Probiotics as means of discus fish (*Symphysodon haraldi*) disease prevention in aquaculture

Sanaya Olga Vladimirovna

Department Aquaculture and Bee-Keeping, Institute of Zootechnics and Biology, Russian State Agrarian University - Moscow Timiryazev Agricultural Academy, Moscow, Russia. Corresponding author: O. V. Sanaya, discus2020@gmail.com

Abstract. This paper presents the study results of the effect of Subtilis-C probiotic containing *Bacillus subtilis* and *Bacillus licheniformis* on the organism of discus fish (*Symphysodon haraldi*) which are quite popular in aquarium husbandry. It was found that feeding the Subtilis-C probiotic at the dose of 1 g/kg food causes an increase in erythropoiesis and cellular immunity response of discus fish. The percentage of erythroblasts in the total number of erythroid cells increases. The proportion of monocytes in the leukogram increases and basophils appear. The content of lysosomal cationic protein in neutrophils increases. Probiotic reduces alanine aminotransferase (ALT) activity to optimal values, increases protein and balances carbohydrate metabolism.
Key Words: hematological and biochemical parameters, lysosomal cationic test, probiotics.

Introduction. In today's world, more and more people are getting involved in ornamental fish culture. It is a branch of aquaculture which serves for aesthetic and scientific purposes. Ornamental aquaculture subjects are used as biomodels (Wen et al 2018; Pronina et al 2019).

Discus (*Symphysodon haraldi* Schultz 1960) belongs to the Cichlidae family and is one of the most sought-after species in the ornamental fish trade (Mesquita et al 2008; Mattos et al 2016).

Most relevant species peculiarities that are important to consider in their breeding, are the following: pair formation and biparental care, rearing of fry in soft water, which is required for larval rearing (Mattos et al 2016, Satoh 2018).

They exhibit complex, evolutionarily evolved behavior in pair formation and rearing offspring (Buckley et al 2010). Discus fish have been characterized by partial spawning as a strategy that increases reproductive success, biparental care of offspring, and feeding of fry with epidermal secretion (Önal et al 2009; Rossoni et al 2010).

The epidermal secretion of the parents of *Symphysodon* spp. fish was found to contain several amino acids. This proves its participation in offspring feeding by analogy with mammalian milk (Chong et al 2006). The peak of protein content of mucus is observed when the offspring enter the free-floating stage. Epidermal secretion includes cortisol, immunoglobulin M (IgM), sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) (Buckley 2012).

Under artificial conditions, fish are exposed to stress factors associated with their artificial breeding that differ significantly from natural breeding. These factors include handling manipulation, increased planting densities, increased reproductive load, and changes in temperature regime. Low temperature has been shown to result in increased formation of reactive oxygen species in the body, which may be related to antioxidant defense (Wen et al 2018). A positive correlation was found between planting density and blood glucose levels in tilapia (Hastuti & Subandiyono 2020).

Long-term artificial breeding increases the risk of inbreeding, which weakens the natural resistance of the organism. Antibiotics are used to fight bacterial infection, which negatively affects the immunity of the fish. Therefore, an important issue is to improve the immunity of farmed fish. One of the options to enhance immune resistance is the use of probiotics as a feed additive.

In a laboratory experiment, it was shown that the increase in dry weight of *Poecilia* spp. receiving probiotic Subtilis-C, was 37.6% compared to the control and 33.4% for Sumatran barbs (*Barbus tetrazona*). In pond conditions, the use of the probiotic preparation together with 0.1% of the carp (*Cyprinus carpio*) feed allowed to increase the fish weight gain by 36.2% (Golovko et al 2009).

Studies of Subtilis-C probiotic application at early stages of fish development have shown that treatment of eggs, embryos and larvae with probiotic increases survival rate and decreases natural mortality of fish at larval stage of development, stimulates fish viability at early stages of ontogenesis and increases natural immunity (Burlachenko & Malik 2007; Rudenko et al 2009; Vlasov et al 2012).

It was found that the *Bacillus subtilis* probiotic determines protein uptake and more intensive use of nitrogen metabolites by gut microflora for the synthesis of their own biomass. This increases the average daily weight gain of carp (*Cyprinus carpio*) and sterlet (*Acipenser ruthenus*) by 25 and 35% (Zuenko et al 2017).

Good results were obtained when using for Nile tilapia (*Oreochromis niloticus*), white weakfish (*Atractoscion nobilis*) and for the whiteleg shrimp (*Litopenaeus vannamei*), with the live microbial supplement *Bacillus subtilis*, which positively modifies the microbial community and its immune system, (Olmos & Paniagua-Michel 2014).

In view of the above, the purpose of this study is learning the effect of the Subtilis-C probiotic on the physiological and immunological status of discus.

Material and Method. The objects of the study were sexually mature discus fish (*Symphysodon haraldi*) raised in a Moscow aquarium complex. The age of the studied fish ranged from 10 to 12 months. Discus fishes were kept in 400-liter tanks with constant environmental conditions (temperature 30±1^{o}C, pH 8.5, photoperiod of 12 hours of light and darkness, water hardness factor of 25°dH).

