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Growth, physiological and immune responses of *Arapaima gigas* (Arapaimidae) to *Aeromonas hydrophila* challenge and handling stress following feeding with immunostimulant supplemented diets

Marcia Kelly Reis Dias[a], Eliane Tie Oba Yoshioka[b], Anselmo Fortunato Ruiz Rodriguez[c], Ricardo Amaral Ribeiro[c], Fernando Sérgio Escócio Drummond Viana Faria[c], Rodrigo Otávio Almeida Ozório[d,e], Marcos Tavares-Dias[a,b,*]

[a] Postgraduate Program in Tropical Biodiversity (PPGBio), Federal University of Amapá (UNIFAP), Macapá, AP, Brazil
[b] Embrapa Amapá, Macapá, AP, Brazil
[c] Postgraduate Program in Biotechnology and Biodiversity (Bionorte), Federal University of Acre (UFAC), AC, Brazil
[d] Interdisciplinary Center for Marine and Environmental Research (CIIMAR), Porto, Portugal
[e] Abel Salazar Institute of Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal

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ABSTRACT

The current study tested the efficacy of a dietary immunostimulant additive (Aquate Fish™®) on the growth performance, and on the physiological and immune responses of *Arapaima gigas*. Two trials were carried out: a feeding trial for 30 days with the experimental diets and a challenge trial for 7 days, in which fish were bacterial challenge (*Aeromonas hydrophila*) following by 60 s handling stress. During the feeding trial, fingerlings were fed diets supplemented with 0 (control), 6, 9 and 12 g Aquate Fish™®/kg diet. Dietary supplementation did not influence feed intake, feed conversion and condition factor, but increased the final biomass, number of erythrocytes, thrombocytes, leukocytes, lymphocytes, monocytes, hemoglobin, glucose, globulins and plasma triglycerides in fish fed at a concentration of 12 g/kg diet. After bacterial infection, mortality occurred only in fish fed control treatment, whereas respiratory burst of leukocytes, number of leukocytes and lymphocytes increased in fish that received 12 g of dietary supplementation. The results indicated that dietary supplementation with 12 g of Aquate Fish™® improved biomass and immunity performance of *A. gigas* fingerlings, without negatively affecting blood biochemical parameters.

1. Introduction

The Amazon region has an important contribution to the cultivation of native and non-native fish species. *Arapaima gigas* Schinz, 1822 (pirarucu), a piscivorous air-breather fish, is among the main native species cultivated due to its high valued zootechnical characteristics such as fast growth, tolerance to the high density of storage and rusticity to the handling, high meat yield, besides having an excellent acceptance in the national and international market [1–4]. However, its production is still limited by the low availability, high cost of fingerlings [4] and economic losses, due to the large mortalities in the initial phase of production. The handling of *A. gigas* during routine practices in aquaculture production can cause stress to the fingerlings, reducing the resistance to diseases outbreaks, leading to mass mortalities [1,3], caused mainly by aeromoniosis [1,5].

Similar to other industries, aquaculture constantly requires new techniques to increase production and productivity, while maintaining fish health [3,6,7]. However, with the development of global aquaculture, several problems have arisen, including the deterioration of water quality, parasitic and bacterial diseases [1,3,4,7]. Diets supplemented with immunostimulants based on *Saccharomyces cerevisiae*, which contain beta-glucan polysaccharide, mannanoligosaccharides and chitin have been recommended for improvement in fish production [7–10]. Diets supplemented with immunostimulants may improve fish performance by establishing the intestinal microbiota and better preparing fish immune system. As a consequence, fish are more resistant to stress, including parasitic and infectious diseases [6–10]. Aquate Fish™® is a commercial feed additive that may play a role in the fish defense systems. Pittman et al. [11] demonstrated that salmon fed diets supplemented with Aquate Fish™® showed a significant increase in mucous

* Corresponding author. Embrapa Amapá, Rodovia Juscelino Kubitschek, km 5, 2600, 68903-419, Macapá, AP, Brazil.
E-mail address: marcos.tavares@embrapa.br (M. Tavares-Dias).

