ISSN 2228-9860 eISSN 1906-9642 CODEN: ITJEA8

International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies

http://TuEngr.com

Biotechnology of Cultivation of Probiotic Lactobacilli

Yury Lysenko[1*], Nadezhda Machneva[2], Anatoly Smirnov[3], Alexander Panin[4], Andrey Koshchaev[2]

[1] *Department of Microbiology, Epizootology and Virology, Kuban State Agrarian University named after I.T. Trubilin, RUSSIA.*

[2] *Department of Biotechnology, Biochemistry and Biophysics, Kuban State Agrarian University named after I.T. Trubilin, RUSSIA.*

[3] *Laboratory of Veterinary Sanitation and Ecological Safety in Beekeeping, All-Russian Scientific Research Institute of Veterinary Sanitation, Hygiene and Ecology, RUSSIA.*

[4] *Department of Immunology and Biotechnology, Moscow State Academy of Veterinary Medicine and Biotechnology - MVA by K.I. Skryabin, RUSSIA.*

**Corresponding Author (scopush@gmail.com)*

Paper ID: 12A4I

Volume 12 Issue 4

Received 24 November 2020
Received in revised form 18 October 2021
Accepted 29 January 2021
Available online 08 February 2021

Keywords:
Lactobacilli cultivation;
Nutrient medium;
Growth requirements;
Reducing sugars;
Microorganism's growth.

Abstract

This article presents the research results on the selection of optimal conditions for the cultivation of probiotic microorganisms isolated from honey bee drone milk for increasing the biomass of the studied lactobacilli. To determine microorganisms' growth requirements, we used the developed molasses medium and conventional nutrient media for lactobacilli. As a result of calculating the number of lactic acid bacteria on agar nutrient medium, it was found that molasses medium has optimal growth requirements, which includes 45.0 g/l of fodder molasses (50% beet molasses and 50% corn molasses), K_2HPO_4 2.0 g/l, yeast extract 0.02 g/l. Simultaneously, the developed medium promoted the increase in the titer of microorganisms up to 5.0 x 10^{10} CFU/ml. The amount of the studied Lactobacillus kunkeei reached 2.3–4.7 x 10^9 CFU/ml on standard nutrient media. When studying the effect of different temperatures and growing times on lactic acid bacteria's biomass growth, the optimal cultivation conditions are 37°C and 24 h. It was proved that the maximum depletion of the nutrient medium's carbon component occurs 24-28 hr from the start of the microorganism growth process.

Disciplinary: Microbiology, Agricultural Biotechnology.

Cite This Article:

Lysenko, Y., Machneva, N., Smirnov, A., Panin, A., Koshchaev, A. (2021). Biotechnology of Cultivation of Probiotic Lactobacilli. *International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies*, *12*(4), 12A4I, 1-9. http://TUENGR.COM/V12/12A4I.pdf DOI: 10.14456/ITJEMAST.2021.72

1 Introduction

One of the crucial stages in producing probiotic supplements is the accumulation of bacterial mass, which largely determines the economic, technological, and biological parameters of the finished product [8, 16]. This stage includes the development of:

- recipes of nutrient media for growing microorganisms during inoculum preparation and accumulation of bacterial mass;

- effective ways to prepare inoculum;

- optimized technological, biological, and time modes of cultivation with bacterial mass accumulation [2, 4, 18].

The requirements imposed by various microorganisms concerning the composition of nutrient media and other conditions are very diverse. In terms of nutritional requirements, lactic acid bacteria are among the most complex microorganisms. Because biotopes with a high content of organic matter (carbohydrates, proteins, peptides, free amino acids, vitamins, etc.) are characteristic of lactobacilli's natural habitat [1, 12, 17].

Lactic acid bacteria show a rather high demand for the presence of vitamins in the nutrient medium. Most of the coccal forms (leukonostoks, lactococci, thermophilic lactic acid streptococci) require niacin, biotin, cyanocobalomin, folic acid for their development, and activity. All lactic acid sticks need pantothenic acid, niacin, and riboflavin [3, 9, 10].

Purine and pyrimidine bases, nucleic acids, and other organic growth factors play an important role in microorganisms' growth and life. Different lactic acid bacteria require different growth factors. So, when purine and pyrimidine compounds are added to milk, an increase in lactobacilli's acid formation is observed. A tremendous effect is observed with the addition of inosine. Some strains were stimulated by adenine, acetic acid, thymidine [7; 13].

