Biological activity of probiotics Bacillus albus and Lactobacillus brevis in the presence of polyvinylpyrrolidone nanofilaments

O Y Berezina¹, N A Sidorova²,³ and A I Savushkin²

¹ Synthesis of Oxide Micro- and Nanostructures Laboratory, Institute of Physics and Technology, Petrozavodsk State University, Lenin Avenue 33, Petrozavodsk 185910, Russia
² Department of Zoology and Ecology, Institute of Biology, Ecology and Agricultural Technologies, Petrozavodsk State University, Lenin Avenue 33, Petrozavodsk 185910, Russia
³ E-mail: fagafon@yandex.ru

Abstract. To substantiate the development of alternative agrarian and food technologies, the article presents the results of a study of the biological activity of potential probiotic cultures Bacillus albus and Lactobacillus brevis in the context of their further use as part of therapeutic and prophylactic additives to feed for various purposes. The biological activity of genuine strains of bacilli and lactobacilli was assessed by the effect of self-aggregation in the presence of polyvinylpyrrolidone nanowires obtained by the electrospinning method. The immobilized cells were cultured in an RTS-1C bioreactor with Reverse-Spin technology for 110 hours, followed by an assessment of the effect of immobilization by the kinetics of growth, pH of the culture medium and preservation of viability. As a result of a series of experiments, it was found that in the presence of PVP, growing cells of Bacillus albus and Lactobacillus brevis during fermentation acquire the ability to maintain a minimum growth rate and an optimal pH range for a long time period. In addition, in the presence of PVP, an increase in the number of viable cells of lactobacilli from $10^4$ to $10^7$ CFU/ml and bacilli from $10^4$ to $10^6$ CFU/ml was found in the reaction mixture. The data obtained are intended for further investigation of the phenomenon of self-aggregation of bacteria in the presence of polyvinylpyrrolidone nanowires and are considered promising for the development of new forms of probiotic products.

1. Introduction

Currently, in the development of alternative agrarian and food technologies, there is a tendency to optimize not only physical and chemical processes, but also biological and biochemical processes, which were not previously considered due to the lack of significance or independence from each other [1]. To substantiate the need to introduce biological and biochemical processes into innovative technologies, various studies are being carried out, confirming the high efficiency of using biotechnological approaches in agrarian food production. For example, in order to control the lack of microelements in the diet of agricultural species of birds and animals, feed components based on L-aspartic amino acid obtained by biocatalytic method with the inclusion of iron, zinc, copper and manganese in organic form have been developed and successfully tested [2]. There is evidence of the
creation of feed additives for poultry, containing an antioxidant complex with dihydroquercetin and arabinogalactan, which has a positive effect on egg productivity, safety, nutrient conversion and feed energy [3]. Biotechnologies continue to develop, associated with the targeted design of complex probiotic preparations of a new generation and the analysis of the rational use of pro- and prebiotics. Enzymatic probiotics containing strains of probiotic bacteria and bacterial cellulases, which cause the breakdown of cellulose and hemicellulose, have been created and are actively used. Forage probiotics based on biofilm-forming bacteria of the genus Bacillus with pronounced proteolytic and endoglucanase activity and Cellulomonas, capable of synthesizing cellulases and amino acids, were obtained [4]. It has been established that multicomponent probiotic preparations have a positive effect on the digestion of rabbits, broiler chickens and on the digestive system of young sturgeon. Using T-RFLP-analysis (terminal restriction fragment length polymorphism), which evaluates the variability of conserved regions of the genome of microorganisms, the mechanism of interaction of introduced probiotic bacteria with the resident microbial community of the gastrointestinal tract of animals [5], broiler chickens and laying hens [6].

The information obtained by the methods of molecular genetic analysis is used to assess the effectiveness of the use of probiotics, to regulate the taxonomic composition of the microflora of the gastrointestinal tract, as well as to theoretically substantiate the expansion of the spectrum of functional capabilities of newly created immobilized forms of probiotic drugs, systemic studies of which have arisen and have begun to rapidly develop only recently. decade [7]. One of the reasons is the industrial demand for technologies that ensure the stability of probiotic bacteria in feed additives for therapeutic and prophylactic purposes and in food. To date, data have been obtained on lactic acid bacteria, which in an immobilized state are capable of reaching a large number (up to 1010 CFU / g), maintain resistance to low and neutral pH values, pepsin and high doses of bile salts [8]. The results of studies on the productivity of immobilized Lactobacillus casei cells used for the production of fermented milk and lactic acid have been published; effective use of the substrate by lactobacilli under immobilization conditions, reducing the risk of microbial contamination of the target product [9] and the intensity of lactic acid fermentation [10]. The listed achievements testify to the prospects of using immobilized microorganisms and their metabolites in agrarian and food technologies. They also confirm the relevance of studies related to the optimization of the technology of immobilization of industrially significant strains of microorganisms, which are actively used both in the production of probiotic products for humans and as part of therapeutic and prophylactic feed additives for agricultural species of animals, birds and aquaculture objects.

