Effect of probiotics Zoonorm and Subalin on the immunophysiological system and microbiocenosis of carp

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Abstract. Aquaculture is a fast-growing sector in Russia. To grow fish to a greater extent than for other agricultural objects, it is necessary to preserve and maintain natural habitat conditions. Minor changes in the aquatic environment, such as changes in pH, temperature fluctuations, and the presence of organic pollutants can lead to a decrease in the number of fish. Use of antibiotic drugs for the prevention and treatment of diseases in fish farming can lead to a change in the microbiocenosis of fish and the hydroecosystem, the appearance of antibiotic-resistant strains of pathogenic microorganisms and a change in the immunophysiological status of the fish organism. To date, a promising and effective therapeutic and prophylactic agent in fish farming is the use of probiotics, which have pronounced antimicrobial activity against pathogenic and conditionally pathogenic microorganisms, immunocorrecting and anti-inflammatory effect. As a result of our research, we have established the effectiveness of the use of preparations Subalin containing Bacillus subtilis and Zoonorm containing live bacteria Bifidobacterium bifidum in the cultivation of carp. A positive effect on the increase in body weight of fish was revealed, the level of contamination of parenchymal organs of fish with bacterial flora decreased, specific and nonspecific resistance increased.

1 Introduction

The effectiveness of the development of freshwater aquaculture depends on many factors: optimal conditions for growing planting material, the quality of feed used, compliance with technological processes, sanitary and hygienic regime of the aquatic environment. The microbiocenosis of fish largely depends on the microbiocenosis of the environment - water, on the level of its contamination with organic substances, on the degree of its aggressiveness, the quality of compound feeds and their bacterial contamination, on the

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immunophysiological status of the fish itself. Unfortunately, at present, the culture of fish farming leaves much to be desired. If low-quality feed is used, oxygen and temperature conditions are violated, fish are constantly exposed to various stress factors, then favorable conditions are created for the development of an infectious process caused by the association of microorganisms (not necessarily pathogenic), and more often even by a complex of saprophytic bacteria that contaminate the fish body as a result of a decrease in its resistance [1]. Attempts to use antibacterial drugs in such cases lead to temporary improvement, but then deterioration occurs again. Often this process can proceed without clinical manifestations, the fish does not lose its presentation, but economic losses occur, since the energy potential of the fish is spent on combating contaminants, and not on increasing body weight. To avoid such losses and to prevent infectious diseases, probiotic drugs are currently widely used in fish farming [2-4].

In medicine and veterinary medicine, probiotics are used to increase the immunophysiological status of the body and especially after a course of treatment with antimicrobial drugs, which include various microorganisms with high antagonistic activity, taking part in the digestive processes, having a whole complex of vitamins, amino acids and enzymes [5].

In recent years, close attention has been paid to the production of probiotic and synbiotic feed additives aimed at stimulating metabolism, nonspecific resistance, prevention of diseases associated with changes in the microbiocenosis of the digestive canal caused by endogenous and exogenous causes. Probiotic drugs and feed additives have a preventive effect, contribute to the restoration of intestinal microbiocenosis after the use of antibiotics and other chemotherapeutic agents, and reduce toxin formation and the negative effect of toxins. As a rule, autochthonous lactic acid bacteria belonging to the genera Lactobacillus and Bifidobacterium, as well as gram-positive spore-forming bacteria of the genus Bacillus, are used as probiotics. The most commonly used drugs are based on Bacillus subtilis, which belong to microorganisms that are not pathogenic to humans and animals, and work with which does not require special precautions [6-8].

