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THE EFFECT OF PROBIOTIC BETAPLUS®ULTRA ON HEMATOLOGICAL PROFILE AND IMMUNE RESPONSE OF YOUNG OF THE YEAR *ACIPENSER STELLATUS*

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Abstract

This experiment was carried out to evaluate the effects of the commercial probiotic Betaplus®Ultra on the haematological profile (red blood cell (RBC) counts, haemoglobin (Hb) concentration, haematocrit (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC), glucose and total proteins) and immune response of young of the year Acipenser stellatus. In this sense, a 93-day experiment was carried out with six experimental variants (five different probiotic concentrations: C_1 - 1-1.28×10^{13} CFU, C_2 - 2.56 ×10^{13} CFU, C_3 - 3.84×10^{13} CFU, C_4 - 5.12×10^{13} CFU, C_5 - 6.4×10^{13} CFU and C_0 - control group). All the trials were performed in duplicates. Based on our results, no significant differences ($p>0.05$) were observed in the values of RBC, PCV, MCV and MCHC, while significant differences ($p<0.05$) were registered in the case of Hb and MCH. Regarding the immune response, no significant differences were registered in the values of leukocytes, lymphocytes neutrophils, monocytes and eosinophils ($p>0.05$) and only, thrombocytes and basophiles were significantly influenced ($p<0.05$) by the probiotic concentration. The results suggest that the probiotic Betaplus®Ultra could be used effectively as a probiotic for the use in aquaculture.

Key words: *haematological parameters, immune response, probiotic, sturgeons.*

INTRODUCTION

Sturgeons are among fish with a very high economic value because of their caviar and meat. Among sturgeons, *Acipenser stellatus* is one of the three most important species for caviar (Frimodt, 1995).

Due to overfishing (Aghilinejhad et al., 2018), poaching (Bloesch et al., 2006), pollution or dam construction (Reinartz and Slavcheva, 2016), the sturgeon population experiencing a severe decline (Lenhardt et al., 2006; Bronzi and Rosenthal, 2014; EUMOFA, 2018).

Although considerable efforts have been made during the last years and several conservation measures have been taken, their status continuously decreased and nowadays almost all sturgeon species are listed as critically endangered (Qiwei, 2010).

In this context sturgeon aquaculture can meet the demand of the population for meat and caviar, thus reducing the pressure on natural sturgeon resources (Bronzi and Rosenthal, 2014; Vasilyeva et al., 2015).

To be profitable aquaculture of sturgeon's farms involves the growth of fish in high stocking densities (Rafatnezhad et al., 2008), which can lead to deteriorating of water quality, increase stress and raise susceptibility to a wide range of diseases (Ni et al., 2016; Long et al., 2019).

Therefore, over recent decades, this increase in productivity has been accompanied by the enlarged use of chemicals, especially antibiotics or other substances (Salah and Aqel, 2014). However, there is a real concern regarding the use of antibiotics in aquaculture, because can modify the intestinal microbiota and give rise to resistant bacteria, which could be harmful to aquatic organisms (Dawood et al., 2018), or can give antibiotic resistance at human and cause severe environmental problems (Mo et al., 2017). In Europe, the use of antibiotics is under strict control and regulatory measures, and only a few antibiotics are approved for use in aquaculture (European Council, 2001).

In this sense, probiotics can be an alternative to substitute antibiotics or other chemicals (Sayes et al., 2018). According to Gatesoupe, 1999, probiotics are defined as microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, to improve health. The use of probiotics in aquaculture proved to have several benefits, such as: improving growth performance by stimulating fish appetite (Lara-Flores and Olvera-Novoa, 2013; Opiyo et al., 2019), enhances the immune response and fish welfare (Nayak, 2010), reduce mortality, increased survival, improved resistance against disease (Safari et al., 2016; Hoseinifar et al., 2018), and improves the water quality by modifying microbial communities of water and sediments (Verschuere et al., 2000).

Bacillus genus is among the most frequently used probiotic microorganisms used in aquaculture (Nwanna, 2015). It is proved that *Bacillus subtilis* produce compounds with antimicrobial properties such as antifungal lipopeptides, being very effective to fungal pathogens and a wide variety of microorganisms (Korenblum et al., 2003; Ongena and Jacquea, 2007).