In general, nutrient media containing lactose or glucose, proteins, peptides, amino acids, vitamins, and other organic growth factors are required for the normal growth, reproduction, development, and metabolism of lactobacilli. Hydrolysates of milk, animal and plant substrates, yeast extracts and autolysates, corn extract, fruit and vegetable juices, preparations from thermophilic methane fermentation products, etc can a source of these ingredients. [14].

Thus, the cultivation of lactic acid microorganisms and obtaining their commercial forms is a complex process influenced by many factors. It is also associated with the need to solve some scientific and technical problems, part of which is to improve the composition of the nutrient medium.

The purpose of the research was to select the optimal conditions for growing probiotic cultures of microorganisms isolated from honey bee drone milk to increase the biomass yield of the studied lactobacilli.

2 Method

The work was carried out in the research laboratory of the Department of Biotechnology, Biochemistry and Biophysics of the Federal State Budgetary Educational Institution of Higher

Education Kuban State Agrarian University. The purpose of which was to determine the nutritional needs and optimal conditions for growing lactic acid microorganisms to intensify the processes of obtaining their microbial biomass.

The research object was Lactobacillus - Lactobacillus kunkeei, which were isolated from a honey bee's drone milk by an independent microbiological method, a quantitative polymerase chain reaction in real-time and metagenomic methods [15].

At the first stage of the research, lactobacilli were grown on various compositions' molasses nutrient medium.

The second stage of the research was to determine the effect of standard nutrient media on the studied cultures' growth.

At the third stage of the experiments, an experiment was laid to identify the maximum thermal tolerance of the strain under study when grown on a molasses medium.

In the process of cultivation on rocking flasks, the dynamics of the consumption of reducing substances with an initial concentration of 4.0% were analyzed. The method for determining reducing substances is based on the reducing ability of sugar mono-forms - glucose and fructose.

For determining the microflora titer, 1.0 ml of each culture grown was taken and placed in a 100 cm^3 flask and filled with 99.0 ml of sterile physiological solutions, left for 1 hour. The dilution was 1:100. After that, a series of successive tenfold dilutions up to 10^{-9} were prepared. Separate sterile tips were used for each dilution. According to GOST 10444.11-89 (item 4.2.2) [5] on Lactobacar from dilutions 10-7, 10-8, 10-9, Sowing in Petri dishes was carried out 10^{-10}. From dilutions 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} with a sterile tip of an automatic dispenser, 1.0 ml of the suspension was transferred into 3 Petri dishes, into which a sterile, melted nutrient medium cooled to 37–40°C was poured. The medium was stirred in a circular motion of the Petri dishes and the agar was left to solidify. Plates with inoculated media were placed in a thermostat and kept at (37 ± 1) °C for 72 hours. The number of grown colonies determined the total titer of microorganisms according to GOST 9225-84 (paragraph 4.5.3) [6]. A simple formula is used to calculate the number of viable cells in 1.0 ml of preparation (X):

$$X = N \times P \quad (1)$$

where

N - is the arithmetic mean of the number of colonies in Petri dishes;

P - is the serial number of the tenfold dilution in which the growth of bacteria is noted.

The results of counting the lactobacilli number on nutrient media were carried out in three replicates to obtain more reliable results.

3 Result and Discussion

3.1 Determination of the Growth Requirements of Microorganisms on a Molasses Medium

The first stage of research included growing bacteria on molasses nutrient medium of various compositions [11]. The culture cultivation time was 48 h, the temperature optimum was 37°C.

Since the main component in the medium is the composition of molasses, the molasses nutrient medium of the following compositions was used to select a nutrient substrate for Lactobacillus kunkeei:

1. Composition of molasses medium No. 1: 45.0 g/l of fodder molasses (100% beet molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l.

2. Composition of molasses medium No. 2: 45.0 g/l of fodder molasses (100% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l.

3. Composition of molasses medium No. 3: 45.0 g/l of fodder molasses (50% beet molasses and 50% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l.

4. Composition of molasses medium No. 4: 45.0 g/l of fodder molasses (25% beet molasses and 75% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l.

5. Composition of molasses medium No. 5: 45.0 g/l of fodder molasses (75% beet molasses and 25% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l.

The results on the number of microbial cultures under study, obtained under laboratory conditions during cultivation in molasses medium, are shown in Figure 1, where the abscissa shows the numbers of the medium molasses compositions ordinate shows the number of microorganisms.