Studies by some scientists show that in addition to the resistance of probiotic Bacillus strains to the action of bile acids, they have the ability to immunostimulate in gastrointestinal disorders. The immunocorregulating role of probiotics has been proven, the practical use of which is due to their ability to regulate microbiological processes in the digestive tract, as well as stimulate growth and development. The ability to synthesize antibiotics, bacteriocins, cyclic lipopeptides and lytic enzymes with antimicrobial activity provides probiotic activity of bacteria of the genus Bacillus. It has also been shown that strains of B. subtilis and B. coagulans as probiotics have a growth-stimulating and preventive effect. Bacteria of the genus Bacillus are able to form endospores, allowing them to remain viable in extreme conditions: at high or low temperatures, radiation, suboptimal pH, pressure, and in the presence of toxic chemicals that damage vegetative cells [9, 10]. The dense multilayer shell of the spores helps the bacilli to remain active during transit through the gastrointestinal tract, some areas of which are a toxic environment for Bacillus due to anoxia conditions, low pH and bile salts, as well as an extremely high concentration of commensal bacteria (up to 10^{12} cells per 1 g of colon contents), which compete for nutrients and ecological space [11-13].

Due to the effect of probiotics and synbiotics on the microbiological status of intestinal contents, they are able to influence the formation of natural resistance and immunological reactivity of the body [7], and this effect is most pronounced in young animals.

The introduction of probiotics into the diet of animals contributes to the protection of the gastrointestinal tract from pathogens, the restoration of symbiotic microflora after therapy with chemotherapeutic agents of various directions of action, strengthening the processes of digestion of food [8].

Currently, probiotic drugs are evaluated not only by their antimicrobial action, but also by the peculiarities of the effect on the body and its microbiota during the development of allergic reactions, autoimmune processes, the need to strengthen the immune response, in the treatment of subacute and chronic infectious diseases [10]. A number of authors have studied the immunomodulatory properties of live probiotic cultures, cell wall components, DNA and individual metabolites on cell cultures, using laboratory animals, as well as when analyzing changes in immunobiological parameters against the background of probiotic administration by doctors and veterinarians.

Studies by a number of authors have demonstrated that the introduction of B. subtilis causes activation of macrophages [7]. In activated macrophages, the synthesis and release of anti-inflammatory cytokines increases: tumor necrosis factor α, interferon-γ (IFN-γ), interleukin (IL) 1β, IL-6, IL-8, IL-10, IL-12, macrophage inflammation protein-2. As a result, a complex inflammatory response develops, aimed at destroying the pathogen. For example, IFN-γ activates macrophages and protects cells from viral infection. IL-6 stimulates the proliferation and differentiation of B-lymphocytes responsible for the synthesis of antibodies. IL-8 is a powerful chemotactic and paracrine mediator for neutrophils. Infiltration by activated neutrophils plays an important role in maintaining inflammation and oxidative stress. IL-12 regulates the growth, activation and differentiation of T-lymphocytes. In one of the works it was shown that exopolysaccharides of probiotic are responsible for the mechanisms of macrophage activation [7, 8].

Studies show that in addition to the resistance of probiotic Bacillus strains to the action of bile acids, they have the ability to immunostimulate in gastrointestinal disorders. The ability to synthesize antibiotics, bacteriocins, cyclic lipopeptides and lytic enzymes with antimicrobial activity provide probiotic activity of bacteria of the genus Bacillus [14, 15].

When analyzing the literature data on the studied use of bacteria of the genus Bacillus, it was demonstrated that after oral administration of spores, their germination in the gastrointestinal tract into vegetative cells is observed. Then there is a re-transformation into spores (resporulation). Such cycles are repeated several times. Ultimately, the spores contained in the feces end up in the external environment. Similarly, after oral administration of vegetative cells, their sporulation in the gastrointestinal tract is observed. The cycles of germination and respiration are repeated several times before excretion from the host body. That is, no matter how probiotic drugs or feed additives based on B. subtilis are taken – in the form of spores or vegetative cells - both forms of the bacterium will be present in the recipient's body and the observed effects and therapeutic effect will be similar [9, 11].

A number of authors have found that 4-5% of its genome of the bacterium B. subtilis encodes the synthesis of various antimicrobial substances. According to the published reviews, by 2005 about 24 such substances were isolated from different strains of B.subtilis, by 2010 – 66, and the list continues to increase constantly. Most antimicrobial substances are represented by ribosomally and non-ribosomally synthesized peptides. Non-peptide substances, such as polyketides, aminosaccharides and phospholipids, are found in smaller quantities. The activity of many is directed against gram-positive and gram-negative bacteria, viruses and fungi, that is, almost all pathogens that can cause intestinal infections are covered [14, 15].

