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Protective effects of the fructooligosaccharide on the growth performance, hematology, immunology indicators and survival of tambaqui (*Colossoma macropomum*, Characiformes: Serrasalmidae) infected by *Aeromonas hydrophila*

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ABSTRACT

Prebiotics are nutraceuticals that can be effective in therapeutic treatments by stimulating the immune system of fish. So, they contribute to reducing the use of antibiotics and synthetic drugs which are harmful to aquatic biota, fish health and human. The main goal of this study was to evaluate the potential effect of fructooligosaccharide (FOS) on zootechnical performance, blood immunology, phagocytic activity of white blood cells and survival of juveniles of *Colossoma macropomum* infected by *Aeromonas hydrophila*. The trials were composed of five diet levels of FOS (0; 0.1; 0.5; 1.0 and 2.0%), in triplicates. The experiments extended over 63 days, 45 days for the zoothecnical performance experiment and plus 18 days for the *A. hydrophila* challenge. An improvement in growth rate, mainly final body mass (BFW), weight gain (WG), feed efficiency (FE) and specific growth rate in animals fed 0.1% and 0.5% FOS supplemented diet was observed. An increase in circulating white blood cells (WBC), among them, a special granulocytic cell (PAS-GL^{+}) in animals fed FOS was also observed. The percentage of phagocytosis was higher in animals fed 0.5; 1 and 2% FOS. Consequently, after experimental infection, a higher survival of animals feeding diets containing FOS was observed, in particular those feeding 0.5% FOS supplemented diet. These results show that the addition of FOS in diets for *C. macropomum* at concentrations of 0.1 and 0.5% has important beneficial effects. Therefore, it is concluded that FOS can be used to promote growth and improve the healthiness of tambaqui, an important commercial Amazonian fish raised worldwide.

1. Introduction

Aquaculture is one of the food production sectors under pressure for growth in the world (The State of World Fisheries and Aquaculture, 2018). The projections indicate the increment of the production modules to meet the increased demand for fish (OCDE, 2015). As a consequence, an indiscriminate use of synthetic drugs for prophylactic and recovery of infested animals in fish farming is proven to be harmful to aquatic biota, fish and human health (Dimitroglou et al., 2011; Carvalho and Santos, 2016; Guerreiro et al., 2017). For this reason, several studies have pointed out to immunomodulatory product as nucleotides, prebiotics, probiotics, and symbiotic as therapeutic treatments aiming at replacing or minimizing the use of antibiotics, for example (Uribe et al., 2011).

The main aspects related to the use prebiotics in aquaculture have been health improvement in animals, validated by the greater resistance to infections by pathogens, increased productivity and improvement of nutrient absorption from diet (Ai et al., 2011; Akter et al., 2015; Firouzbakhsh et al., 2014). The mechanisms of action of prebiotics are yet to be fully elucidated, but their activities can be divided into nutritional, physiological and antimicrobial effects (Song et al., 2014).

Prebiotics can promote the proliferation of bacteria responsible for the production of antimicrobial compounds (bacteriocins) which act in reducing the number of pathogenic bacterial cells competing for adhesion sites. They may also alter microbial metabolism by increasing or decreasing enzyme activity and enhancing host immunity by acting on

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antibodies and macrophages (Ringø and Gatesoupe, 1998; Balcazar et al., 2006). Fructooligosaccharide (FOS), for example, is selectively fermented by beneficial commensal bacteria (*Bifidobacterium* and *Lactobacilli*) which increases its metabolism in the presence of this fiber. Most of the bacteria using FOS have specific transporters and intracellular β-fructofuranosidase for FOS catabolism with a low degree of polymerization. The use of FOS appears to occur through two catabolic pathways: (a) the substrate is transported intact and hydrolyzed by a cytoplasmic glycosyl hydrolase (GH32β-F phase), or (b) is hydrolyzed by extracellular substrates followed by catalysis by a GH32β-phase associated with the cell surface. Thereafter it undergoes absorption of the hydrolyzed products (i.e., fructose, sucrose and glucose associated with molecule) associated with one or more carriers (Goh and Klaenhammer, 2015). Several studies have reported positive influences of FOS on fish growth and hemato-immunology (Guerreiro et al., 2017; Firouzbakhsh et al., 2014; Ibrahem et al., 2010).