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cell density and cell sizes when compared with fish fed non-supplemented diets, and such effects disappeared after discontinuation of the treatment. *Oreochromis niloticus* fed diets supplemented with Aquate Fish™® had lower ectoparasites levels, higher survival rate and improved in intestinal integrity [12,13]. Therefore, it is necessary to investigate the ideal concentrations of this commercial immunostimulant for the addition in the diet of this Amazonian fish, aiming the improvement of its immunity. This study investigated the effects of the Aquate Fish™®, a commercial immunostimulant, in the growth performance, physiology and immunity of *A. gigas*, evaluating the resistance against *Aeromonas hydrophila*.

2. Materials and methods

This study was carried out in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee on Ethics in the Use of Animals (CEUA/UFAC: 08/2014).

2.1. Experimental diets

Aquate Fish™® (Alltech, USA) is a feed additive containing *Saccharomyces cerevisiae* extracts, zinc proteinate, seaweed meal, selenium and ascorbic acid. The experimental diets were prepared using commercial feed for carnivorous fish with a minimum of 45% crude protein (Presence, USA) and supplemented with four concentrations (0, 6, 9 and 12 g Aquate Fish™®/kg diet) defined from the manufacturer's recommendations. The chemical composition of the diets was performed in triplicate, according to the guidelines of the Association of Analysts of Analytical Chemistry [14] (Table 1).

2.2. Fish and experimental design

Arapaima gigas (12.0 ± 8.0 cm) fingerlings were purchased from a commercial fish farm in Rio Branco (AC, Brazil) and transported to the Aquaculture and Fisheries Laboratory of Embrapa Amapá (AP, Brazil). During the acclimation period, fish were kept in 1000 L tanks with aeration and continuous flow of water and fed four times a day *ad libitum* with commercial feed for carnivores containing 55% crude protein (Presence®, São Paulo, Brazil).

During the feeding trial, 96 fingerlings (59.3 ± 21.3 g and 20.7 ± 2.1 cm) were distributed into 12 tanks of 100 L (n = 3 tanks/treatment; 8 fish/tank) and fed the experimental diets four times a day (8 a.m., 11 a.m., 2 p.m. and 5 p.m.). Fish were fed at 6% of biomass for 30 days. Water renewal and aeration within each tank were kept continuously, and dissolved oxygen (6.6 ± 0.3 mg L^{-1}), temperature (29.3 ± 0.1 °C) and pH (5.3 ± 0.3) were daily measured using a multiparameter probe (Horiba U52 model, Kyoto, Japan).

2.3. Parameters of fish growth

For the evaluation of growth performance, the following parameters were used:

a) Initial biomass (kg/m^3) = initial average weight x total number of fish;
b) Final biomass (kg/m^3) = final mean weight x total number of fish;
c) Daily feed intake (%BW/day) = average feed intake (kg)/average biomass gain (kg/m^3);
d) Daily feed consumption = feed quantity (g)/days;
e) Daily weight gain = Weight gain (g)/time (days);
f) Weight gain = (final weight - initial weight);
g) Specific growth rate (SGR) = (ln final weight - ln initial weight) x 100/(days);
h) Food efficiency (FE) = 100 × [weight gain (g)/amount of ingested feed (g)];
i) Relative condition factor, according to method recommended by Le Cren [15];
j) Hepatosomatic index (HSI, %): liver weight (g)/body weight (g) x 100;
k) Viscerosomatic index (VSI, %): viscera weight (g)/body weight (g) x 100.