As a result of the study, molasses medium No. 3 turned out to be the most effective, where 50% beet molasses and 50% corn molasses were used as fodder molasses, the titer of Lactobacillus kunkeei was 4.8×10^{10} CFU/ml, while in other variants of the used nutrient compositions medium titer of cultures was lower. So on molasses nutrient medium No. 1, the amount of Lactobacillus kunkeei was 2.1×10^{10} CFU/ml; in option No. 2, the titer of Lactobacillus kunkeei was 2.7×10^{10} CFU/ml; option No. 4 - 3.3×10^{10} CFU/ml and option No. 5 - 3.6×10^{10} CFU/ml.

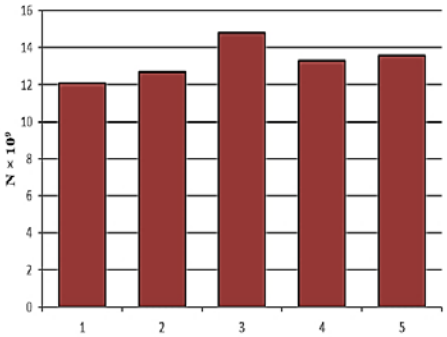

Figure 1: The number of Lactobacillus kunkeei on molasses medium with different levels of beet and corn molasses, where N - is the number of microorganisms (CFU/ml), No. 1-5 composition of the molasses medium

Thus, when growing Lactobacillus kunkeei on molasses medium, the medium with 50% beet molasses and 50% corn molasses had the best growth requirements.

3.2 Determination of the Growth Requirements of Microorganisms on Standard Nutrient Media

The second stage of research was to determine the effect on the growth of the studied cultures of various nutrient media, which are often used to cultivate lactic acid bacteria [11]. The culture cultivation time was 48 h, the temperature optimum was 37°C.

The selection of standard nutrient media was carried out taking into account the well-known media recommended by the Research Institute of the Dairy Industry (Uglich), and also used the classic generally accepted media:

1. Composition of molasses medium: 45.0 g/l of fodder molasses (50% beet molasses and 50% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l.

2. Medium for lactic acid bacteria (Uglich): yeast extract - 0.2%; corn extract - 0.3%; glucose syrup - 2.0%; ascorbic acid (sodium citrate) - 4.0 g/l; KH_2PO_4 - 2.0 g/l.

3. Composition of the MRS medium, g/l: peptone - 10.0; yeast extract - 20.0; glucose - 20.0; dipotassium hydrogen phosphate - 2.0; sodium acetate - 5.0; triammonium citrate - 2.0; magnesium sulfate - 0.2; manganese sulfate 4-water - 0.05.

4. Composition of glucose-peptone medium (GPS), g/l: Na_2HPO_4 12-water - 3.2; KH_2PO_4 - 0.3; $MgSO_4$ - 0.5; NaCl 0.5; peptone - 2.0; yeast extract - 0.05; glucose - 25.0.

The number of microbial cultures under study, obtained under laboratory conditions during cultivation in various nutrient media, is shown in Figure 2, where the abscissa indicates various nutrient media, and the ordinate indicates the number of microorganisms.

As a result of the study, medium No. 1 (molasses medium) became the most effective since the Lactobacillus kunkeei titer was 5.0×10^{10} CFU/ml. Medium 3 (MRS medium) - 2.3×10^9 CFU/ml became less effective. When growing lactobacilli on a medium for lactic acid bacteria in Uglich, the following results were obtained: Lactobacillus kunkeei - 4.7×10^9 CFU/ml, and on HPS the titer of Lactobacillus kunkeei was 4.2×10^9 CFU/ml.

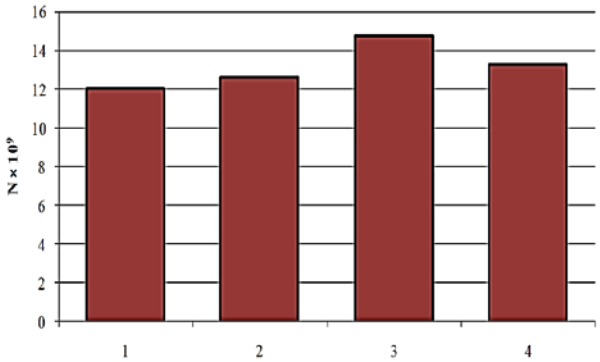

Figure 2: The amount of Lactobacillus kunkeei on various nutrient media, where N is the number of microorganisms (CFU/ml), No. 1-4 is the composition of the nutrient medium (No. 1 - molasses medium, No. 2 - Uglich medium, No. 3 - MRS medium, No. 4 - environment GPS)

Thus, the most optimal growth requirements for the studied microorganisms is a nutrient medium based on fodder molasses (45.0 g/l fodder molasses (50% beet molasses and 50% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0, 02 g/l), providing the largest increase in the biomass of lactobacilli.