In connection with the above, the purpose of the studies performed was to study the biological activity of the bacteria Bacillus albus and Lactobacillus brevis in the presence of polyvinylpyrrolidone (PVP) nanofilaments synthesized in the installation by the method for electrospinning.

2. Material and research method

PVP-poly-(N-vinylpyrrolidone) refers to synthetic polymers-gamma-vinyl lactams of N-aminobutyric acid obtained by homopolymerizing an aqueous solution of N-vinylpyrrolidone at a temperature of 50-80 °C under the action of 0.05-2.5% H2O2 in the presence of NH3. Due to the complex of physical, chemical and biological properties, polyvinylpyrrolidone is a unique compound, biocompatible and capable of interacting with cell membranes, non-toxic, chemically stable, capable of dissolving in water and organic solvents [11]. PVP aqueous solutions obey Newton's laws; PVP forms films well, which makes it an effective polymer for coatings under stable temperature conditions [12]. The possibility of using PVP and its modified forms to create structured clusters for the immobilization of microorganisms is enhanced by its transformation into nanofibers, which have a high specific surface area with a small diameter.

The synthesis of PVP nanowires was carried out in the laboratory for the synthesis of oxide micro- and nanostructures of the Physico-Technical Institute of Petrozavodsk State University. For the synthesis of nanowires, a solution of high molecular weight PVP (Mr = 1.3×106 g/mol) was used. A medical syringe (needle diameter 0.7 mm) was used in the electrospinning device. The supply of PVP
solutions from the syringe was carried out with an NE-300 syringe pump at a rate of 0.5 ml / h. An electric field of 1.8 kV / cm was created between the needle and the metal substrate using an INVR-30/5 high-voltage source. A photograph of the obtained PVP nanowires is shown in figure 1.

![Electron photo of PVP nanowires](image)

**Figure 1.** Electron photo of PVP nanowires, magnification × 80.

Depending on the characteristics of the carrier, the immobilization process was simulated using physical immobilization methods (without the formation of covalent bonds) due to self-aggregation. The process of self-aggregation of microbial cells in the presence of PVP is schematically shown in figure 2.

![The phenomenon of self-aggregation of bacteria](image)

**Figure 2.** The phenomenon of self-aggregation of bacteria.

To analyze the effect of PVP on the biological activity of cultures in the experiment and control (bacterial culture without a carrier), we used genuine strains of *Bacillus albus* VMPA.1 and *Lactobacillus brevis* 1K.LBA, isolated from environmental objects. Isolation of microorganisms into a pure culture was carried out in the microbiology laboratory of Microbiome LLC, Petrozavodsk State University, using phenotypic traits regulated in Bergi’s Keys to Bacteria [13]. The isolated strains were genotyped in the laboratory of molecular diagnostics of the Center for Collective Use «Bioengineering» of the Federal Research Center «Fundamentals of Biotechnology» (Moscow) according to the method of Sanger F. [14] using the BigDye Terminator v. 3.1 reagent kit (Applied Biosystems, Inc., USA) on the ABIPRIZM 3730 genetic analyzer (AppliedBiosystems, Inc., USA) according to the manufacturer's instructions.

The studied strains of bacilli and lactobacilli were cultured in a sterile 250 ml Erlenmeyer flask containing 50 ml of a nutrient base, 0.5 g of a carrier, and 1 ml of a microbial culture at a concentration of 10⁹ CFU/ml. A cell concentration of at least 10⁹ CFU/ml meant the number of viable bacteria capable of immobilization in the presence of 1 g of carrier. To create homogeneous cultivation conditions and forced aeration, a shaker of the «S: S -3L. A20» series with a digital control system and orbital motion of the platform at a rotation speed of 110 rpm was used.