2.4. Blood parameters and respiratory activity of leukocytes

After the feeding trial, blood from four fish from each replicate tank (8 per treatment) were sampled by puncture of the caudal vessel in syringes containing sodium heparin (5000 UI mL^{-1}). Blood was divided into two aliquots. An aliquot of blood was used to determine the hematocrit by the microhematocrit method, total erythrocytes count in the Neubauer chamber, and the hemoglobin concentration by the cyanometahemoglobin method. With these data, the hematometric indexes of Wintrobe were calculated: mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). Blood extensions were made and panchromically stained with a combination of May Grünwald-Giemsa-Wright, for differential leukocyte count in up to 200 cells of interest, at each extension. These blood extensions were also used to determine the number of leukocytes and total thrombocytes [16]. The nomenclature of leukocyte populations followed the recommendations of Tavares-Dias et al. [17]. The respiratory burst activity was determined according to method of Biller-Takahashi et al. [18]. Briefly, 100 µL of heparinized blood was added to 100 µL of 0.2% nitroblue tetrazolium solution (Sigma, St. Louis, MO, USA) and the final solution was homogenized and incubated for 30 min at 25 °C. After incubation and a second homogenization, 50 µL from the solution were added to 1 ml of *N*, *N*-dimethyl formamide (Sigma, St. Louis, MO, USA). This solution was then homogenized and centrifuged at 3000 g during 5 min. The optical density of supernatant was determined on spectrophotometer (Biospectro SP-220, Curitiba, Brazil) at 540 nm.

The second aliquot of blood was centrifuged at 75G for 5 min (Mod.

Table 1
Basal composition of commercial ration and proximate composition of the experimental diets.

Parameters	Manufacturer's data	Concentration of diets			
		0 g/kg	6 g/kg	9 g/kg	12 g/kg
Crude protein (%)	45	48.4 ± 0.6[a]	47.4 ± 0.1[a]	47.6 ± 0.6[a]	47.7 ± 0.8[a]
Dry matter (%)	87	93.5 ± 0.07[a]	91.6 ± 0.5[b]	91.2 ± 0.09[b]	91.0 ± 0.1[b]
Ethereal extract (%)	9	2.8 ± 0.5[a]	5.6 ± 1.7[b]	4.3 ± 0.05[b]	4.1 ± 0.1[b]
Ashes (%)	16	10.6 ± 0.1[a]	10.7 ± 0.2[a]	10.6 ± 0.04[a]	10.5 ± 0.1[a]
Phosphorous (%)	1	1.3 ± 0.1[a]	1.2 ± 0.04[a]	1.5 ± 0.3[a]	1.6 ± 0.07[a]
Calcium (%)	2–3	0.2 ± 0.5[a]	0.2 ± 0.02[a]	0.2 ± 0.02[a]	0.1 ± 0[a]
Vitamin C (mg/kg)	1500	–	–	–	–
Vitamin E (mg/kg)	400	–	–	–	–

Mean values ± standard deviation. Values followed by different letters, on the same line, indicate difference between treatments by the Tukey test ($p < 0.05$).

5424, Hamburg, Germany) to obtain plasma and determine the concentration of glucose, total protein, albumin, total cholesterol and triglycerides using commercial reagent kits (Biotécnica, MG, Brazil), with absorbance readings performed in a spectrophotometer (Biospectro SP-220, Curitiba, Brazil). Globulins content was determined subtracting albumin from total protein levels.

2.5. Challenge with inoculation of Aeromonas hydrophila and stress

After 30 days of feeding trial, other four fish from each replicate (8 fish/treatment) were challenged with *Aeromonas hydrophilla* (ATCC:7966). The fish were inoculated intraperitoneally with 1.8×10^8 UFC mL^{-1}, the lethal concentration (LC_{50-96h}) for *A. gigas* [1]. Mortality and clinical signs of bacteriosis were observed for 7 days, after which the surviving fish were subjected to handling stress. The fish were captured individually and subjected to stress by allowing them to struggle out of the water for 60 s [19,20]. After 6 h of handling stress and challenge with *A. hydrophilla*, blood was sampled from each fish as previously mentioned and divided into two aliquots for determination of hematocrit, hemoglobin concentration, number of total erythrocytes, thrombocytes, leukocytes and leukocyte respiratory activity, levels of glucose, total protein, albumin, cholesterol, triglycerides and globulins, as described in item 2.4.

