Results of the study on the antimicrobial activity of probiotic strains of lactobacilli and their potential application in feed formulations for aquaculture species

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Abstract. A comparative analysis of the antibacterial activity of authentic strains of lactobacilli was conducted. Primary data on the antibacterial activity of *Lactobacillus acidophilus*, *L. brevis*, and *L. plantarum* were obtained, as well as the impact of L. brevis in the composition of the extruded feed «BioMar» on the physiological condition of juvenile rainbow trout. The supplement was orally introduced into the main diet at a concentration of 2×10^8 cells of *L. brevis* per milligram of feed for 30 days. The obtained results were evaluated based on changes in the growth indicators, survival rates, and the diversity of the background microflora in the intestinal contents of trout fry in the experimental and control groups. According to the experiment results, the physiological indicators of trout fry receiving the premix with lactobacilli in the feed were higher than those in the control group. In the intestinal contents of the experimental group, a high proportion of lactobacilli (9.4%) was maintained, unlike the control group, which was on a diet without any additives (average values of lactobacilli in the intestinal contents did not exceed 0.6%). The studied biological properties of the Lactobacillus brevis strain make it a potential probiotic culture for use in therapeutic and prophylactic feed formulations for aquaculture.

1 Introduction

One of the unique properties of probiotic strains of lactobacilli is their ability to biosynthesize a wide range of low-molecular-weight biologically active compounds with significant potential for combating pathogens with multiple drug resistance. Key characteristics of probiotics contribute to this biological effect, including survival in the gastrointestinal tract, the ability of strains to autoaggregate and grow, the formation of biofilms for protection against environmental stressors, and the effective manifestation of probiotic action in the intestine [1]. According to the results of long-term studies, it has been demonstrated that the positive effect of lactobacilli is also closely related to a broad spectrum of biologically active molecules, organic acids, competition for adhesion sites, and nutrients [2]. The use of column chromatography on silica gel followed by HPLC has revealed the chemical characteristics

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and structure of several antibacterial compounds produced by lactobacilli. For instance, the probiotic strain Lactobacillus plantarum LJR13 has been found to produce tert-butyl-4-(4-oxo-(2-((2-oxo-1-(p-tolyl)-2-(p-tolyloxy) ethyl) carbamoyl) pyrrolidin-1-yl) butanoate [3]. This compound showed no cytotoxicity towards mouse embryonic fibroblast cells (NIH-3T3) and inhibited the development of colorectal carcinoma cells, indicating its potential anti-cancer activity. By now, the composition of probiotic cultures is known, which have found application as substitutes for currently used antibiotics. Lactobacterium acidophilus, L. plantarum, L. fermentum, L. delbrueckii subsp. bulgaricus, L. lactis subsp. lactis are effectively used for therapeutic and preventive feeding of fish. These include mono- and polycomponent preparations such as «Azogilin», «Bacell», «Alchem Poseidon», «Lactobacterin», «Prolam» etc. Typically, these drugs are employed to optimize the physiological state, improve hematological and fish-breeding indicators, as well as to normalize the bacterial component of the gastrointestinal tract and enhance overall resistance [4, 5, 6, 7].

In order to study the antibacterial activity of lactobacillus strains and assess the effectiveness of introducing promising strains into the feed, several tasks have been outlined: to investigate the antibacterial activity of lactobacilli and perform a comparative analysis of the effectiveness of young trout consumption of extruded feed «BioMar» with an additive of lactobacilli to the main diet.

2 Materials and Methods

For a comparative investigation of the antibacterial activity of lactobacilli, authentic strains of *Lactobacillus acidophilus*, *L. brevis* and *L. plantarum* from the Collection of Lactobacilli with probiotic and antimicrobial properties at the Microbiology Laboratory of Microbiome LLC (Limited Liability Company), Petrozavodsk State University, were employed. The standard pharmacopoeial method of agar diffusion [8] was utilized for quantitative determination of the active substance.