Previous studies were conducted to determine the effects of *Bacillus subtilis* and *Bacillus licheniformis* for several fish species (Azarin et al., 2015; Romanova et al., 2020) but, from our knowledge so far, there is no published information concerning the effects of these probiotics on *A. stellatus* welfare.

Therefore, this study was performed to investigate the effect of probiotic BetaPlus®Ultra on the haematological profile and immune response of stellate sturgeon.

MATERIALS AND METHODS

Experimental design. This study was conducted in a commercial sturgeon farm located in Horia, Tulcea County, Romania. The experimental design was randomized, composed by six treatments in two replications: one control group, feeding continuously only with basal diet (C_0), and five groups feeding with a basal diet supplemented with probiotic in different concentration: C_1 - 1.28 × 10^{13} CFU (colony-forming units), C_2 - 2.56 × 10^{13} CFU, C_3 - 3.84 × 10^{13} CFU, C_4 - 5.12 × 10^{13} CFU, C_5 - 6.4 × 10^{13} CFU. The probiotic BetaPlus® (BioChem Co., Germany) was in a powder form and contained a 1:1 ratio of *Bacillius subtilis* (DSM5750) spores, and *Baciulius licheniformis* (DSM 5749) spores.

Water quality parameters included: temperature 23.81 ± 2.43°C, pH 8.41 ± 0.15, dissolved oxygen 6.46 ± 0.32 mg L^{-1}, nitrite ($N\text{-}NO_2^-$) 0.03 ± 0.01 mg L^{-1}, nitrate ($N\text{-}NO_3^-$), and ammonium ($N\text{-}NH_4^+$) 0.22 ± 0.16 mg L^{-1} were in the optimal range for sturgeon’s growth, Mims et al., 2002.

Fish. The experiment started with 6000 fish of 35-day post hatched larvae (500 fish per each tank) with a mean individual weight (± SD) of 0.35 ± 0.02 g and a total length between 3-5 cm. The experimental conditions and the growing system were presented in our previous paper (Stroe et al., 2019). The experiment lasted for 93 days and was performed in duplicate.

Collection of the blood samples. At the end of the trial, feeding was stopped for 24 hours and blood samples were collected from seven fish from each experimental variant, from the vein of the caudal region of anesthetized fish. Seven fish per tank (14 fish per treatment) were randomly sampled, anesthetized with 2-phenoxyethanol (8 mL 40 L^{-1} of water for 5 minutes) to reduce handling stress.

For the determination of red blood cells (RBC, 10^6 μl^{-1}), haematocrit (PCV, %), haemoglobin concentration (Hb, g dL^{-1}), blood samples were taken using heparinized syringes, while for the determination of blood glucose (GLU, g dL^{-1}) and proteins (TP, mg dL^{-1}) we used syringes without anticoagulant and allowed to clot for 4 h, then centrifuged in microtube centrifuge at 3,000×g for 5 min.

At the same time, blood smears were made, fixed with absolute Methanol, and allowed to air-dry. The fixed smears were then coloured using May-Grünwald and Giemsa method and examined microscopically using immersion ×100 objective. In total, we analyse 84 blood smears. All areas in each smear were scanned and the percentage of leukocyte cells (monocyte, lymphocyte, neutrophils, and eosinophils) was determined by counting a total of 200 leukocyte cells, using Zeizz Axio Imager microscope. The absolute number of circulating blood leukocytes and thrombocytes were determinate concerning 1000 erythrocytes and converted to unit blood volume.

Immediately after blood extraction, the red blood cells were counted using a haemocytometer (Improved Neubauer Weber Scientific Ltd.). The haematocrit percentage (PCV, %) was determined using a heparinized haematocrit capillary tube which was filled with blood (30 µl) and centrifuged for 5 minutes at 12000 rpm in a microhematocrit centrifuge. Haemoglobin (Hb, g dL^{-1}) concentration was determined using the cyanmethemoglobin method. For this purpose, 20 µL of whole blood was added to 5 ml of Drabkin's solution and then after an incubation of 30 minutes, the mixture was read at 540 nm. Lately, the haemoglobin (Hb), haematocrit (Ht), and red blood cell (RBC) values were used to calculate the following constants: the mean corpuscular volume (MCV, μm^3), mean corpuscular haemoglobin (MCH, pg), and the mean corpuscular hemoglobin concentration (MCHC, g dl^{-1}).