The tambaqui (*Colossoma macropomum*) was chosen as the experimental model because of its high economic value in Brazil (Instituto Brasileiro de Geografia e Estatística - IBGE, 2016) and worldwide. The tambaqui is the second most important farmed in Brazil and is now farmed in several Asia countries. It occurs naturally in the Amazon basin and in the main rivers of the Orinoco Basin in Venezuela (Araújo-Lima and Goulding, 1998).

Finally, the main goal of the present study was to evaluate the effects of fructooligosaccharide (FOS) supplemented diets on zootechnical performance, hemato-immunology, phagocytic activity of white blood cells and the survival of juveniles of *C. macropomum* to infection by *Aeromonas hydrophila*. In this sense, it was hypothesized that FOS presents a potential prebiotic role, improving growth performance and immunological resistance of juveniles of *C. macropomum*.

2. Material and methods

2.1. Experimental design 1

For the present experiment, 105 juveniles of *C. macropomum* (initial mass = 35 ± 024 g; length = 1037 ± 005 cm) obtained from Balbina fish experimental station (Presidente Figueiredo, Amazonas, Brazil) were used. The fish were transferred to 15 experimental conical tanks of 80 liters located in the Laboratory of Ecophysiology and Molecular Evolution - LEEM, at the Brazilian National Institute for Research of the Amazon - INPA. The experimental design was set to analyze the effects of 0.1, 0.5, 1.0 and 2.0% FOS supplemented diets, plus a base diet without FOS, in triplicates. The FOS enrichment levels were based on (Akrami et al. (2013) and (Wu et al. (2013).

The juveniles of *C. macropomum* were acclimatized for 10 days in conical tanks with a capacity of 80 liters (7 fish/tank). They were fed base diet (Table 1) twice a day at 8:00 a.m. and 3:00 p.m., receiving 2.5% of the biomass. After acclimatization, the base diet was suspended, except for the control, and replaced by the test diets, which were administered for 45 days. At 0, 15, 30 and 45 days, all animals were weighed in an analytical digital scale (Model Marte BL3200H, Minas Gerais, Brazil) with an accuracy of 0.1 g and measured with an ichthyometer, with a precision of 0.1 cm, considering the standard length (from head to caudal fin bifurcation) to adjust the amount of food.

In addition, at the end of 45 days, 9 animals/treatment and control (3 fish/experimental unit) were randomly collected. These animals were anesthetized by immersion in a solution of 0.5 g L^{-1} tricaine methanesulfonate (MS222), and blood samples were collected for hematological analysis. The variables related to water quality were analyzed throughout the experiment. The mean values (mean ± SD) were: dissolved oxygen = 6.20 ± 0.14 mg L^{-1}, temperature = 26.08 ± 0.65 °C, pH = 5.85 ± 0.08, electric conductivity = 15.92 ± 2.1 μs cm^{-1} and total ammonia = 34.09 ± 9.41 μM. All procedures were authorized by the Ethics Committee for Animal Use of INPA under protocol number 052/2017.

Table 1
The composition of experimental diets (g kg^{-1}).

Experimental diets	Base diet	FOS			
		0.1%	0.5%	1%	2%
Comercial diet[a]	975	975	975	975	975
FOS[b]	0	1	5	10	20
Cellulose[c]	20	19	15	10	0
Chromium oxide[d]	5	5	5	5	5
Proximate compositions (% dry matter)[e]					
Dry matter	92.6	90.8	92.3	91.1	89.2
Crude protein (%)	30.4	29.4	30.9	29.5	29.6
Crude Lipid (%)	3.7	5.3	5.3	4.3	3.9
Moisture (%)	7.4	9.2	9.2	8.9	9.1
Ash (%)	12.8	12.9	12.9	12.1	12.3