2.6. Statistical analyses

All data were evaluated on the assumptions of normality and homoscedasticity using Shapiro-Wilk and Bartlett, respectively. Data that showed a normal distribution were analyzed using variance analysis (ANOVA - One Way) followed by the Tukey test, to compare the means. Data that not showed a normal distribution were analyzed using Kruskal-Wallis test, and Dunn test were used to assess differences among medians [21].

3. Results

After the feeding trial, most of the growth performance parameters (body length, feed intake, feed conversion, daily weight gain, condition factor and VSI) did not vary with the dietary treatments ($p > 0.05$). There was, however, an increase in the final biomass in fish fed the diet with 12 g of the additive, and a decrease in daily weight gain, weight gain, SGR and HSI in the fish fed 6 g of the additive (Table 2).

Hematocrit, MCHC, respiratory burst, total protein, albumin and number of eosinophils did not vary with the dietary treatment, whereas the number of erythrocytes, leukocytes, lymphocytes, monocytes, thrombocytes, hemoglobin, glucose, plasma globulins and triglycerides were significantly increased ($p < 0.05$) with the addition of 12 g Aquate Fish™®/kg diet. Plasma cholesterol showed a significantly increase in fish fed 6 g and 12 g Aquate Fish™®/kg diet when compared to fish fed the control diet and 9 g Aquate Fish™®/kg diet. Conversely, MCV decreased ($p < 0.05$) in fish fed 12 g Aquate Fish™®/kg diet when compared with the fish fed 9 g Aquate Fish™/kg diet. The number of neutrophils decreased ($p < 0.05$) in fish fed 9 g Aquate Fish™®/kg diet when compared to the other treatments (Table 3).

After pathogen challenge and handling stress, detection of fish mortality occurred only in fish fed non-supplemented diets (control). Post infection values of Kn, VSI, HSI, glucose, albumin, hematocrit, MCHC, number of monocytes, neutrophils and eosinophils did not vary among the dietary treatments ($p > 0.05$). Nevetheless, protein and globulin levels were significantly higher and MCV was lower ($p < 0.05$) in fish fed with 6 g Aquate Fish™/kg diet than fish fed control diet. Plasma cholesterol was higher in fish supplemented with 6 g Aquate Fish™®/kg than in fish fed with 9 g and 12 g Aquate Fish™®/kg diet ($p < 0.05$). Plasma triglyceride levels were higher in fish fed 9 g Aquate Fish™®/kg diet than in fish fed the control diet. Respiratory burst increased ($p < 0.05$) in fish fed diet supplemented with 12 g Aquate Fish™® when compared with the other treatments, whereas the number of thrombocytes increased compared to control fish ($p < 0.05$). The number of total leukocytes and lymphocytes increased in fish fed with 6 g Aquate Fish™® ($p < 0.05$) when compared with fish fed control diet (Table 4).

4. Discussion

Currently, new feeding practices play an important role in global aquaculture. The addition of feed additives to improve dietary quality is currently a common practice by many feed manufacturers to achieve better fish performance [7]. In the current study, the dietary supplementation Aquate Fish™® for a duration of 30 days did not influence the survival, body length, feed intake, feed conversion, condition factor in *A. gigas*, fish, but increased biomass in fish supplemented with 12 g of this immunostimulant. Some zootechnical results varied with the level of Aquate Fish™® supplementation. Some parameters showed improvement and other inhibition of performance with the dietary inclusion of Aquate Fish™®. Pádua et al. [13] demonstrated that *O. niloticus* fed diets supplemented with Aqute Fish™® had a higher survival rate, but occurred no effect on specific growth rate and weight gain. MycosorbA+®, a commercial immunostimulant containing mannanoligosaccharides derived from yeasts and algae did not have an effect on growth performance in *A. gigas* [2]. Conversely, *Epinephelus coioides* fed diet supplemented with *S. cerevisiae* showed improved weight gain and feed efficiency [22]. Supplementation with 0.1% of *S. cerevisiae* in the

Table 2
Growth performance parameters of *Arapaima gigas* fed the experimental diets for 30 days.