Statistical analysis. The results are presented as means ± SD, the difference between experimental variants was analysed by ANOVA and statistical assessment of the result was carried out using SSPS software 21 version. The Kolmogorov-Smirnov test was used to confirm the normality and homogeneity variances and if significant differences were found the Post-hoc comparisons were conducted with the help of Duncan's test. The differences were considered to be significant at $p<0.05$.

RESULTS AND DISCUSSIONS

Research presented in this paper is corroborated with those obtained for growth performance. From the analysis of growth performance, it was concluded that fish from the C_2 variant recorded the best growth performance (Stroe et al., 2019). In Table 1 are presented the results of the haematological and biochemical parameters of *A. stellatus* fed with different concentrations of probiotics. From the statistical analysis, significant differences (ANOVA, $p<0.05$) were recorded in the concentration of haemoglobin and MCH. The addition of probiotic BetaPlus®Ultra to the diet had no significant influence (ANOVA, $p>0.05$) on erythrocyte count, haematocrit, the mean corpuscular volume, mean corpuscular haemoglobin concentration, total proteins, and glucose.

The post hoc Duncan test showed that the haemoglobin concentration from the group C_1, C_4, and C_5 was significantly higher than those from the C_0, C_2, and C_3. The increase of Hb in fish blood may indicate the immunostimulant effects and antiinfection properties of this probiotic. Haemoglobin is an indicator for oxygen transportation capacity of fish and according to Talpur and Ikhwanuddin (2012) increase in haemoglobin concentration has an important role in improving the well-being of fish and enhancing the immunity and growth. Increasing haemaglobin after the administration of a mixture of probiotics which contains *Bacillus subtilis* in the diet of *Acipenser baerii* fingerlings was also reported by Sayed Hassani et al., 2020.

Regarding the MCH values, the post hoc analyses showed three different groups. The lowest MCH concentration was obtained in the C_3 and C_4 ($p>0.05$), followed by the variant C_2 and C_1 ($p>0.05$) and C_5, C_0 respectively ($p>0.05$).

The blood biochemical indicators did not show significant differences (ANOVA, $p>0.05$) between the experimental variants. Although the TP did not register significant differences, an increase of their values was observed in the C_2 variant, where also was obtained the best growth performance. Some authors suggested that TP decrease under long-term exposure to stressful conditions (Sala-Rabanal et al., 2003). However, all the haematological results obtained by us for *A. stellatus* are in line with those obtained by other authors (Table 2).

Microscopic examination of blood smears shows no morphologic changes among leukocytes. After microscopic examination of blood smears, it was observed that lymphocytes dominated in comparison with the other types of leukocytes, being present in a very large number (Figure 1).

Table 1. Haematological and biochemical parameters of *A. stellatus* fed different concentrations of probiotics

Experimental variants	RBC, (10^6 µL^{-1})	PCV (%)	Hb (g dL^{-1})	MCV (µm^3)	MCH (pg)	MCHC (g dL^{-1})	TP (g dL^{-1})	GLU (mg dL^{-1})
C_0	0.71±0.11[a]	23.43±2.76[a]	6.24±0.79[b]	333.37±54.14[a]	88.70±13.81[b]	26.7±2.01[a]	4.41±0.14[a]	58.11±6.13[a]
C_1	0.95±0.26	24.14±2.27	6.60±0.48	270.22±73.32	74.07±20.67	27.41±1.72	4.37±0.66	56.14±6.71
C_2	0.90±0.17	24.00±1.53	6.32±0.36	275.46±51.99	72.17±11.46	26.38±1.60	4.66±0.18	55.90±4.63
C_3	1.00±0.23	23.43±2.15	5.95±0.40	245.87±64.16	62.42±15.57	25.53±2.08	4.38±0.17	56.95±5.43
C_4	1.01±0.21	25.29±2.56	6.81±1.54	258.68±49.07	69.02±15.35	26.71±3.57	4.45±0.17	52.71±3.30
C_5	0.85±0.23	26.14±1.86	7.54±0.98	331.03±109.73	93.62±26.31	28.96±4.30	4.16±0.20	57.00±4.96
ANOVA	0.11	0.17	0.03	0.10	0.02	0.31	0.14	0.52

