Biotechnological potential of *Lactobacillus brevis* 2k.Gv for aquaculture purposes

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**Abstract.** The article presents the results of a study of the biological properties of the *Lactobacillus brevis* 2k.Gv strain (morphological, cultural, biochemical, physiological and probiotic) in order to assess the biotechnological potential and create alternative forms of probiotics for highly productive fish farming. Under the conditions of an aquarium experiment, a positive effect of the strain on the physiological parameters of the rainbow trout *Parasalmo mykiss* Walbaum was found. *L. brevis* 2k. Gv was orally administered to fish for 30 days at a concentration of 2×10⁸ cells/mg feed as a supplement to the main diet. It was found that the studied strain has a multifactorial effect on the fish organism. Throughout the entire period of feeding juvenile trout with extruded feed enriched with lactobacilli, no signs of infectious and somatic diseases were recorded in the experimental group of trout; hepatosomatic and splenosomatic indices of organs, as well as hematological parameters of blood remained normal. The results obtained prove the presence of probiotic properties in the *Lactobacillus brevis* 2k. Gv strain and the possibility of its further use as part of feed additives for therapeutic and prophylactic purposes for aquaculture objects.

**1. Introduction**
Currently, more than ever, new technologies for aquaculture are in demand, associated with an increase in fish resistance to unfavorable environmental factors. The successful introduction of such technologies into the practice of highly productive fish farming largely depends on the composition of extruded feed, which includes a complex complex of ingredients of biological origin that have an immunostimulating effect. Premixes based on probiotic bacteria and products of their metabolism are a good example of therapeutic and prophylactic additives. As a rule, these are representatives of the genera *Azomonas* sp., *Lactobacillus* sp., *Bacillus* sp., *Ruminococcus* sp., as well as *Saccharomyces cerevisiae*, capable of suppressing the infectious process due to the synthesis of inhibitory compounds, which in turn suppress the proliferation of pathogenic and opportunistic species microorganisms [1–3]; modulate the biological systems of the host and improve fish-breeding performance [4–6]. According to the studies performed, the effectiveness of such biological premixes largely depends on the method of administration of the probiotic, dosage, duration of use and the form of the introduced probiotic microorganism [7]. Most often, live strains of bacteria are introduced into the main diet for fish [8, 9], but the use of lyophilized cells [10], spores, and cell-free supernatants is also known [11].

Among a wide variety of probiotic microorganisms, *Lactic acid bacteria* (LAB) are the most common and lead in complex processes of restoring homeostasis of a macroorganism (especially post-infectious ones) due to reparative intracellular mechanisms and biologically active substances in the form of bacteriocins, hydrogen peroxide, proteases [12] or biosurfactants, which inhibit the adhesion...
of pathogens [13]. A typical representative of LAB - *Lactobacillus brevis* is a gram-positive, immobile, non-sporogenous rod-shaped bacterium with a characteristic morphotype in the form of polymorphic cells measuring 3.5-6.0 × 1.2 μm, single or collected in short chains. *L. brevis* belongs to the division *Firmicutes*, class *Bacilli*, family *Lactobacillaceae*, genus *Lactobacillus* [14]. The main metabolites of the microorganism are lactic acid and ethanol. It has also been established that some *L. brevis* strains are capable of producing biogenic amines, tyramine and phenylethylamine [15]. Most *L. brevis* strains have adhesive properties to cells of the gastrointestinal tract, high antibiotic and proteolytic activity.

Taking into account the various positive effects of LAB on the macroorganism, it is extremely important to create and study original forms of probiotics that contribute to the expansion of the spectrum of adaptive reactions of aquaculture objects. In connection with the above, in the presented study, the following goal was put forward and realized: to assess the biotechnological potential of lactic acid bacteria *Lactobacillus brevis* and their influence on some physiological parameters of the rainbow trout *Parasalmo mykiss Walbaum* for the subsequent creation of new forms of therapeutic and prophylactic additives to feed used in industrial fish farming.

2. Material and research methods
The study was carried out at the Research Center for Aquaculture of Petrozavodsk State University in the period from 2019 to 2020. The study of the biotechnological potential of the LAB was carried out using the example of the *Lactobacillus brevis: 2k.Gv* strain (from the collection of the Microbiology Laboratory of the Scientific Center for Aquaculture of PetrSU) isolated from the enrichment culture of acetic acid fermentation. Cultivation of LAB was carried out in a sterile 250 ml Erlenmeyer flask containing 50 ml of MRS nutrient medium modified with a mineral carrier and 1 ml of microbial mass at a concentration of 10⁹ CFU / ml. For stable mixing of the ingredients, a shaker of the S:S-3L.A20 series, («Biosan», Latvia), with a digital control system and orbital motion of the platform at a rotation speed of 110 rpm was used. The physiological activity of lactobacilli was assessed using the «RTS-1C, Biosan» bioreactor with software and the function of monitoring the growth of microorganisms in real time. For incubation of cells, sterile 50 ml «TPP TubeSpin» vessels with a membrane filter were used. The amount of nutrient medium introduced was 29 ml, and the culture volume was 1 ml. The indices of the optical density that the microorganism is capable of and the growth kinetics were measured at a given measurement range of 0-8 OD at λ-850 nm, measurement accuracy of ±0.3 OD and a constant incubation temperature of 37 °C (temperature stability was ± 0.1 °C). In parallel, the biochemical activity of L. brevis in relation to mono-, di- and trisaccharides, as well as viability and biotiter, were evaluated.