Parameters	Concentration of diets			
	0 g/kg (N = 24)	6 g/kg (N = 24)	9 g/kg (N = 24)	12 g/kg (N = 24)
Initial length (cm)	20.6 ± 1.8^a	20.6 ± 1.7^a	20.6 ± 1.6^a	21.1 ± 1.7^a
Final length (g)	33.2 ± 2.1^a	31.6 ± 1.7^b	31.6 ± 2.1^b	33.9 ± 2.0^{a,b}
Initial biomass (kg/m^3)	2.7 ± 5.7^a	2.6 ± 5.6^a	2.9 ± 6.0^a	2.9 ± 5.8^a
Final biomass (kg/m^3)	9.4 ± 8.1^a	7.7 ± 6.9^a	9.8 ± 8.2^a	10.6 ± 8.8^b
Daily weight gain (g)	5.5 ± 0.1^b	3.9 ± 0.8^a	4.6 ± 0.7^b	5.3 ± 0.4^b
Weight gain (g)	148.8 ± 59.3^a	116.7 ± 41.4^b	137.7 ± 45.6^a	160.0 ± 48.2^a
Daily feed intake (g)	38.3 ± 0.6^a	36.8 ± 0.5^a	34.9 ± 2.8^a	38.1 ± 0.5^a
AFC	1.1 ± 0.1^a	1.3 ± 0.3^a	1.1 ± 0.2^a	1.0 ± 0.06^a
SGR	4.2 ± 1.5^b	3.5 ± 0.9^b	4.0 ± 1.1^b	5.4 ± 0.2^a
Feed efficiency (%)	94.4 ± 10.7^a	88.8 ± 19.6^a	88.0 ± 14.1^a	98.2 ± 5.6^a
Condition factor	1.00 ± 0.02^a	1.00 ± 0.01^a	1.00 ± 0.02^a	1.00 ± 0.02^a
HSI (%)	2.0 ± 0.2^b	1.7 ± 0.4^a	2.2 ± 4.5^b	2.0 ± 0.2^b
VSI (%)	9.2 ± 1.3^a	9.1 ± 1.3^a	9.3 ± 0.3^a	8.8 ± 1.1^a

Mean values ± standard deviation. HSI: Hepatosomatic index, VSI: Viscerosomatic index, AFC: Apparent feed conversion, SGR: Specific growth rate. Values followed by different letters, on the same line, indicate difference between treatments by the Tukey test ($p < 0.05$).

Table 3
Plasma biochemical parameters and blood immune parameters of *Arapaima gigas*, after 30 days feeding the experimental diets.