Note: Values are presented as mean±SD;

Table 2. Reference values of haematological and biochemical parameters of sturgeons

References	RBC, (10^6 µL^{-1})	PCV (%)	Hb (g dL^{-1})	MCV (µm^3)	MCH (pg)	MCHC (g dL^{-1})	TP (g dL^{-1})	GLU (mg dL^{-1})
Grecu et al., 2019	0.555÷1.23	14.46±3.69	5.37±0.69	176.21±40.03	-	-	-	-
Khara et al., 2013	1.36±0.86	6.55±0.46	26.6±0.86	517±11 (fl)	126±0.22	24.5±0.34		
Dorojan et al., 2015	-	-	-	-	-	-	1.96±0.25	44.00±2.00

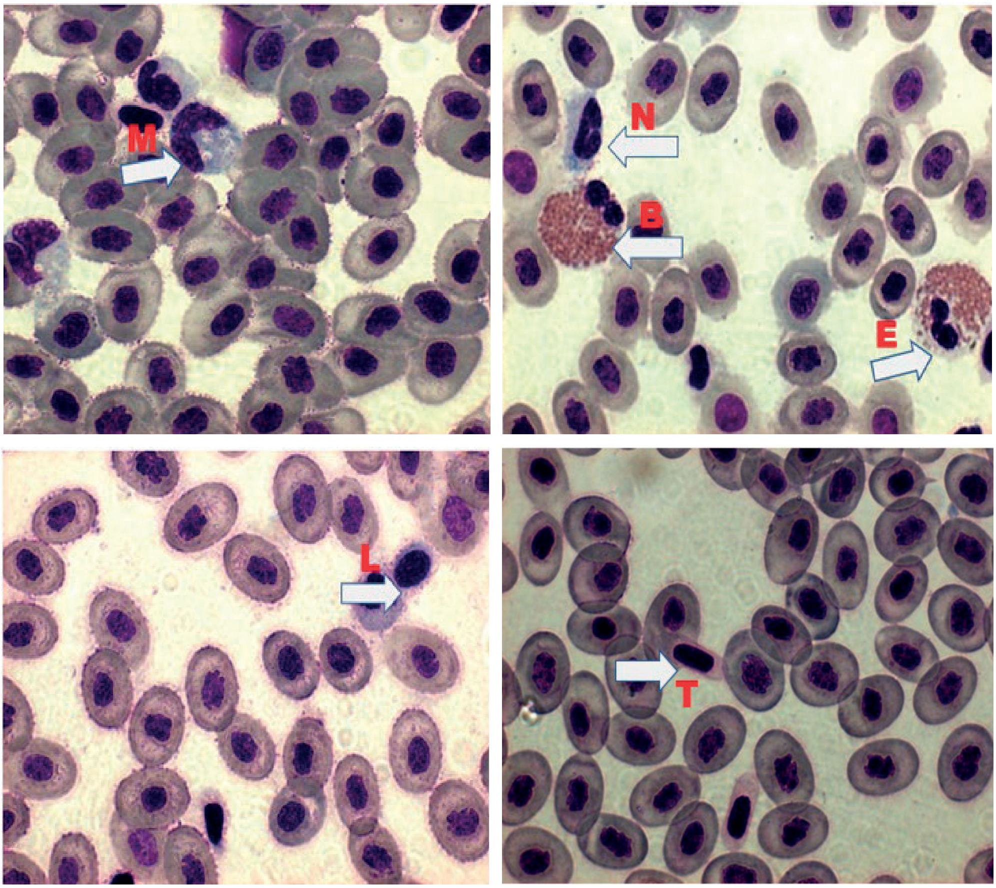

Figure 1. The microstructure of peripheral blood cells of *A. stellatus ×100*
(M - monocytes; N - neutrophils; B - basophils; E - eosinophils; L - lymphocytes; T - trombocytes)

Leucocytes formula of the stellate sturgeon reflects the prevalence of the percentage of lymphocytes reported on all leukocytes, followed by neutrophils, eosinophils and monocytes (Table 3). The statistical analysis ANOVA, revealed no significant differences (p>0.05) in the percent of lymphocytes, monocytes, neutrophiles and eosinophils, while

significant differences ($p<0.05$) were recorded in the percentage of basophils.
Regarding the absolute number of leukocytes (Table 4), no significant differences were registered in the values of leukocytes, lymphocytes neutrophils, monocytes, and eosinophils ($p>0.05$) and only, thrombocytes and basophiles were significantly influenced ($p<0.05$) by the probiotic concentration.