The effect of immobilization was considered positive when stabilizing physiological activity, achieving optimal conversion of the substrate into a product instead of biomass, expanding the pH optimum, and maintaining cell viability [15]. The kinetics of cell growth was recorded in real time on a «RTS-1C» bioreactor manufactured by «BioSan Ltd» (Latvia). For incubation of bacteria, sterile 50 ml «TPP TubeSpin 50» vessels were used for aerobic cultivation with a membrane filter. The volume of the culture medium was 29 ml, and the volume of the introduced reaction mixture (PVP and
bacterial cells) was 1 ml. The optical density indices of the reaction mixture and the growth kinetics of the studied microbial cultures were measured at a given measurement range of 0-8 OD at λ - 850 nm, measurement accuracy ± 0.3 OD and constant incubation temperature of 37 °C (temperature stability ± 0.1 °C ). The acidity of the culture solutions was measured using a pH meter ionometer with an error (± 0.005pX, ± 0.2 mV) and an extended EMF measurement range (± 4000 mV) every 10 hours from the start of fermentation.

To assess the viability of microorganisms, direct methods of cultivation of *Bacillus albus* and *Lactobacillus brevis* using growth media were used. As a growth medium for *Bacillus albus*, we used meat-peptone agar (MPA), consisting of 11 of meat-peptone broth (MPB) and 15 g of agar. For *Lactobacillus brevis*, a nutrient medium of the following composition (g / l) was used: pancreatic fish protein hydrolysate - 20.0; baker’s yeast extract - 5.0; meat extract - 5.0; C₆H₁₂O₆ - 20.0; KH₂PO₄ 2.0; CH₃COONa - 5.0; «Twin-80» - 1 ml; (NH₄)₃C₆H₅O₇ - 2.0; MgSO₄ - 0.1; MnCl₂ - 0.05; agar - 13.0 ± 2.0. The cultures were incubated in a thermostat at 37 °C for 24 hours. Viability was calculated by the number of colony-forming units (CFU) formed by inoculating 1 ml of the culture mixture on agar culture medium (CFU / ml). Working cultures of bacilli were maintained on BCH, and acidophilic lactobacilli - on media using sterile skim milk. The results were statistically processed using Microsoft Office Excel 2016 for Windows 10.

3. Research results

Bacterial growth kinetics and optical density of the culture solution. The results of measuring the optical density of the culture solution and the growth kinetics of immobilized *Bacillus albus*.VMPA.1 and *Lactobacillus brevis* 1K.LBA cells during incubation in the bioreactor are shown in typical graphs (figure 3-4). Under the conditions of static cultivation, the lactobacilli population developed according to patterns reflecting changes in cell biomass and in the environment. In the presence of PVP in the first 10 hours of fermentation, the growth rate of lactobacilli decreased from 0.12 h⁻¹ to 0.02 h⁻¹ (2% of μₘ), while the population remained homogeneous until 48 hours of the experiment.

![Figure 3](image.png)

**Figure 3.** Kinetics of growth - μ (h⁻¹) (a) and optical density - OD, λ = 850 (b) *Lactobacillus brevis* 1K.LBA in the presence of PVP by 110 h of incubation.

![Figure 4](image.png)

**Figure 4.** Kinetics of growth - μ (h⁻¹) (a) and optical density - OD, λ = 850 (b) *Bacillus albus*. VMPA.1 (b) in the presence of PVP for 110 h of incubation.
A decrease in the growth rate to 0.01 h\(^{-1}\) (1\% of \(\mu_m\)) was accompanied by the appearance of signs of heterogeneity, the growth rate decreased to zero values, and a critical period of biomass decline began (figure 3 a). It should be noted that the minimum growth rate of lactobacilli was maintained until the end of the experiment. The length of the stationary phase was 40 hours and lasted from 50 to 90 hours of fermentation (figure 3 b). According to the available data [16], in the state of the stationary growth phase, the resistance of cells to damaging environmental factors increases. This occurs mainly due to the biosynthesis of secondary metabolites, which begins at the beginning of the stationary phase with a decrease in the concentration of the nutrient substrate and a change in environmental conditions. In this case, the producer genes responsible for the expression of secondary metabolites are grouped into specific clusters [17]. The maximum growth rate of 0.08 h\(^{-1}\) (8\% of \(\mu_m\)), Bacillus albus in the presence of PVP reached 38 h of fermentation in the bioreactor, by 42 h the growth rate of bacilli decreased to 0.03 h\(^{-1}\) (3\% of \(\mu_m\)), to 52 h again increased to 0.07 h\(^{-1}\) (7\% of \(\mu_m\)) and then began to irreversibly decrease (figure 4 a). The main reason for this kinetics of Bacillus albus growth is related to their ability to form spores. According to Dawes I. W and Thornley J. H. M. [18], bacilli are characterized by a negative correlation between the process of sporulation and growth rate. At the same time, it is important to note that, as in the case of lactobacteria, in the presence of PVP, growing cells of bacilli during fermentation acquired the ability to maintain a minimum growth rate for a long time period. The spore germination also explains the prolonged growth retardation or lag period in the Bacillus albus population, which lasted 28 hours. The length of the stationary phase was 40 hours and lasted from 72 to 110 hours of fermentation. It can be assumed that the decrease in the optical density of the culture during the stationary phase from 2.2 to 1.7 OD (figure 4b) is associated with the partial differentiation of cells into resting cells, which are characterized by a temporary cessation of growth and the presence of a lag period before the onset of induction of biosynthesis products.