Discus fish are kept in schools, without placing other animals, without plants with a diet based on food of animal origin: in the morning they have been fed with minced meat (beef heart, salmon, spinach) at the rate of 1% of the fish weight and in the evening the feeding consisted of chironomid (larvae of the family *Chironomidae*).

The fish were divided into two groups according to the principle of analogues: intact control group and experimental group, with 12 specimens in each group. The experimental fish were administered Subtilis-C feed additive in the amount of 1 g powder per 1 kg of minced meat. Subtilis-C probiotic contains *Bacillus subtilis* and *Bacillus licheniformis.*

Blood sampling was performed intravital from the caudal vein observing aseptic rules. Blood sampling in fish is a difficult task. In discus fish, it is even more difficult given the strongly flattened shape and small size (Figure 1).

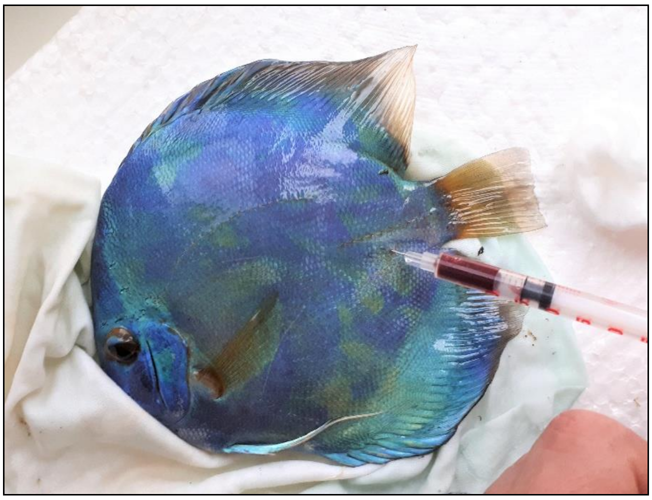

Figure 1. Discus fish blood sampling (original image).

Blood smears were done immediately after blood sampling, two for each fish, one for the leukogram and one for the cytochemical reaction of cationic protein determination.

Physiological and immunological evaluation of fish was carried out according to hematological and cytochemical indices.

Hematological parameters are labile and subject to changes, including seasonal changes. It was noted that the highest value of thrombocytes and total leukocyte count in tilapia blood is registered in autumn at the expense of monocytes, while in spring and summer there is a decrease in total leukocyte count (Jerônimo et al 2011). A direct correlation between the *Fusarium moniliforme* fungal toxins coming with food and the total number of leukocytes was shown (Grant 2015).

The ratio of immunocompetent cells: white blood cell (WBC) differential test reflects the physiological state and immune status of the fish.

Leukocyte formula was determined by differential counting in Pappenheim stained peripheral blood smears. The level of hematopoiesis in fish was determined by the proportion of immature forms of erythrocytes and leukocytes.

An important marker of immune response is the cytochemical index of the cellular immunity factor. Non-enzymatic cationic proteins of lysosomes (defensins) have a direct killer effect on pathogens (Pronina 2017; Chowdhury 2020).

The phagocytic activity of fish neutrophils was evaluated using the lysosomal-cationic test adapted for hydrobionts by Pronina (2014) using the cytochemical method with bromophenol blue. The content of non-enzymatic cationic protein in the lysosomes of peripheral blood neutrophils was determined. The studied cells were divided into 4 groups according to the degree of phagocytic activity, as follows: zero-degree, no cationic protein granules; first-degree, single granules; second-degree, the granules occupy about 1/3 of the cytoplasm; third-degree, granules occupy 1/2 of the cytoplasm and more.

The mean cytochemical coefficient (MCC) was calculated according to the formula:

$$MCC = \frac{0 \times H_0 + 1 \times H_1 + 2 \times H_2 + 3 \times H_3}{100}$$

where H0, H1, H2, H3 are the number of neutrophils with activity of 0, 1, 2, and 3 points, respectively H0 + H1 + H2 + H3 = 100.

The analysis of biochemical blood parameters is the most valuable modern research method because their physiological values have been shown to be species-specific. Factors such as: age, sex, environmental conditions, and diet can significantly affect the biochemical and hematological blood parameters of fish (Patriche et al 2011).

To obtain serum, fish blood was drawn with a syringe into a dry tube. The tube with blood was left in a rack for 1 hour at room temperature. After blood clot formation, the serum was carefully separated from the formed clot using a Pasteur pipette and placed in the refrigerator at +3^{o}C for 3-5 hours to complete serum formation. The serum was then sucked off with a fine needle syringe or pipette and transferred to an Eppendorf tube. Serum for biochemical analysis was frozen at -24^{o}C and transported to the laboratory frozen in thermocontainers.

Biochemical analysis of blood serum was performed on the ChemWell Awareness Technology instrument, using VITAL reagents.

Mathematical processing of digital materials was performed by Student's method of variation statistics using MS Excel software.