As test microorganisms, *Escherichia coli* K12 ATCC 209P and *Staphylococcus aureus* ATCC 209P from the RCIM (all-Russian collection of industrial microorganisms) were chosen. Each indicator strain was inoculated into HFM-agar (Hydrolysate of Fish Meal-agar) based on fishmeal hydrolysate (Obolensk). The experimental selection of the seeding dose for the test microorganism was 10^5 CFU (Colony-Forming Units)/ml to ensure continuous growth of the microbial lawn during testing. Holes were punctured in the medium and filled with 50 µl of culture. The cultures were incubated for 18 hours at a temperature of 37±2 °C. The diameter of the inhibition zones of the test cultures around the holes was measured in millimeters, and all cultures were performed in triplicate.

Strains with the most pronounced antibacterial activity were tested as a premix added to the main diet for rainbow trout fry (*Parasalmo mykiss* Walbaum). As an aquaculture species, rainbow trout is characterized as a cold-water species with a relatively high growth rate. For the trout feeding experiment, 30 fry aged 3 months were selected (15 for the experiment and 15 for the control).

The aquarium experiment on the effectiveness of using a lactobacillus premix in the main diet of trout fry was conducted over a period of 30 days. The trout were kept in aquariums at a temperature of (14.2±0.5 °C), dissolved O_2 concentration (8.3±0.2 mg/l), pH (7.1±0.5), total NH_3 (0.010±0.004 mg/l), and natural lighting. Fish feeding in the experimental and control groups began after a 10-day adaptation period.

The control group received extruded feed «BioMar» (produced in Denmark). The additive to the main diet was administered orally at a concentration of 2×10^8 lactobacillus cells/mg of feed. In the control, fish received traditional feed without probiotics, branded as «BioMar». At the end of the experiment, changes in body mass and length of the fry in the experimental

and control groups were recorded. Throughout the experiment, the viability of the tested groups of trout fry was monitored. Daily observations were made on the fish's behavior and its response to feed.

To assess the effectiveness of feed consumption spent on growth, absolute and relative average daily weight gain of fish were calculated in the experimental and control groups, considering survival rates [9, 10]. All fish weight measurements were performed according to SS (State Standard) 1368-2003.

The absolute average daily weight gain of fish was calculated using the formula:

$$C\ (g/day) = (M_i - M_0)/t, \quad (1)$$

where:
M_0 – initial fish weight at the start of feeding (g),
M_i – fish weight at the end of the feeding period (g),
t – duration of the feeding period (days).

The calculation of relative average daily weight gain was performed using the formula:

$$Cw\ (\%) = 2\ (M_i - M_o) \times 100/(M_i + M_0) \times t, \quad (2)$$

where:
M_0 – initial fish weight at the start of feeding (g),
M_i – fish weight at the end of the feeding period (g),
t – duration of the feeding period (days).
Survival was calculated using the formula:

$$Survival\ (\%) = (N_i / N_0) \times 100, \quad (3)$$

where:
N_i – final number of fish in the experiment,
N_0 – initial number of fish in the experiment.

To investigate the background microflora of the trout intestinal contents in the experimental and control groups, samples were collected at intervals of 15 and 30 days in sterile tubes with buffered peptone water, following aseptic techniques. The taxonomic composition of the microflora was assessed according to methodological recommendations (1991) and based on phenotypic criteria regulated in the Bergey's Manual of Systematic Bacteriology (1997).

The analysis of the obtained data was conducted using approaches from variational statistics commonly applied in biology [11] and methods for determining bacterial quantities and statistical processing of results [12].

3 Results

The results of the comparative study of the antibacterial activity of authentic strains of *Lactobacillus acidophilus*, *L. brevis* and *L. plantarum* are presented in Table 1. The highest antibacterial activity was observed in the L. brevis strain, in the presence of which the average diameter of the inhibition zone for *Escherichia coli* was 9.1 mm, and for *Staphylococcus aureus*, it was 11.6 mm. Based on the antibacterial activity of lactobacilli, the *L. brevis* culture was selected for use as a premix in the compound feed.