3.1. Acidity values of the culture solution of Lactobacillus brevis and Bacillus albus

To assess the effect of immobilization of lactobacilli and bacilli, an analysis of changes in the acidity of the culture solution in the presence of PVP was performed (table 1). During 100 hours of fermentation, the degree of acidification of the culture medium of lactobacilli and bacilli in the presence of PVP gradually decreased. For L. brevis, the pH decreased 2.13 times, and for B. albus-1.17 times. The obtained pH ranges for these types of microorganisms: 2.21- 4.70 for lactobacilli and 6.15-7.23 for bacilli can be considered optimal under the conditions of the experiment performed.

| Measurement option | Exposure time (h) | pH of the culture solution |
|--------------------|------------------|---------------------------|
|                    |                  | L. brevis | B. albus |
| 1                  | 10               | 4.70 ± 0.31 | 7.23 ± 0.21 |
| 2                  | 20               | 3.20 ± 0.48 | 7.56 ± 0.12 |
| 3                  | 40               | 3.58 ± 0.40 | 6.43 ± 0.27 |
| 4                  | 60               | 3.16 ± 0.43 | 6.82 ± 0.25 |
| 5                  | 70               | 3.81 ± 0.42 | 6.46 ± 0.28 |
| 6                  | 80               | 2.42 ± 0.14 | 6.33 ± 0.27 |
| 7                  | 100              | 2.21 ± 0.13 | 6.15 ± 0.41 |

3.2. The viability of Lactobacillus brevis and Bacillus albus

The analysis of changes in the viability of the investigated microorganisms in the presence of PVP is presented in table 2.
Table 2. The number of viable cells at different periods of exposure.

| Measurement option | Exposure time (h) | Viable cell count (CFU / ml) |
|--------------------|------------------|-----------------------------|
|                    |                  | L. brevis | B. albus                  |
| 1                  | 10               | $5 \times 10^4$ | $0.5 \times 10^4$         |
| 2                  | 20               | $4 \times 10^5$ | $6 \times 10^5$          |
| 3                  | 40               | $6 \times 10^6$ | $10 \times 10^8$        |
| 4                  | 60               | $2 \times 10^7$ | $4 \times 10^3$         |
| 5                  | 70               | $5 \times 10^6$ | $8 \times 10^2$         |
| 6                  | 80               | $7 \times 10^6$ | $6 \times 10^4$        |
| 7                  | 100              | $2 \times 10^7$ | $3 \times 10^6$        |

In all variants of the experiment, the number of viable cells remained fairly stable. Despite the revealed heterogeneity of the kinetics of growth of populations of lactobacilli and bacilli, the change in the number of cells of lactobacilli from $10^4$ to $10^7$ and of bacilli from $10^4$ to $10^6$ makes it possible to assert that, despite the duration of fermentation (for 100 hours), biologically active variants are retained that are capable of self-aggregation. synchronize the physiological functions of cells.

4. Conclusion
As a result of the studies performed on the comparative analysis of the biological activity of potential probiotics *Bacillus albus* and *Lactobacillus brevis*, which these cultures exhibit in the presence of polyvinylpyrrolidone nanofibers, it was found that in the state of self-aggregation bacteria are able to maintain their proliferative function both immediately after immobilization and after prolonged use during fermentation. In bacilli and lactobacilli, the kinetic characteristics of growth were significantly different, which can be described as unbalanced with a predominance of biosynthesis of secondary metabolites. Taking into account that growth for microorganisms is an autocatalytic process, its regulation under given cultivation conditions (temperature 37 °C) depended on many reasons, including the presence of nutrients and metabolic products: the accumulation of metabolites in the reaction medium and a decrease in the concentration of nutrient substrate inhibited the growth of the tested strains in different ways. The highest level of metabolic activity associated with a decrease in the acidity of the reaction mixture by 2.5 units and an increase in the number of viable cells by a factor of 1.75 was found for lactobacilli (tables 1, 2). Experimental data are consistent with the results of Efremenko E. N. [19], who believes that the carrier significantly affects the kinetic characteristics of the growth of immobilized cells by performing the function of a protective barrier, "shielding" them from the negative effects of metabolites and preventing penetration into the matrix. Despite the fact that the material presented in the article is primary, it can be used as an information base for future research in the biology of probiotic cultures, in order to be used in the future to optimize the technology of targeted synthesis of certain compounds and biologically active substances that are important for the design of new forms of probiotic multicomponent biological products for therapeutic and prophylactic purposes.