The innate cellular immune system is formed by a series of cells which are vital to the survival of the host (Nakandakare et al., 2013). Generally, fish thrombocytes are involved on the animal's defence mechanism, in phagocytosis, blood clotting, and other possible immunologic functions (Nagasawa et al., 2014; Bozzo et al., 2007).

Table 3. The relative number of leucocytes at the end of the experimental period

Relative number (%)	Experimental variants						
	C_0	C_1	C_2	C_3	C4	C_5	ANOVA
Lymphocytes	86.99±2.49	89.91±4.77	91.32±1.66	90.67±2.73	87.09±3.75	88.41±4.10	0.18
Monocytes	0.58±0.08	0.35±0.04	0.16±0.25	0.33±0.25	0.29±0.43	0.35±0.36	0.70
Neutrophiles	7.71±1.89	4.59±2.25	4.58±1.61	4.83±0.98	4.58±1.27	6.31±3.22	0.05
Eosinophils	4.47±1.8	3.71±1.97	3.47±1.15	3.75±2.32	3.47±3.19	4.59±1.88	0.45
Basophiles	0.25±0.27	1.54±0.95	0.48±0.41	0.42±0.25	0.48±0.26	0.63±0.46	0.001

Note: Values are presented as mean±SD;

Table 4. The absolute values of leucocytes at the end of the experimental period

Absolute number ($x10^3$ μL^{-1} blood)	Experimental variants						
	C_0	C_1	C_2	C_3	C_4	C_5	ANOVA
Leukocytes	38.17±7.59	58.04±20.90	59.03±16,46	47.51±19,52	47.20±11.92	52.96±28.31	0.43
Lymphocytes	33.23±6.81	51.47±15.53	53.93±15,11	46.82±25,10	40.73±9.13	47.19±25.86	0.42
Monocytes	0.21±0.23	0.29±0.54	0.11±0,19	0.15±0,15	0.15±0.25	0.18±0.27	0.25
Neutrophiles	2.96±1.03	2.87±2.51	2.71±1,39	2.24±0,90	3.28±1.28	2.50±1.13	0.92
Eosinophils	0.48±0.23	0.49±0.31	0.46±0.16	0.31±0,17	0.61±0.53	0.58±0.39	0.65
Basophiles	0.02±0.03	0.18±0.12	0.07±0.06	0.04±0,03	0.03±0.03	0.07±0.06	0.002
Thrombocytes	9.95±2.02	20.48±16.28	22.82±11.07	31.79±9.42	30.65±10.09	13.53±8.86	0.006

Note: Values are presented as mean±SD;

Increasing of fish thrombocytes in variants where probiotic was administrated can suggests an improvement of immune system of fish. Stimulation of the immune response of sturgeons through dietary supplements represents a great interest for aquaculture (Seyed et al., 2014). Improvement of fish immune response after dietary supplementation with *Bacillius subtilis* was also reported in the case of Persian sturgeon (*Acipenser persicus*) fingerlings (Darafsh et al., 2020). The authors reported a significant impact on the RBC count, PCV, and percentage of neutrophils. Also, Faeed et al. (2016) showed that out of all the blood parameters, only haematocrit and MCV are significantly increased when *Enterococcus faecium* is added to the diets of *Sander lucioperca* as a probiotic. According to Kumar et al. (2008) probiotics interact with the immune cells such as monocytes and neutrophils cells to enhance innate immune responses.

CONCLUSIONS

According to the results obtained from the hematological examination of *A. stelatus*, it can be concluded that the addition of feed with the commercial probiotic BetaPlus®Ultra could improve both the haematological and immunological responses. Obviously, these probiotics boost the immune system to defend the body against pathogenic organisms, but further researches must focus on the effect of this probiotic to digestive enzymes, and challenge test in order to validate the effectiveness of this probiotic on fish resistance to disease.

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