[a] Ingredients: broken rice, fatty rice bran, soybean meal, wheat bran, meat and bone meal, blood meal, fish meal, ground whole corn, sodium chloride, iron sulphate, copper sulphate, Manganese sulphate, zinc oxide, calcium iodine, cobalt sulfate, sodium selenite, choline chloride, propionic acid, ammonium hydroxide, ethoxyquin, dicalcium phosphate, diacetylated tartaric acid ester, mono-diglyceride, butylated anisole hydroxide (BHA), butylated toluene hydroxide (BHT). Contains (as mg/kg in diets): Calcium, 5000; Phosphate, 6000; Sodium, 2000; Iron, 30; Copper, 5; Manganese, 30; Zinc, 60; Iodine, 1; Cobalt, 0.10; Selenium, 0.30; Vitamin K3, 6; Vitamin B1, 12; Vitamin B2, 25; Niacin, 125; Pantothenic acid, 62; Vitamin B6, 12; Folic acid, 5; Biotin, 0.30. Contains (as IU/Kg in diets): Vitamin A, 15,000; Vitamin D3, 3000; Vitamin E, 62, Vitamin C, 300; Choline, 500; Methionine 3950. Contains (as mcg/Kg): Vitamin B12, 50. NUTRIPISCIS, São Paulo, Brazil.
[b] Fructooligosaccharide. ADS Nutrition Laboratory Ltda, São Paulo, Brazil.
[c] Carboxy methyl cellulose. EMFAL - Alcohol supplier company, Minas Gerais, Brazil.
[d] Sigma-Aldrich Korea Yongin, Republic of Korea.
[e] Means of three analyses.

2.2. Diet preparation

The respective concentrations of fructooligosaccharide (Natue® - São Paulo, Brazil) were incorporated into a commercial diet (NUTRIPISCIS®, Brazil) following a methodology similar to other studies (Dimitroglou et al., 2010; Amirkolaie and Rostami, 2015). The commercial diets were ground and homogenized and the mixtures were pelleted in a pelletizer (CAF-22 Inox 1/25 CV, Rio Claro, Brazil) and dried in a greenhouse for 12 h at 40 °C. The base diet was FOS-free. The centesimal composition of the diets was determined according to AOAC (Association of official analytical chemists - A.O.A.C, 1995).

2.3. Growth performance and biometric parameters

The evaluation of the zootechnical performance of juveniles of *C. macropomum* submitted to the experimental conditions was based on data collected at the end of the experimental period. The final mass (FBW), final length (FL), mass gain (WG), feed efficiency ratio (FE), condition factor (CF) and specific growth rate (SGR) were calculated as follow:

WG (%) = [(final mass - initial mass) x 100]/initial mass

FE (%) = (mass gain/dry feed intake) x 100

$CF = mass/length^3$

SGR (% day) = [(ln final mass - ln initial mass)]/rearing time (days) x 100

2.4. Hematological indicators

Blood samples (0.5 mL) were collected from the caudal vessel using 3 mL heparinized syringes and 27 Gx ½ needles (Sodium heparin 5000 IU) and stored in 2 mL Eppendorf bullets and kept in iced water. Plasma

was obtained by blood centrifugation at 3.000 *g* for 10 min in a 5430R centrifuge (model 5430R, Hamburg, Germany). For erythrocyte counts (RBC), whole blood was diluted in formaldehyde citrate solution (3.8 *g* sodium citrate, 2.0 ml formalin 40% and distilled water q.s.p. 100 ml) in the ratio 1: 201. The RBC was done in a Neubauer counting chamber using an optical microscope Leica (model DM1000, Wetzlar, Germany), under magnification of 400 ×. Leukocyte counting was performed using blood extensions, after staining by the May Grünwald-Giemsa Wright (MGGW) method (Ranzani-Paiva et al., 2013), under an optical microscope Leica (model DM1000, Wetzlar, Germany), with a magnification of 400 ×. Hematocrit was determined by the microhematocrit method as described by (Goldenfarb et al. (1971)). The hemoglobin concentration was determined by the cyanmethemoglobin method, as described by (van Kampen and Zijlstra (1961). The corpuscular constants MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) were calculated according (Brown (1976).