Results and Discussion. Discus fish of the control and experimental groups had no significant differences in body weight. Obviously, *Bacillus subtilis* has no effect on fish growth (Table 1).

Table 1

Size and weight indicators, hematological and cytochemical index of discus fish in experiment

Index	*Control*	*Experiment*
	Size and weight	
Body mass (g)	73.9±4.9	85.7±5.2
Body length TL (cm)	10.7±0.2	11.3±0.2
	Erythropoiesis (%)	
Hemocytoblast, Erythroblast	1.0±0.1	2.0±0.2*
Normoblast	4.0±0.4	5.7±1.0
Basophilic erythrocytes	12.0±2.0	6.1±0.3*
Normocyte	83.2±1.9	86.2±2.2
	Leukocyte formula (%)	
Myeloblasts	1.0±0.1	1.0±0.1
Promyelocytes	1.2±0.3	1.2±0.1
Myelocytes	1.3±0.3	1.0±0.1
Metamyelocytes	2.0±0.4	2.0±0.1
Banded neutrophils	1.5±0.5	1.8±0.1
Segmented neutrophils	1.4±0.3	1.8±0.1
Eosinophils	1.0±0.1	1.0±0.1
Basophils	-	1.0±0.1*
Monocytes	2.2±0.2	3.8±0.3*
Lymphocytes	88.4±1.4	86.4±1.4
	Lysosomal cation test	
MCC, units	1,20±0,05	1,31±0,01*

Note: hereinafter* - the differences are significant ($p<0.05$).

Probiotic activated erythropoiesis: in discus fish of the experimental group the content of blast forms of erythrocytes was almost two times higher than in the control. The proportion of more mature basophilic erythrocytes in the total number of erythroid cells respectively decreased.

The appearance of basophils in the leukogram of discus fish of the experimental group confirms the strengthening of nonspecific cellular defense.

A significant increase in the proportion of monocytes in the leukogram of fish treated with probiotic can be explained by the activation of phagocytic activity of macrophages.

The results of the lysosomal cationic test showed that feeding probiotic increased the content of cytotoxic cationic protein in neutrophils lysosomes, indicating an increase in cellular immunity.

According to several biochemical parameters, the discus treated with probiotic showed significant differences from the control (Table 2).

After 2 months of experiment, ALT activity decreased almost twofold. The increase in the level of the enzyme is observed in the destruction of cardiac muscle and liver cells, probably the probiotic increased cellular resistance of myocytes and hepatocytes of discus fish (Shen et al 2015; Zoppini et al 2016). Significant increase in AST activity has been shown. According to our observations, immunostealthing fish have rather high level of AST because AST takes part in amino acid transamination, so we can assume that the increased activity of the enzyme relates to protein metabolism intensification.

Blood glucose level in discus fish of the experimental group was significantly lower than in the control group as an indicator of balanced carbohydrate metabolism.

The lactate level in fish at the end of the experiment decreased, which indicates the normalization of glucose metabolism (glycolysis), since the increase of this index indicates metabolic acidosis and distortion of acid-base balance.

Table 2

Biochemical parameters of discus fish

Index	*Control (n=12)*	*Experiment (n=12)*
Alanine Aminotransferase (ALT), units L^{-1}	63.7±16.4	37.5±7.3*
Aspartate Aminotransferase (AST), units L^{-1}	60.3±13.8	153.9±32.3*
Glucose, mmol L^{-1}	4.6±0.4	3.4±0.2*
Creatine kinase (CK), units L^{-1}	1246±701	2065±614*
Lactate, mg d L^{-1}	16.6±1.2	7.9±1.0*
Alkaline phosphatase (ALP), units L^{-1}	153.5±37.6	103±41
Urea, mg d L^{-1}	9.4±5.3	13.0±2.8
Uric acid, mg d L^{-1}	5.6±1.1	2.9±0.3*
Total protein, g L^{-1}	60.8±12.6	52.2±3.2
Albumin, g L^{-1}	21.6±5.1	21.8±0.8
Triglycerides, g L^{-1}	502.8±28.1	472±7
Cholesterol, mg d L^{-1}	439.2±121.5	457±44

Note: hereinafter* - the differences are significant ($p<0.05$).

A significant decrease in uric acid content in the blood of the fish treated with probiotic at the end of the experiment is associated with the good functioning of their excretory system, eliminating this product of purine metabolism.

Conclusions. The Subtilis-C immunomodulator probiotic activates erythropoiesis of discus fish and increases immune protection: the share of macrophages (monocytes) in the leucocytic formula increases, basophils appear and the content of cytotoxic non-enzymatic cationic protein in neutrophil lysosomes of blood increases. Biochemical blood tests showed that the probiotic enhances cellular resistance (ALT), protein metabolism and balances carbohydrate metabolism.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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Authors:
Sanaya Olga Vladimirovna, Department Aquaculture and Bee-Keeping, Institute of Zootechnics and Biology, Russian State Agrarian University - Moscow Timiryazev Agricultural Academy, Moscow, Russia, e-mail: discus2020@gmail.com

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