Acknowledgments
The research was carried out within the state assignment of the Ministry of Science and Higher Education of the Russian Federation (theme No. 0752-2020-0007).

Bibliography
[1] Panfilov V A 2019 Agricultural and food innovations and synergetics *J Agrarian and food innovations* 4 (8) 7-19
[2] Novikov A D, Voronin S P, Gubanova T A, Gumenyuk A P, Larikova G A, Sinolitsky M K, Sheiko I P and Yanenko A. S 2012 Abstracts of Int. Reports Scientific-Practical Conf. (St. Petersburg: Lenexpo) pp 97-8
[3] Gorlov I F, Slozhenkina M I, Anisimova E Yu and Knyazhechenko O A 2019 Innovative developments for the development of agrarian food technologies J Agrarian food innovations 4 (8) 19-29
[4] Ushakova N A, Kozlova A A, Ratnikova I A and Gavrilova N N 2012 Abstracts of Int. Reports Scientific-Practical Conf. (St. Petersburg: Lenexpo) pp 97-8
[5] Laptev G Yu. Regulation of the microflora of the gastrointestinal tract of animals 2012 Abstracts of Int. Reports Scientific-Practical Conf. (St. Petersburg: Lenexpo) p 100
[6] Nikonov I N, Laptev G Yu and Egorov I 2012 Abstracts of Int. Reports Scientific-Practical Conf. (St. Petersburg: Lenexpo) p 100
[7] Denkova Z, Slavchev A, Blazheva D and Krastanev A 2014 The Effect of the Immobilization of Probiotic Lactobacilli in Chitosan on their Tolerance to a Laboratory Model of Human Gut J Biotechnology and Biotechnological Equipment 21 (4) 442-50
[8] Mitropoulou G, Nedovic V, Goyal A and Kourkoutas Y 2013 Immobilization Technologies in Probiotic Food Production J of Nutrition and Metabolism Article ID 716861 1-15
[9] Kourkoutas Y, Xolias V, Kallis M, Bezirtzoglou E and Kanellaki M 2005 Lactobacillus casei cell immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production J Process Biochemistry 40 (1) 411-6
[10] Champagne C, Lee B and Saucier L 2010 Encapsulation Technologies for Active Food Ingredients and Food Processing, ed N J Zuidam and V Nedovic (New York: Springer NY) p 345-65
[11] Teodorescu M, Bercea M. 2015 Poly(vinylpyrrolidone) - a versatile polymer for biomedical and beyond medical applications J Polymer-Plastics Technology and Engineering 54 923-43
[12] Bleicher L, Lorenz D H, Lowd H L, Wood A S and Wyman D P 1980 Handbook of Water-Soluble Gums and Resins (New York: McGraw-Hill) pp 211-7
[13] Brief guide to bacteria Bergi 1980 ed J Hoult (Moscow: Mir) pp 304-6
[14] Sanger F, Nicklen S and Coulson A R 1977 DNA sequencing with chain-terminating inhibitors J Proc. Natl. Acad. Sci. 74 (12) 5463-7
[15] Beshay U, Enshasy H El, Ismail I M K, Moawad H and Ghany S 2011 ABD-El, Pol J Microbiol. 2 (60) 133-8
[16] Pirt S J 1978 Principles of Microbe Cultivations ed I L Rabotnova (Moscow: Mir) 321
[17] Andryukov B G, Mikhailov V V and Besednova N N 2019 Antimicrobial Activity of Secondary Metabolites of Marine Bacteria J Antibiotics and Chemotherapy 64 7-8
[18] Dawes I W, Thornley J H M Sporulation in Bacillus subtilis. Theoretical and experimental studies in continuous culture systems 1970 J. gen. Microbiol. 62 49
[19] Efremenko E N 2009 Heterogeneous Biocatalysts Based on Immobilized Cells of Microorganisms (Moscow: Inst. biochem. physics to them. N.M. Emanuel RAN) p 53