Parameters	Concentration of diets			
	0 g/kg (N = 12)	6 g/kg (N = 12)	9 g/kg (N = 12)	12 g/kg (N = 12)
Glucose (mg dL^{-1})	24.2 ± 12.6^{b}	35.0 ± 11.2^{b}	20.0 ± 12.3^{b}	44.9 ± 14.8^{a}
Total protein (g dL^{-1})	3.0 ± 0.7^{a}	2.8 ± 0.3^{a}	3.0 ± 0.5^{a}	3.1 ± 0.7^{a}
Total cholesterol (mg dL^{-1})	92.2 ± 33.3^{a}	146.8 ± 47.1^{b}	113.1 ± 44.7^{a}	172.3 ± 30.2^{b}
Triglycerides (mg dL^{-1})	85.3 ± 28.6^{a}	92.1 ± 41.8^{a}	123.6 ± 45.0^{a}	177.4 ± 102.1^{b}
Albumin (g dL^{-1})	0.9 ± 0.6^{a}	1.0 ± 0.46^{a}	1.2 ± 0.7^{a}	0.6 ± 0.3^{a}
Globulin (g dL^{-1})	2.1 ± 0.6^{a,b}	1.8 ± 0.6^{a}	1.8 ± 0.6^{a}	2.5 ± 0.7^{b}
Hematocrit (%)	28.6 ± 2.2^{a}	28.9 ± 1.4^{a}	27.7 ± 1.9^{a}	29.4 ± 2.4^{a}
Hemoglobin (g dL^{-1})	10.2 ± 1.1^{a,b}	9.9 ± 0.9^{a,b}	9,2 ± 1,2^{a}	10,9 ± 1,1^{b}
Erythrocytes (x 10^{6} μL^{-1})	1.40 ± 0.40^{a,b}	1.30 ± 0.30^{a,b}	1.20 ± 0.40^{a}	1.70 ± 0,30^{b}
MCV (fL^{-1})	221.1 ± 60.2^{a,b}	225.8 ± 52.3^{a,b}	249.1 ± 61,7^{a}	179.3 ± 37.5^{b}
MCHC (g dL^{-1})	35.7 ± 4.5^{a}	34.2 ± 2.7^{a}	33.1 ± 2.7^{a}	37.1 ± 3.6^{a}
Respiratory burst	0.23 ± 0.06^{a}	0.22 ± 0.07^{a}	0.19 ± 0.03^{a}	0.24 ± 0.07^{a}
Thrombocytes (x 10^{3} μL^{-1})	19.2 ± 5.6^{a}	27.5 ± 6.7^{a}	24.4 ± 7.8^{a}	40.3 ± 9.6^{b}
Leukocytes (x 10^{3} μL^{-1})	152.4 ± 42.4^{a}	160.3 ± 36.0^{a}	145.3 ± 45.8^{a}	212.9 ± 41.8^{b}
Lymphocytes (x 10^{3} μL^{-1})	74.7 ± 24.7^{a}	78.2 ± 20.9^{a}	82.7 ± 29.1^{a}	122.3 ± 30.9^{b}
Monocytes (x 10^{3} μL^{-1})	4.5 ± 3.2^{a}	4.3 ± 3.2^{a}	4.9 ± 2.9^{a}	10.0 ± 4.8^{b}
Neutrophils (x 10^{3} μL^{-1})	70.9 ± 18.7^{b}	74.8 ± 16.6^{b}	55.3 ± 16.6^{a}	76.6 ± 15.5^{b}
Eosinophils (x 10^{3} μL^{-1})	2.3 ± 2.5^{a}	3.0 ± 1.7^{a}	2.4 ± 2.0^{a}	4.0 ± 4.1^{a}

Mean values ± standard deviation. MCV: Mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration. Values followed by different letters, on the same line, indicate difference between treatments by the Dunn test ($p < 0.05$).

diet of *O. niloticus* [23] and 2% in the diet of *Huso huso* [24], as well as 2% of β-glucan combined with mannanoligosaccharides in the diet of *Piaractus mesopotamicus* [9] improved fish growth performances. The previous studies indicated that *S. cerevisiae*-based products were efficient in promoting fish growth and potentially viable for aquaculture use from the production viewpoint.