3.3 Study of the Temperature-time Optimm for Growing Microorganisms

Next, the optimal temperature and time of cultivation of Lactobacillus kunkeei on molasses medium were determined to increase the cell titer as soon as possible. The above results of cultivation were obtained at a cultivation temperature of 37°C.

A series of experiments was laid to determine the maximum thermal tolerance of the studied lactobacilli when grown on molasses medium (45.0 g/l of fodder molasses (50% beet molasses and 50% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l) for 48 h. The dependence of the number of lactic acid bacilli cells on temperature and time is in Figures 3 and 4, where the abscissa indicates the time of microorganism cultivation, and the ordinate indicates the number of microorganisms.

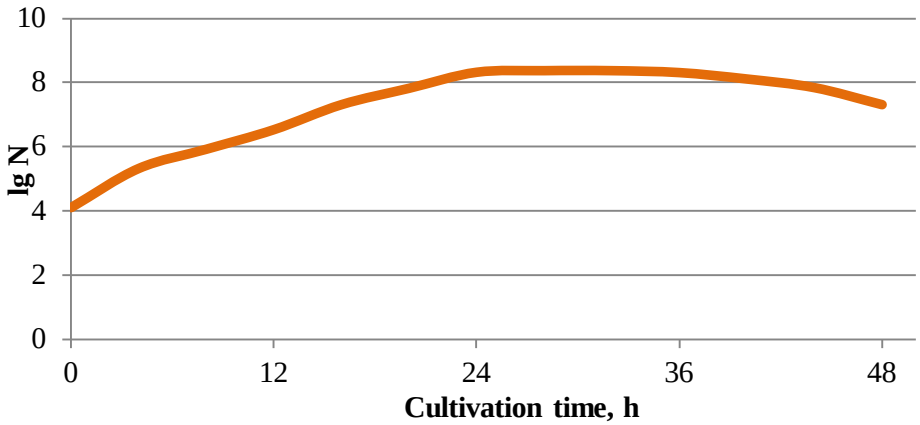

Figure 3: Dynamics of changes in the titer of Lactobacillus kunkeei at 37°C, where lg N - is the decimal logarithm of the number of microbial cells, CFU/ml

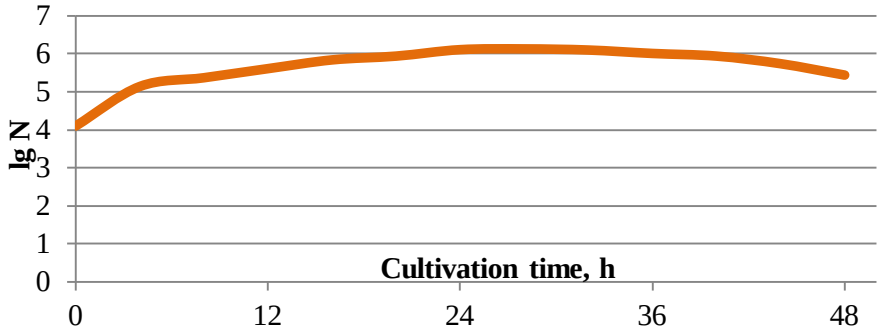

Figure 4: Dynamics of changes in the titer of Lactobacillus kunkeei at 38°C, where lg N is the decimal logarithm of the number of microbial cells, CFU/ml

Based on the above results of cultivation on molasses medium at different temperatures and growth times, it was found that the highest cell titer was reached by 24 hours from the start of

cultivation. More prolonged cultivation and higher temperatures lead to a decrease in titer and, as a rule, to increase the number of non-viable cells. The result of cultivation at 39°C is not presented, since, at this temperature, the culture under study did not reveal any growth relative to the inoculum titer. However, after decrease the cultivation temperature to 37°C, growth recovered in the same volume, which indicates that the culture did not die off but passed into a phase of growth retardation, which continued until the temperature dropped by 2-3°C.

3.4 Studying the Dynamics of the Consumption of Reducing Substances by Lactobacilli

Further, the dynamics of the use of reducing substances by microorganisms with an initial concentration of 4.0% was assessed. The results are in Figure 5.