Table 1. Comparative data on the antibacterial activity of the studied lactobacillus strains

Studied strains	*Escherichia coli* K12	*Staphylococcus aureus*
	diameter of inhibition zones, mm	
L. acidophilus	5.1 ± 0.2	8.2 ± 0.3
L. brevis	9.1 ± 0.6	11.6 ± 1.4

L.plantarum	4.1 ± 0.3	6.3 ± 0.7

Results of the experiment on the impact of *Lactobacillus brevis* strain in the «BioMar» feed on the growth and survival of rainbow trout fry are presented in table 2. It was found that despite some differences in the initial weight of the fish (for the experimental group – 87.2 g, and for the control group – 90.3 g), by the end of the experiment, the average weight values of the experimental group not only equalized with the control values but also exceeded them by 26.7 g. In addition, the rainbow trout fry in the experimental group showed higher values of absolute and relative growth – 1.2 g/day and 0.9%, respectively. The obtained data correlate with the survival results of fry in the experiment and control. the survival of rainbow trout fry in the experimental group at the end of the experiment was 73%, which is 1.6 times higher than similar values in the control group.

Table 2. Growth and survival rates of *P. mykiss* juveniles in the experiment and control (average values)

№	Indicator	Investigated Groups	
		Experimental (n = 15)	Control (n = 15)
1	Initial Weight, g	87.2 ± 4.8	90.3 ± 5.9
2	Final Weight, g	116.4 ± 12.8	108.2± 10.6
3	Absolute Growth, g/day	1.2 ± 0.4	0.7 ± 0.2
4	Relative Growth, %	0.9 ± 0.3	0.5 ± 0.1
5	Survival, %	73.3	46.6
6	Total Growth, g	29.2	17.9

In the intestinal content of the control group of rainbow trout fry, Proteobacteria dominated. A variety of pathogenic and conditionally pathogenic Gram-negative bacteria were found, including representatives of the genera *Escherichia*, *Salmonella*, *Vibrio*, *Yersinia*, *Pseudomonas*, causing mixed bacterial infections with diverse clinical manifestations, affecting numerous fish species, and varying in the severity of outbreaks [13]. With the inclusion of lactobacilli in the diet of rainbow trout fry, the total percentage of Proteobacteria decreased by 2.8 times. By day 20, species of the genera *Serratia* and *Proteus* were not detected, and the proportion of *Alcaligines*, *Escherichia* and *Klebsiella* decreased from 5.6% to 2.4%. In the control group, throughout the experiment, the qualitative and quantitative diversity of pathogenic and conditionally pathogenic bacteria remained stable at the level of 31–36%. It is worth noting that in the intestinal content of the experimental group of rainbow trout fry, a high proportion of lactobacilli was maintained by the end of the experiment—9.4%, unlike the control group, where the average values of lactobacilli in the intestinal content did not exceed 0.6%.

4 Conclusion

Following the assessment of the antibacterial activity of genuine lactobacillus strains, cultures of Lactobacillus brevis were identified as particularly effective in inhibiting the growth of *Escherichia coli* K12 and *Staphylococcus aureus*. Upon analyzing the impact of introducing selected L. brevis cultures into the diet of juvenile trout, successful colonization in the intestine was observed, accompanied by a reduction in the proportion of pathogenic and opportunistic members of Proteobacteria. The experimental findings regarding the administration of a probiotic premix to juvenile trout align with the results of various studies investigating the influence of probiotics on rainbow trout physiology [14]. While feeding the experimental group of rainbow trout juveniles with «BioMar» feed containing lactobacilli, these microorganisms remained viable within the fish's gastrointestinal tract, withstanding passage through the stomach and persisting in the intestinal contents. It can be inferred that

the studied culture maintains metabolic stability in an environment characterized by enzymatic activity and extensive feed digestion, enhancing its probiotic potential. The enumerated biological properties of *Lactobacillus brevis* could be further optimized through cultivation conditions, including adjustments to acidity and nutrient medium composition. This optimization aims to maximize the yield of beneficial lactobacilli biomass while preserving and enhancing the ability to biosynthesize antibacterial compounds.

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