Physiological indicators of health [6,16] and immune status [2,6,10] are often used to evaluate the efficacy of dietary supplementation of feed additives. In the current study, VSI and HSI of *A. gigas* fed with different Aquate Fish™® levels were not altered, although plasma triglycerides, cholesterol and glucose increased in fish fed 12 g Aquate Fish™®. These results indicated that there was no change in the use of lipid and glycogen reserves, since fish did not undergo any food deprivation during the test. In contrast, no alteration in plasma levels of cholesterol, triglycerides and glucose of *H. huso* supplemented with 1–2% of *S. cerevisiae* in the diet were reported [24]. In the current study, dietary supplementation of Aquate Fish™® showed mixed effects on plasma biochemical and blood immune parameters. On one hand had no effect on hematocrit, MCHC, MCV, respiratory burst of leukocytes, total protein, albumin and number of eosinophils. Similarly, *P. mesopotamicus* supplemented with a combination of 1–8% of β-glucan and mannanoligosaccharides for 30 days, did not change the number of erythrocytes, hematocrit, hemoglobin, MCHC, VCM, and total plasma protein levels [9]. In *H. huso* supplemented with 1–2% of *S. cerevisiae* in the diet, for 42 days, no changes in plasma levels of total proteins, glucose, erythrocytes, hematocrit, hemoglobin, MCHC, MCV and

Table 4
Mortality, body, blood and immune parameters of *Arapaima gigas* fed for 30 days with the experimental diets following a bacterial challenge and handling stress.

	Concentration of diets			
Parameters	0 g/kg (N = 11)	6 g/kg (N = 12)	9 g/kg (N = 12)	12 g/kg (N = 12)
Mortality (%)	8.3	0	0	0
Kn	1.00 ± 0.01^{a}	1.00 ± 0.01^{a}	1.00 ± 0.01^{a}	1.00 ± 0.01^{a}
HSI (%)	2.0 ± 0.4^{a}	1.7 ± 0.4^{a}	1.7 ± 0.5^{a}	1.5 ± 0.3^{a}
VSI (%)	9.5 ± 1.4^{a}	9.0 ± 1.2^{a}	9.1 ± 1.1^{a}	8.2 ± 1.0^{a}
Glucose (mg dL^{-1})	46.3 ± 27.8^{a}	44.3 ± 17.2^{a}	43.7 ± 7.1^{a}	64.9 ± 18.6^{a}
Total protein (g dL^{-1})	2.8 ± 0.2^{b}	3.6 ± 0.4^{a}	3.2 ± 0.3^{a,b}	3.0 ± 0.6^{b}
Total cholesterol (mg dL^{-1})	148.7 ± 27.8^{a,b}	193.1 ± 30.9^{a}	137.7 ± 64.2^{b}	98.0 ± 39.6^{b}
Triglycerides (mg dL^{-1})	36.4 ± 16.8^{a}	65.4 ± 42.3^{a,b}	86.4 ± 31.7^{b}	55.5 ± 21.4^{a,b}
Albumin (g dL^{-1})	1.1 ± 0.3^{a}	1.1 ± 0.4^{a}	1.1 ± 0.4^{a}	0.9 ± 0.4^{a}
Globulin (g dL^{-1})	1.7 ± 0.4^{b}	2.7 ± 0.5^{a}	2.1 ± 0.6^{a,b}	2.0 ± 0.6^{a,b}
Hematocrit (%)	28.6 ± 3.4^{a}	27.5 ± 2.8^{a}	28.7 ± 2.2^{a}	29.0 ± 2.0^{a}
Hemoglobin (g dL^{-1})	10.2 ± 1.4^{b}	11.0 ± 2.2^{a,b}	10.3 ± 2.8^{b}	13.2 ± 3.1^{a}
Erythrocytes (x 10^{6} μL^{-1})	2.20 ± 0.40^{b}	2.30 ± 0.01^{a}	2.60 ± 0.2^{a,b}	2.50 ± 0.4^{a,b}
MCV (fL^{-1})	134.7 ± 16.8^{b}	101.6 ± 28.5^{a}	113.2 ± 13.8^{a,b}	120.7 ± 25.3^{a,b}
MCHC (g dL^{-1})	35.8 ± 3.5^{a}	39.8 ± 6.1^{a}	35.9 ± 10.2^{a}	44.5 ± 11.2^{a}
Respiratory burst	0.16 ± 0.05^{b}	0.20 ± 0.04^{b}	0.18 ± 0.03^{b}	0.29 ± 0.09^{a}
Thrombocytes (x 10^{3} μL^{-1})	40.7 ± 9.4^{c}	55.1 ± 19.4^{a,c}	59.4 ± 10.8^{a,b}	73.2 ± 15.9^{b}
Leukocytes (x 10^{3} μL^{-1})	258.5 ± 44.9^{a}	355.0 ± 125.6^{b}	310.0 ± 26.8^{a,b}	329.7 ± 55.9^{a,b}
Lymphocytes (x 10^{3} μL^{-1})	167.2 ± 28.9^{a}	236.1 ± 83.9^{b}	202.4 ± 22.8^{a,b}	218.8 ± 54.4^{a,b}
Monocytes (x 10^{3} μL^{-1})	10.8 ± 6.4^{a}	13.5 ± 8.4^{a}	16.7 ± 20.1^{a}	18.0 ± 6.1^{a}
Neutrophils (x 10^{3} μL^{-1})	77.3 ± 19.7^{a}	99.6 ± 47.1^{a}	84.9 ± 20.1^{a}	88.1 ± 36.8^{a}
Eosinophils (x 10^{3} μL^{-1})	3.2 ± 2.1^{a}	5.8 ± 8.0^{a}	6.0 ± 4.1^{a}	4.8 ± 4.3^{a}