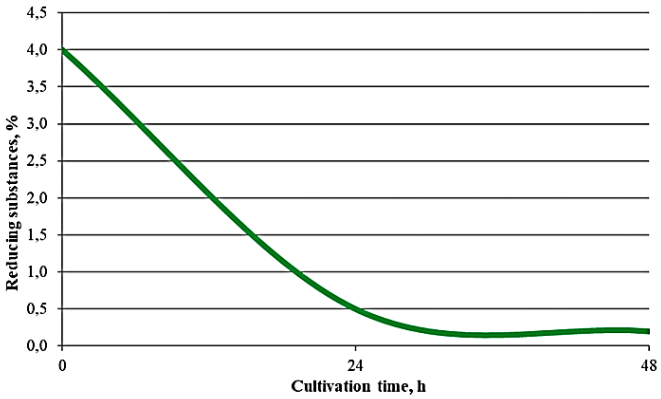

Figure 5: Dynamics of consumption of reducing substances by the culture of Lactobacillus kunkeei on a molasses medium

Figure 5 shows that the most significant depletion of the carbon component falls on 24–28 h from the beginning of growing microorganisms. Therefore, in this time interval, it is advisable to complete the cultivation of the microorganism under study.

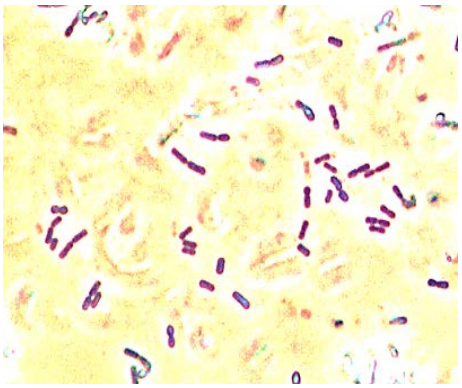

Figure 6: Morphological characteristics of Lactobacillus kunkeei in a smear at 24 h growth, Gram stain, (magnification 1200 ×)

Separately, during the cultivation of Lactobacillus kunkeei, microscopic control of the state of cells, changes in their morphological properties, and the presence of foreign microorganisms were studied. For microscopic control, research microscopes of the Carl Zeiss company of the Axio Imager series were used in both brightfield and phase-contrast modes. According to morphological characteristics, the lactobacilli cells had a constant size and were short rods from 3-4 µm, thickness

up to 0.5 μm. Polymorphism was absent. As the strain is stored, the cells have an insignificant tendency to decrease in size and become indicated (Figure 6).

Thus, the optimal growing time for Lactobacillus kunkee is 24 hours, at an optimum temperature of 37°C.

4 Conclusion

As a result of biotechnological (microbiological) studies, it was established that when growing Lactobacillus kunkeei isolated from drone milk of a honey bee on molasses medium, the medium of the same name with 50% beet molasses and 50% corn molasses had the best growth needs. When studying the developed medium with well-known lactic acid bacteria, it was proved that the most optimal growth requirements for the studied microorganisms are a nutrient medium based on fodder molasses (45.0 g/l fodder molasses (50% beet molasses and 50% corn molasses), K_2HPO_4 - 2.0 g/l, yeast extract - 0.02 g/l), providing the highest biomass yield of lactobacilli. It was also revealed that when Lactobacillus kunkeei was cultivated on molasses medium at different temperatures and growth times, the highest cell titer was reached by 24 hours from the start of cultivation, while the optimal growth temperature was 37°C. In general, the selected growing conditions and nutrient molasses medium can be recommended to develop a biological product that can be used in particular for beekeeping.

5 Availability of Data And Material

Data can be made available by contacting the corresponding authors.

6 Acknowledgment

This work was supported by a grant from the President of the Russian Federation for state support of young Russian scientists - candidates of sciences (agreement No. 075-15-2020-253 of March 17, 2020).

7 References

[1] Bannikova, L. A. (1975). Selection of lactic acid bacteria and their application in the dairy industry. *Food industry*, 148 p.

[2] Bannikova, L. A., Koroleva, N. S., Semenikhina, V. F. (1987). *Microbiological foundations of dairy production*. Agropromizdat, 400 p.

[3] Bakhnova, N. V., Anischenko, I. P. (2005). Direct addition bacterial concentrates for dairy products. Collection of materials of the international specialized scientific and practical seminar "*Bacterial starters and biological agents used in the production of fermented dairy products in Russia*." Uglich, p. 80-81.

[4] Gorbatova, K. K. (2003). *Physicochemical and biochemical bases of the production of dairy products*. GIORD, 352p.

