V. Suarez, Resistance of functional *Lactobacillus plantarum* strains against food stress conditions, Food Microbiology 48, 2015, 63-71.
21. B.M. Corcoran, C. Stanton, G.F. Fitzgerald, R.P. Ross, Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars, Appl. Environ. Microbiol., 71, 2005, 3060-3067.
22. Y. Shao, S. Gao, H. Guo, H. Zhang, Influence of culture conditions and preconditioning on survival of *Lactobacillus delbrueckii* subspecies *bulgaricus* ND02 during lyophilization, J. Dairy Sci., 97, 2013, 1270-1280.
23. S. Song, D. Bae, K. Lim, M. W. Griffiths, S. Oh, Cold stress improves the ability of *Lactobacillus plantarum* L67 to survive freezing, International Journal of Food Microbiology, 191, 2014, 135-143.
24. R. Haindl, A. Neumayr, A. Frey, U. Kulozik, Impact of cultivation strategy, freeze-drying process, and storage conditions on survival, membrane integrity, and inactivation kinetics of *Bifidobacterium longum*, Folia Microbiologica, 65, 2020, 1039-1050.
25. S. Kulkarni, S.F. Haq, S. Samant, S. Sukumaran, Adaptation of *Lactobacillus acidophilus* to Thermal Stress Yields a Thermotolerant Variant Which Also Exhibits Improved Survival at pH 2, Probiotics & Antimicro. Prot., 10, 2018, 717-727.
26. E. Sanhueza, E. Paredes-Osses, C.L. González, A. García, Effect of pH in the survival of *Lactobacillus salivarius* strain UCO_979C wild type and the pH acid acclimated variant, Electronic Journal of Biotechnology, 18, 2015, 343-346.
27. J. Silva, A.S. Carvalho , R. Ferreira , R. Vitorino, F. Amado, P. Domingues, P. Teixeira, P.A. Gibbs, Effect of the pH of growth on the survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* to stress conditions during spray-drying, Journal of Applied Microbiology, 98, 2005, 775-782.
28. M.H. Saarela, H.-L. Alakomi, A. Puhakka, J. Ma ̈tto, Effect of the fermentation pH on the storage stability of *Lactobacillus rhamnosus* preparations and suitability ofin vitroanalyses of cell physiological functions to predict it, J. Applied Microbiology, 106, 2009, 1204-1212.
29. V. Patrone, T. Al Surrayai, F. Romaniello, A. Fontana, G. Milani, V. Sagheddu, E. Puglisi, M. L. Callegari, H. Al Mansour, M. W. Kishk, L. Morelli, Integrated Phenotypic Genotypic Analysis of Candidate Probiotic *Weissella* Cibaria Strains Isolated from Dairy Cows in Kuwait, Probiotics and Antimicrobial Proteins, 13, 2021, 809-823.
30. J. Wang, L. Chen, Impact of a Novel Nano-Protectant on the Viability of Probiotic Bacterium *Lactobacillus casei* K17, Foods, 10, 2021, 529, https://doi.org/10.3390/ foods10030529
31. M. Kowalczyk, A. Znamirowska-Piotrowska, M. Buniowska-Olejnik, M. Pawlos, Sheep Milk Symbiotic Ice Cream: Effect of Inulin and Apple Fiber on the Survival of Five Probiotic Bacterial

Strains during Simulated In Vitro Digestion Conditions, Nutrients, 14, 2022, 4454, https://doi.org/10.3390/nu14214454

32. J. Gautier, S. Passot ,C. Pénicaud, H. Guillemin, S. Cenard, P. Lieben, F. Fonseca, A low membrane lipid phase transition temperature is associated with a high cryotolerance of *Lactobacillus delbrueckii* subspecies *bulgaricus* CFL1, J. Dairy Sci., 96, 2013, 5591-5602.
33. F. Shamekhi, M. Shuhaimi, A.B. Ariff, A.M. Yazid, Optimization of a cryoprotective medium for infant formula probiotic applications using response surface methodology, Ann. Microbiol., 62, 2012, 911-921.