For the experiment on feeding with extruded food enriched with lactobacilli, underyearlings (age 0+) of rainbow trout were used in the amount of 60 individuals with an average weight of 104 ± 6.82 g. To assess the physiological reaction of juvenile trout to food, in fish before the start of the experiment and after its completion the weights of the liver and spleen were determined, and the hepatosomatic (HPSI,%) and splenosomatic (SSI,%) indices were calculated [16]. In addition, hematological parameters were assessed: the percentage of erythrocytes, immature lymphocytes, mature lymphocytes, monocytes, granulocytes and platelets and the change in ESR (ml/hour). All hematological studies were performed according to standard methods accepted in ichthyology [17; 18].

The experiments carried out with juvenile rainbow trout are consistent with international ethical standards set forth in the European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes, as well as with the requirements set forth in the Order of the USSR Ministry of Health dated 12.08.1977 № 755 «On measures for further improvement of organizational forms of work with the use of experimental animals» and other regulatory documents
(conclusion of the Ethics Committee in the field of animal research at Petrozavodsk State University № 274 of May 7, 2020).

2.1 Experimental conditions
During the experiment, juvenile trout were kept under natural light in 100 m³ aquariums equipped with a closed water supply system (water exchange rate 15 l/min) at a temperature of 13.2 ± 0.5 °C, the concentration of dissolved O₂ is 8.8 ± 0.4 mg/l, pH 7.3 ± 0.3, NH₃ 0.010 ± 0.007 mg/l.
Before the start of the feeding experiment, juveniles of trout were kept in a common aquarium for 10 days in order to adapt to the conditions of detention. Then the fish were divided into two representative groups, taking into account the mass, and planted in 4 aquariums, forming two independent recirculating water systems (closed water supply installations) between themselves:
1. Fry weighing less than 100 g (average weight of individuals 74 ± 1.7 g): 10 individuals - control (aquarium № 1) and 10 individuals - experiment (aquarium № 3);
2. Fry weighing more than 100 g (average fish weight 114 ± 2.22 g): 10 individuals - control (aquarium № 2) and 10 individuals - experiment (aquarium № 4).

The remaining 20 specimens were evaluated according to the studied parameters before the start of the experiment for comparative analysis with its results.
The fish in the control was fed with the extruded food «BioMar» (made in Denmark) balanced in all nutrients for juvenile trout with a pellet size of 3 mm. For the experiment, the same food was used, but enriched with the genuine L. brevis strain: 2k. Gv, the content of which in the daily portion was at least 10⁸ CFU/mg. Immediately before use, the feed pellets and the bacterial concentrate were slowly mixed and left to pickle for 12 hours at room temperature 21 ± 3 °C. The daily feeding rate was calculated based on 1.5% of the fish body weight. The amount of coma given in the control and experimental groups was the same.

3. Results
As a result of the studies performed, the cultural, morphological and tinctorial characteristics of the studied strain of lactobacilli are described, which are presented in Table 1.

| Investigated strain Growth | Growth on agar medium | Growth on a liquid medium | Morphological, tinctorial signs |
|---------------------------|-----------------------|---------------------------|--------------------------------|
| L. bravis: 2k. Gv         | Colony diameter 1.2 ± 0.2 mm, rounded, matte, convex, with a cream shade | Uniform opacity | Sticks located singly and in short chains, Gr (+) |

It was found that the L. brevis 2k. Gv strain isolated from the enrichment culture of acetic acid fermentation possesses important technological properties and is capable of reaching the optical density of the culture solution −1.6 OD (Figure 1). Growing in a fermenter made it possible to obtain a biomass of L. bravis:2k.Gv with a cell concentration of up to 1×10⁹ CFU/ml and higher.
Figure 1. The results of measurements of the physiological activity of \textit{Lactobacillus brevis: 2k.Gv} in fermentation conditions for the bioreactor RTS-1C, «Biosan».