The introduction of probiotics into the diet contributes to the protection of the gastrointestinal tract from pathogens, the restoration of symbiotic microflora after therapy with chemotherapeutic agents of various directions of action, strengthening the processes of digestion of food. They, actively fighting with pathological microflora, contribute to the development of normoflora. These are colibacterin, acidophilic milk «Narine», lactobacterin, bifidumbacterin, bificol, acylact, etc. One of the most important functions of

probiotics is the production of a complex of biologically active substances capable of neutralizing bacterial toxins, dangerous metabolites [16-18].

The aim of our research was to study the effect of probiotics Zoonorm and Subalin on the immunophysiological system, features of microbiocenosis and the level of contamination of carp.

2 Materials and methods of research

Zoonorm and Subalin preparations were tested in the ponds of the experimental breeding farm «Yakot» of All-Russian Scientific Research Institute of Fisheries and Oceanography (VNIRO). The experiment included clinically healthy carp: two-year-olds with an average weight of 161 g and three-year-olds with an average weight of 345 g.

The Zoonorm preparation consists of a lyophilized microbial mass of living bacteria of the *Bifidobacterium bifidum strain № 1*, sorbed on particles of crushed activated carbon and lactose filler. One dose of the drug contains 10 million colony-forming units of bifidobacteria ($1x10^7$ CFU). The drug Subalin contains *Bacillus subtilis* and filler as a base. One dose of the drug contains $1x10^9$ CFU.

In the spring, five ponds were stocked according to the standards for 1 fish farming zone (2.5 thousand fish/ha). From the same group, 10 fish specimens were randomly selected, from which blood was taken for immunological studies and parenchymal organs were seeded on erythritagar and Endo nutrient medium to determine the microbiocenosis and the level of contamination of fish. To feed the fish, full-fledged feeds for two-year-olds and three-year-olds of carp were used. 4 experimental groups and one control group were formed: 1 experimental group (O_1) – used feed with minimal addition of the drug Zoonorm throughout the growing season; 2 experimental group (O_2) – used feed with the addition of the drug Zoonorm courses; 3 experimental group (O_3) – used feed with minimal addition of the drug Subalin throughout the growing season; 4 experimental group (O_4) – used feed with the addition of the drug Subalin courses; control group (C) – drugs were not used (table 1). Feeding with probiotic preparations was carried out from June to August.

Table 1. Calculation of the amount of compound feed and probiotics

№ pond	Group	Introduction of the drug	Number of fish	Months								
				June			Jule			August		
				1*	2	3	1	2	3	1	2	3
1	Control	-	275	3.6	4.6	5.7	6.8	7.8	11.2	12.0	12.0	12.4
2	Zoonorm, doses	Daily	175	1.8/ 2.5	2.5/ 2.5	3.2/ 2.5	3.9/ 2.5	4.6/ 2.5	7.7/ 2.5	8.3/ 2.5	8.6/ 2.5	8.8/ 2.5
3	Zoonorm, doses	Courses	200	2.3/ 20	3.1/ 20	3.9	4.7/ 25	5.5/ 25	8.6	9.2/ 30	9.6/ 30	9.4
4	Subalin, doses	Daily	200	2.5/ 0.8	3.2/ 1.1	4.1/ 1.3	4.9/ 1.6	5.6/ 1.8	8.7/ 2.9	9.3/ 3.1	9.6/ 3.2	9.4/ 3.1
5	Subalin, doses	Courses	190	2.5/ 3.2	3.2/ 4.1	4.0	4.7/ 6.1	5.4/ 6.9	8.5	9.0/ 11.5	9.3/ 11.9	9.4

Note: 1* – ten-day feeding courses; numerator – amount of feed per day (kg); denominator – number of doses per day.

Monitoring of the epizootic condition of ponds was carried out every 10 days with control catches. To study the immunophysiological parameters and pathoanatomic picture of the carp of the control and experimental groups, 10 fish were randomly selected.

To assess the effect of probiotics on the immunophysiological system of fish, we studied the bactericidal activity of blood serum, the titer of agglutinating antibodies, the level of contamination of parenchymal organs of fish, the survival rate of *Bacillus subtilis* and *Bifidobacterium bifidum* in the intestines of fish.