[5] GOST 10444.11-89 (2010). Food products. Methods for the determination of lactic acid microorganisms. *Gosstandart of Russia*, 15 p.

[6] GOST 9225-84 (2009). Milk and dairy products. *Microbiological analysis methods*. Standartinform, 15 p.

[7] Erzinkyan, L. A. (1971). *Biological features of some lactic acid*, 235 p.

[8] Koschaev, A. G., Lysenko, Yu. A., Mishchenko, V. A., Luneva, A. V. (2017). Intensification of the process of cultivation of physiologically adapted lactobacilli as the basis for creating biological products of microbial

origin for poultry farming. *Polythematic network electronic scientific journal of the Kuban State Agrarian University*, 128, 1102-1115.

[9] Kvasnikov, E. I., Nesterenko, O. A. (1975). Lactic acid bacteria and ways of their use. Nauka, 384 p.

[10] Anoshina, O. M., Melkina, G. M., Sidorenko, Yu. I. (2007). Laboratory workshop on a general and special technology of food production. Koloss, 183 p.

[11] Lysenko, Yu.A., Koschaev, A.G., Donnik, I.M., Luneva, A.V. (2019). Nutrient medium for the cultivation of lactobacilli. Patent No. 2686326 Russian Federation, 11 p.

[12] Perfiliev, G. D., Sviridenko, Yu. Ya. (2006). Production and use of bacterial concentrates. *Cheese making and butter making*. 3, 24 p.

[13] Koschaev, A.G., Lysenko, Yu.A., Luneva, A.V., Radchenko, V.V. (2017). Development of an optimal method for producing lactic acid bacteria hydrolyzate. *Polythematic network electronic scientific journal of the Kuban State Agrarian University*. 132, 1464-1476.

[14] Sorokina, N.P. (2004). Bacterial concentrates. Cheese making and butter making, 3, 16 p.

[15] Lysenko, Yu. A., Koshchaev, A. G., Lysenko, A. A., Omarov, R. S., Shlykov, S. N. (2020). Biological properties of microorganisms isolated from drone milk of honeybees. *International Transaction Journal of Engineering, Management and Applied Sciences and Technologies*, *11*(12), 11A12K.

[16] Koshchaev, A. G., Lysenko, Y. A., Semenenko, M. P., Kuzminova, E. V., Egorov, I. A., Javadov, E. J. (2018). Engineering and development of probiotics for poultry industry. *Asian Journal of Pharmaceutics*. 12(4), S1179-S1185.

[17] Koshchaev, A. G., Lysenko, Y. A., Lysenko A. A., Luneva, A. V., Saleeva, I. P., Fisinin, V.I. (2017). Screening of microorganism symbiont strains as a base of probiotics for poultry industry. *Journal of Pharmaceutical Sciences and Research*, 9(8), 1373-1379.

[18] Koshchaev, A. G., Lysenko, Y. A., Luneva, A. V., Gneush, A. N., Aniskina, M. V., Fisinin, V. I., Saleeva, I. P. (2018). Studying Biological Activity of Lactobacillus Hydrolysates. *Journal of Pharmaceutical Sciences and Research*, 10(10), 2475-2479.

Yury Lysenko is a doctor of biological sciences, associate professor of the department of microbiology, epizootology and virology, Kuban State Agrarian University named after I. T. Trubilin. Research interests: development and pharmacological substantiation of the use of new feed additives of microbial origin in poultry meat farming.

Nadezhda Machneva is a candidate of biological sciences, associate professor of the department of biotechnology, biochemistry and biophysics, Kuban State Agrarian University named after I.T. Trubilin. Research interests: bioconversion of agricultural products and processing waste.

Anatoly Smirnov is a doctor of veterinary sciences, professor, academician of the Russian Academy of Sciences, head of the laboratory of veterinary sanitation and ecological safety in beekeeping, All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology. Research interests: development of environmentally friendly systems of veterinary and sanitary services for animal husbandry and beekeeping.

Alexander Panin is a doctor of veterinary sciences, professor, academician of the Russian Academy of Sciences, professor of the department of immunology and biotechnology, Moscow State Academy of Veterinary Medicine and Biotechnology - MVA by K.I. SKRYABIN. research interests: modern problems of industrial biotechnology and bioengineering.

Andrey Koshchaev is a doctor of biological sciences, a corresponding member of the Russian Academy of Sciences, professor of the department of biotechnology, biochemistry, and biophysics, Kuban State Agrarian University named after I. T. Trubilin. Research interests: biochemistry of feed raw materials, dietary supplements, and industrial microorganisms.