With respect to four monosaccharides (arabinose, glucose, ribose and fructose), two disaccharides (maltose and lactose) and trisaccharide (raffinose), the \textit{L. brevis: 2k.Gv} strain showed biochemical activity (Table 2). The data on the enzymatic activity of the studied culture are consistent with the description of the biochemical profile of \textit{L. brevis}, regulated in the Bergi Bacteria Guide (1980).

\begin{table}[h]
\centering
\begin{tabular}{ l l l }
\hline
Substrate & \textit{Lactobacillus brevis: 2k.Gv} & \textit{Lactobacillus brevis} * \\
\hline
Arabinose & + & + \\
Glucose & + & + \\
Lactose & + & 90\% + \\
Maltose & + & + \\
Mannose & – & – \\
Raffinose & + & 90\% + \\
Ribo & + & + \\
Fructose & + & + \\
\hline
\end{tabular}
\caption{Enzymatic activity of the \textit{Lactobacillus brevis strain: 2k.Gv}.}
\end{table}

Note: * - data according to Bergi Bacteria Guide (1980)

When feeding juvenile trout with «BioMar» enriched with a culture of lactobacilli, a positive reaction of the liver of juvenile trout to the use of a biological additive in the diet was established (Table 3). In the studied groups («small» and «large» fry), fish with a hepatosomatic index corresponding to the norm for a given age (HPSI: 1.23 and 1.18, respectively) were introduced into the experiment. According to the studies of a number of authors Kumar V, Makkar H P S, Becker K [19], the normal liver weight of juvenile rainbow trout in artificial cultivation is from 1.0 to 1.4\% of body weight (HPSI <1.5\%).

During the experiment, the parameters of the fry HPSI increased, both in the experiment (by an average of 16.5\%) and in the control (by an average of 20.5\%), which indicates the accumulation of reserve nutrients in the liver of trout in the form of glycogen and fat. Remaining within the normal range, the relative weight of the fish liver in the experimental groups was lower than in fish from the control groups - by an average of 5\%, which may be due to the inclusion of lactic acid bacteria in the general metabolism of the trout organism. This, in turn, does not exclude microflora control over the process of lipid accumulation in the liver.
Table 3. Hepatosomatic (HPSI) and splenosomatic index (SSI) of juvenile rainbow trout in the experiment and control.

| Group of fish                  | HPSI, % | SSI, % |
|-------------------------------|---------|--------|
|                               | Fry with an initial weight < 100 g | Fry with an initial weight > 100 g | Fry with an initial weight < 100 g | Fry with an initial weight > 100 g |
| Before the experiment         | 1.23 ± 0.04 | 1.18 ± 0.10 | 0.07 ± 0.01 | 0.11 ± 0.01 |
| Experience (end of experiment)| 1.48 ± 0.11 | 1.40 ± 0.08 | 0.13 ± 0.03 | 0.18 ± 0.02 |
| The control (end of experiment)| 1.55 ± 0.06 | 1.47 ± 0.12 | 0.11 ± 0.01 | 0.16 ± 0.03 |

Despite the fact that the indices of the spleen throughout the entire experiment also remained within the normative values (0.17–0.28% of the total weight of the individual), the indices of the SSI in the experimental groups differed in higher values compared to the control, which may be due to multifactorial effect of lactobacilli on the immune system of fish in conditions of aquarium keeping [20]. It should also be noted that short-term feeding with «BioMar» extruded feed with bioadding did not affect the external state of the internal organs of juvenile trout. Clinical examination and ichthyopathological examination of *P. mykiss* juveniles revealed no characteristic signs of infectious and somatic diseases.

In addition to calculating physiological indices, the state of trout in the experiment and control was checked using a blood test, which is of great importance in fish farming for early diagnosis of the disease. In the hematological study, the number of erythrocytes, the erythrocyte sedimentation rate, the number of leukocytes and the leukocyte formula were taken into account. Data on the hematological parameters of the blood of trout receiving a given concentration of LAB and trout with a standard ration as an additive to the basic diet are presented in Table 4.

Table 4. Morphotic parameters of blood cells of juvenile rainbow trout in the experiment and control.

| Group of fish | Shaped elements, % |
|---------------|--------------------|
|               | reticulocytes  | mature | erythrocytes | immature | lymphocytes | mature | lymphocytes | monocytes | granulocytes | platelets |
| 1*            | 5.5              | 94.49  | 24.5         | 25.612   | 5.75        | 3.93   | 2.8         |           |              |          |
| 2**           | 14.87            | 85.13  | 67.25        | 26.11    | 2.65        | 3.98   | 2.8         |           |              |          |