To conduct these studies, a blood sample was taken from the caudal artery with a sterile syringe from carp from each examined group of fish in vivo. The blood was kept for 40 minutes in a thermostat at 37^0 C, then the formed clot was circled with the drawn end of a Pasteur pipette and the samples were placed in the refrigerator for 24 hours to seal the clot and accumulate serum. Further, for the purpose of additional purification, it was centrifuged for 15 minutes at 3000 rpm. The blood serum of the studied fish obtained in this way was used to stage a microagglutination reaction and determine its bactericidal activity. The methodology for assessing the bactericidal activity of blood serum is based on the ability of fish blood serum to inhibit the reproduction of microorganisms due to the humoral immune factors contained in it. To determine the bactericidal activity of the blood serum, 0.5 ml of the test serum was mixed with 9.5 ml of sterile meat-peptone agar, to which 0.2 ml of a one-billion suspension of the test microbe was added. As a control, in parallel with each series of the studied serums, the test microbe was seeded in 10 ml of broth that did not contain blood serum. After thorough mixing, half of the mixture was taken from each tube with a sterile pipette and its optical density was determined on a photocolorimeter with a microprocessor system. The tubes with the remaining material in them were incubated in a thermostat at 26^0C for 3 hours. After the expiration of the incubation period, the optical density was re-determined. The bactericidal activity of blood serum was calculated by the formula:

$$(1 - (ODo^* - ODo):(ODc^* - ODc)) \times 100\% \quad (1)$$

where ODo – optical density of the test serum before incubation;

ODc – optical density of the serum-free mixture (control) before incubation;

ОПо* – optical density of the test serum mixture after incubation;

ОПс* – optical density of the serum-free mixture (control) after completion of incubation.

The essence of the microagglutination method is the detection of antibodies contained in the blood serum, capable in vitro in the presence of electrolytes to enter into a specific reaction with antigens located on the surface of bacterial cells, leading to the bonding of the latter and the formation of a characteristic precipitate.

To set up the reaction, micropanels from 96 wells were used, in which a number of double dilutions of the tested serum with isotonic sodium chloride solution with a pH of 7.2 were prepared using automatic micropipettes.

The same amount of antigen was introduced into each well with dilution of serum, which is a suspension of formalinized cells in isotonic sodium chloride solution with a concentration of $1x10^9$ microbial bodies in ml. After that, the panels were kept at room temperature for 24 hours.

The number of antibodies specific to this antigen in the test serum was expressed in the form of their titer, which was taken into account by the last well of the serum dilution series, in which a specific loose precipitate in the form of an umbrella (complete agglutination) was observed.

The reaction of completed phagocytosis serves to determine the ability of phagocytes contained in the peripheral blood of carp to inactivate the cells of the microorganism in vitro. As a test microbe, the same strain of mobile aeromonads was used as when evaluating the bactericidal activity of blood serum.

In a test tube containing 0.4 ml of a sterile 2% sodium citrate solution, 0.2 ml of a sample of freshly frozen blood from the examined fish and 0.4 ml of a one-billion

suspension of a test microbe were added. In parallel, an equal amount of bacterial suspension was added to test tubes containing 0.4 ml of sodium citrate and 0.2 ml of isotonic sodium chloride solution. The mixtures were thoroughly mixed and incubated in a thermostat at 26^0C for two hours, after which each sample of the material was diluted with a sterile isotonic sodium chloride solution in a ratio of 1:2000000 seeded on an Endo nutrient medium and thoroughly rubbed with a glass spatula. The results were recorded after incubation at 37^0C for 24 hours. The number of colonies grown in crops of control mixtures demonstrates a zero level of phagocytosis and serves for a more accurate assessment of the initial number of viable bacteria in the prepared suspension of the test microbe.

The activity of phagocytosis (the digesting ability of phagocytes) was taken into account by the formula:

$$(1 - No : Nk) \times 100\% \quad (2)$$

where No – the number of colonies in the mixture containing the test blood sample,

Nk – arithmetic mean number of colonies in the control mixture seeding.