Mean expression values ± standard deviation. Mean values ± standard deviation. MCV: Mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration. Values followed by different letters, on the same line, indicate difference between treatments by the Dunn test ($p < 0.05$). HSI: Hepatosomatic index, VSI: Viscerosomatic index, Kn: Relative factor of condition.

number of leukocytes were reported [24].

On the other hand, Aquate Fish™® increased the number of several blood cells, such as the total erythrocytes, leukocytes, lymphocytes, monocytes, thrombocytes, indicating an improvement of the immune defense response in the *A. gigas*. Amin et al. [8] observed an increase in the activity of phagocytosis and lysozyme, number of leukocytes and percentage of monocytes and neutrophils in O. *niloticus* supplemented for 42 days with 0.3% glucan, indicating an improvement in the innate immunity.

Although handling is a routine practice in fish farming of any kind, it can cause stress during *A. gigas* production, resulting in alterations in fish resistance to diseases [1,3]. In the current study, the use of 12 g Aquate Fish™® caused a reduction of fish mortality after pathogen challenge and improved cellular immune response and respiratory burst of leukocytes in *A. gigas* after experimental infection with *A. hydrophila* and management stress. Similarly, in *P. mesopotamicus* has been demonstrated that supplementation with 0.1 or 1.0% β-glucan, respiratory burst of leukocytes, complement hemolytic activity, lysozyme activity and number of leukocytes increased after challenge with *A. hydrophila*, reducing fish mortality [25]. The addition of 0.2 and 0.4% of Glucan-MOS® was also sufficient to positively influence these immune parameters in *P. mesopotamicus* after challenge of stress and infection with *A. hydrophila* [10]. In *E. coioides* supplemented with different concentrations of *S. cerevisiae* and challenged with inoculation of *Streptococcus* sp. and iridovirus, phagocytic activity, respiratory burst of leukocytes, number of leukocytes in the kidney, lysozyme activity and alternative complement activity increased in fish fed with 10^5 and 10^7 UFC kg^{-1} of diet, reducing fish mortality [22]. Therefore, these immunostimulatory supplements improved the innate immunity of the fish, increasing their survival after challenge of immune resistance.

5. Conclusions

The results of this study indicate that supplementation with 12 g of Aquate Fish™/®kg of diet improved the biomass and immunity performance of *A. gigas* fingerlings, without negatively impacting the hematological parameters investigated. Finally, the 30-day supplementation period was sufficient to stimulate growth performance and to minimize handling stress and *A. hydrophila* infection, modulating innate immunity responses.

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