Note: * – experimental group, ** – control group
The ratio of the number of reticulocytes and mature erythrocytes in the experimental group of trout was 1:17.12, and in the control group - 1:5.72%. Analysis of the mechanism of increasing the number of reticulocytes in the blood of trout from the control group allows us to state the fact of increased destruction of erythrocytes, therefore, their level increases and exceeds the same values in the experimental group by almost 2.7 times. It should also be noted that the presence of precursors of reticulocytes - normoblasts - was constantly noted among the cells of the erythrocyte series in the control. A blood smear made it possible to differentiate the shape of erythrocytes: mature erythrocytes can be characterized as oval, flattened cells with eosinophilic cytoplasm and a centrally located elliptical nucleus (Figure 2a). Among the erythrocytes of the control groups of trout, there were various sizes (anisocytosis) - the frequency of occurrence of 4.1%, abnormal cell forms in the form of poikilocytosis - the frequency of occurrence of 16.6% (Figure 2b), dacryocytes - the frequency of occurrence of 3.2% (Figure 2c), nuclear invaginations - the frequency of occurrence is 6.2%. The leukocytes of the experimental and control groups had some differences in the set of morphotic elements. Thus, in the blood of the experimental groups of trout, the number of mature lymphocytes was 56.12%, which is 2.15 times more than in the control groups. Monocytes also predominated, their share in the experiment was 5.75%, and in the control - 2.65%. The proportion of polymorphonuclear leukocytes (PMN) from the total number of leukocyte cells did not differ, and there were no significant differences among platelets (3.1% in the experiment and 2.8% in the control).

Based on the assessment of the erythrocyte sedimentation rate of the two groups of *Parasalmo mykiss*, some change in these indicators was revealed when feeding fish with a dietary supplement to the main diet, and without it. The ESR range among fish in the control groups varied from 2.36 to 4.22 ml/h, and in the experimental ones - from 2.67 to 4.09 ml/h. All the obtained values are close to the norm for trout, but in the control, the erythrocyte sedimentation rate was 1.2 times higher than in the experiment.

4. Conclusion
As a result of the research, primary experimental data were obtained that describe the biotechnological potential of the strain of lactic acid bacteria *Lactobacillus brevis: 2k.Gv*. To assess the effect of lactobacilli on the morphophysiological state of *P. mykiss* juveniles, some fish-breeding and hematological parameters were analyzed. A comparative analysis of the efficiency of consumption of the extruded feed «BioMar» with and without supplements to the main diet of juvenile trout revealed a
positive effect of feed with lactobacilli on the physiological state of fish. Under conditions of therapeutic and prophylactic nutrition, no pronounced clinical and ichthyopathological changes were found in the trout organism; hepatosomatic (HPSI) and splenosomatic indices (SSI) varied within normal limits.

On the basis of the studies performed, the evidence of hematological parameters of the circulating blood of fish was confirmed as objective criteria for changes caused by environmental factors, including the dynamics of the feed ration - standard and with a therapeutic and prophylactic feed additive in the form of lactobacilli. When studying the blood of the experimental and control groups of *P. mykiss*, it was found that the average ESR value for the control groups averaged 3.57 ml/h, and for the experimental ones - 2.92 ml/h. Erythrocytes are attributed to the most numerous blood cells of juvenile trout, the proportion of mature erythrocytes was 94.49% (in the experiment) and 85.13% (in the control). Moreover, in the blood of the experimental groups of trout, a predominance of mature erythrocytes and the absence of abnormal forms of erythrocyte cells were found. The presence of reticulocytes and normoblasts was noted in the blood of control fish groups. Such differences can be caused by the conditions of fish feeding and determined by the functional activity of the hematopoietic tissue [21]. In the blood of the experimental groups of trout, the number of mature lymphocytes averaged 56.12%, which is 2.15 times more than in the control.

Thus, as a result of the studies carried out, it can be assumed that the isolated *L. brevis: 2k.Gv* strain has important technological parameters for reasonable use in the process of creating new forms of probiotics for industrial fish farming objects. The *L. brevis: 2k.Gv* strain is able to successfully colonize intestinal cells and stimulate the resistance of the microorganism to damaging environmental factors. During the feeding of the experimental groups of *P. mykiss* juveniles with «BioMar» with lactobacilli, the latter remained viable in the microflora of the fish gastrointestinal tract, transferred the passage through the stomach and remained in the intestinal contents. According to the results of the study, the culture of lactic acid bacteria remains metabolically stable in the aggressive environment of the digestive tract of fish, coupled with enzymatic activity and a high degree of food digestion, which increases its probiotic potential. It should be emphasized that the listed properties of *L. brevis: 2k.Gv* are strain characteristics of the species, which does not exclude the optimization of its technological parameters by changing the cultivation regime, the composition of the nutrient medium and the use of immobilization technology on biologically compatible carriers to ensure the maximum yield of useful biomass of lactic acid bacteria.

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