To determine the survival rate of *Bacillus subtilis*, scraping from the intestinal mucosa was placed in a test tube with isotonic sodium chloride solution and heated in a water bath for 20 minutes, after which seeding was done on cups with erythritagar with kanamycin. To determine the survival rate of *Bifidobacterium bifidum*, 1 ml of scrapings from the intestinal mucosa were introduced into test tubes with 9 ml of preheated bifidum medium, after which a number of tenfold dilution were made. Incubated at 37^0C for a day, after which the results were recorded. *Bacillus subtilis* colonies were counted on erythritagar, *Bifidobacterium bifidum* was determined by the presence of characteristic colonies in an anaerobic environment.

The reliability of the difference in average values was assessed using the Student's t-test at significance levels of 95% and 99%. When processing statistical materials, a Microsoft Excel computer program was used.

3 Result of research

In September, after the fishing of all ponds, the carp of the control and experimental groups were weighed (table 2).

Table 2. Weight indicators of fish of the control and experimental groups during autumn fishing

Group	Spring stocking of the pond, pieces.	M_1 of fish initial, g	m_2 of fish at the catch, g	Weight gain	
				g	%
C	275	168.7	671.4	502.7	298.0
O_2	175	152.6	789.7	621.2	407.1
O_3	200	149.5	671.5	522.0	349.2
O_4	200	161.0	708.4	547.4	340.0
O_5	190	170.0	791.8	621.8	365.8

It should be noted that the increase in the body weight of fish in the experimental groups lies within the limits of statistical reliability relative to the control group.

The second survey of the epizootic situation in the experimental breeding farm «Yakot» of the All-Russian Research Institute of Fisheries and Oceanography (VNIRO) was conducted in May. For bacteriological research, water samples were taken from ponds, 10

fish that came out after wintering, and 10 fish from each group of the previous year's experience by random sampling. Blood samples were taken from fish for immunological studies and parenchymal organs to assess the level of bacterial contamination (tables 3 and 4).

Table 3. The level of contamination of parenchymal organs of fish

Group	Contamination, abs./%			
	Absent	Single	Moderate	Abundant
C	12/60.0	7/35.0	1/5.0	-
O_1	18/90.0	2/10.0	-	-
O_2	18/90.0	2/10.0	-	-
O_3	17/85.0	1/5.0	2/10.0	1/5.0
O_4	17/85.0	3/15.0	-	-

Table 4 shows that the immunophysiological status of the fish of the experimental groups after wintering remains quite high.

Table 4. Immunological indicators of fish

Group	Bactericidal activity of blood serum	Titer of agglutinating antibodies	
		to the antigen 77-18	to the antigen 360-2
C	74.6±18.1	1:8	1:4
O_1	99.6±0.0002	1:16	1:8
O_2	99.4±0.0004	1:16	1:16
O_3	85.1±2.0	1:8	1:16
O_4	99.4±0.0004	1:16	1:16

Water sampling from ponds in which fish of the experimental and control groups were grown was carried out from June to August. A total of 20 samples were taken. The level of bacterial contamination of water ranged from 840-13360 CFU/ml in June, 2020-74560 CFU/ml in July, from 1540 CFU/ml to abundant in August.

The etiological structure of aeromonads was very motley. *A.caviae, A.eucrenophila, A.hydrophila, A.sp.5, A.sp.9* were most often isolated, and bacteria of the *E. coli* group (coliform bacteria) were present in almost all crops.

The indicators of fish growth in the control and experimental groups for the entire study period are shown in table 5.

Table 5. Indicators of fish body weight gain

Group	Initial weight, g		Final weight, g		Weight gain, g		Weight gain, times	
	previous period	current period	previous period	current period	previous period	current period	previous period	current period
C	169	350	673	961	504	611	4.0	2.8
O_1	153	323	688	955	535	632	4.5	3.0
O_2	150	353	664	940	514	587	4.4	2.7
O_3	161	350	686	752	525	402	4.3	2.2
O_4	170	343	657	893	487	550	3.9	2.6

It should be noted that there was a high bacterial pressure in the ponds where fish were grown, which could not but affect the level of contamination and the rate of body weight gain of fish (table 6).

Table 6. The level of contamination of fish in the control and experimental groups

Group	Contamination, abs./%			
	Absent	Single	Moderate	Abundant
SPRING				
C	16/80.0	2/10.0	2/10.0	-
SUMMER				
C	18/90.0	2/10.0	-	-
O_1	19/95.0	1/5.0	-	-
O_2	20/100	-	-	-
O_3	18/90.0	-	1/5.0	1/5.0
O_4	17/85.0	3/15.0	-	-
AUTUMN				
C	16/80.0	4/20.0	-	-
O_1	19/95.0	1/5.0	-	-
O_2	18/90.0	-	1/5.0	1/5.0
O_3	20/100	-	-	-
O_4	18/90.0	2/10.0	-	-

The experimental group (O_3), who received Subalin with compound feed throughout the season, had a 100% survival rate of bacilli, which indicated good adaptability of the probiotic in the intestines of fish. Adaptability of the Zoonorm in both groups was noted in 100% of fish, but with the course application of the Zoonorm was sown in higher dilutions (table 7).

Table 7. Adaptability of bifidobacteria in the intestines of fish

Zoonorm, minimum		Zoonorm, courses	
Fish number	Result	Fish number	Result
1	$3x10^5$	1	$1x10^9$
2	$2x10^4$	2	$5x10^8$
3	$5x10^3$	3	$1x10^7$
4	$3x10^4$	4	$2x10^6$
5	$2x10^4$	5	$1x10^8$
6	$3x10^5$	6	$3x10^6$
7	$3x10^5$	7	$4x10^6$
8	$1x10^4$	8	$1x10^3$
9	$1x10^5$	9	$3x10^4$
10	$2x10^7$	10	$2x10^5$

When using feed with probiotics, specific and nonspecific resistance increased in fish. The protective effect of the drugs was assessed by pathoanatomic signs, the level of contamination of the internal organs of fish with bacterial flora, bactericidal activity of blood serum and titer of agglutinating antibodies (table 8).

Table 8. Immunological indicators of fish

Group	Bactericidal activity of blood serum	Titer of agglutinating antibodies	
		to the antigen 77-18	to the antigen 360-2
C	72.6±13.4	1:64	1:8
O_1	90.5±0.95	1:128	1:16
O_2	96.3±0.04	1:256	1:16
O_3	97.1±0.007	1:128	1:32
O_4	92.1±0.27	1:256	1:32

4 Conclusions

The conducted studies have shown the effectiveness of the use of probiotics Subalin and Zoonorm to strengthen the immunophysiological system of fish. Already after a week of feeding with compound feed with Subalin, the normalization of the condition of the fish was noted: the dryness of the skin disappeared, the body of the fish was evenly covered with shiny mucus, ulcers were scarred, parenchymal organs acquired a normal color and consistency, and the intestinal walls were elastic, not tearing. As a result of the use of Subalin in farms, its positive effect on the output of fish from wintering, growth rate, its immunophysiological and organoleptic indicators was noted. Bacterial contamination of internal organs was significantly reduced, despite the significant pressure of the aquatic microflora. The analysis of the actual material shows that the effect of the use of probiotics does not depend much on the specific characteristics of the microbiocenosis of pond water, and is primarily associated with the colonization activity of viable *Bacillus subtilis* spores. We have found that under the action of probiotics, the production of agglutinating antibodies increases, which is manifested by an immunostimulating effect. However, it was noted that a significant increase in bacterial pressure on fish affects immunological parameters, in particular, the titer of agglutinating antibodies, since a significant expenditure of energy resources of fish is spent on combating contaminants. Thus, the use of probiotic drugs should be combined with disinfection measures in ponds and the creation of optimal living conditions for fish. At the same time, timely fish-breeding and ichthyopathological control of fish-breeding and clinical indicators will allow assessing the quality of the fish grown and identify negative factors affecting it.

Thus, the use of probiotic agents, which are an alternative to antibiotics, allows ensuring environmental safety and leveling the processes of antibiotic resistance drift of bacterial pathogens in aquaculture.

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