2.5. Biochemical indicators

Plasma glucose, triglycerides and cholesterol were determined using the In Vitro kit (In Vitro Diagnostic Ltda, Minas Gerais, Brazil), following the manufacturer's instructions, adapted for the plate spec Spectramax 348 PLUS spectrophotometer (Molecular Devices, USA). This method was also used by Kelly and Wood (2001).

2.6. Leukocyte phagocytic function

Immediately after blood collection, 200 microliters were placed in Eppendorf tubes and 100 microliters containing 1×10^8 CFU mL^{-1} of *A. hydrophila* were added. The tubes were incubated at 28 °C in a water bath for 30 min and homogenized for 10 min. Blood samples were then smeared, in duplicate. After drying, the slides were stained and fixed by Giemsa/May-Grunwald. To determine the phagocytotic percentage, 200 leukocytes were counted under the microscope Leica (model DM1000, Wetzlar, Germany), with a magnification of 400 ×. The phagocytic activity was determined according to the mathematical expression below, as Cai et al. (2004):

Phagocytotic percentage (%) = Phagocytotic leukocyte number/ Observed total leukocyte number

2.7. Experimental design 2 - challenge against Aeromonas hydrophila

Juveniles of *C. macropomum* (12 animals/treatment) fed base diet and FOS (0.1, 0.5, 1.0 and 2%) supplemented diets for 45 days were microchipped with transponders (MicroChip Brazil, São Paulo, Brazil) and were placed in a 300-liter aquarium. After 2 days of acclimation, they were injected intraperitoneally with 0.5 mL of saline solution (0.85% NaCl) containing 6×10^8 CFU of *A. hydrophila* (serial number: 0315318). The concentration close to this one was tested by Ribeiro et al. (2016). Soon after the injections, the fish were exposed to hypoxia (O_2 = 0.8 mg L^{-1}). Hypoxia was induced by suppressing aeration of the aquarium. The animals took about 30 min to show signs of hypoxia (expansion of the lower lip and rise to the surface of the water column). Thereafter, a further 3 h under hypoxia were observed. After this period, normoxia was restored (O_2 = 6.8 mg L^{-1}). The experiment lasted 18 days when all the animals in the control treatment died. Confirmation of pathogenicity occurred through the appearance of typical clinical signs of fish infected by *A. hydrophila*, such as erratic swimming, peeling, hemorrhagic septicemia and belly swelling as previously described Ribeiro et al. (2016); Fečkaninová et al., 2017).

2.8. Bacterial preparation

Strains of *A. hydrophila* were cultured in Muller Hunter agar and stored in saline solution (0.85% NaCl) containing 20% glycerol (v/v) at −70 °C to provide stable inoculation throughout the experiment (Yarahmadi et al., 2016). The optical density of 1.0 observed at 640 nm corresponds to the bacterial concentration of 1×10^9 CFU mL^{-1} (Ribeiro et al., 2018). Previously, we confirmed the pathogenicity of the bacterium in the concentration of 6×10^8 CFU mL^{-1}, which caused 100% mortality of *C. macropomum* juveniles fed base diet (mass: 45.12 ± 1.3) in 19 days.

2.9. Statistical analysis

All results are presented as a mean ± standard error (SEM). To ensure homogeneity of fish mass (g) and length (cm) at the beginning of the experiments, the Cochran test was performed at the 5% level of significance. It was adopted n = 6, considering the individuals belonging to the same population group. In addition, for the same data we perform a Quadratic Polynomial regression ($f = y0 + a*x + b*x^2$) to find the best FOS inclusion concentration according to the mathematical equation. All other data were analyzed by one-way ANOVA followed, in case of significant differences, by the Tukey test, considering a confidence interval of 5%. SigmaStat 3.5 and SigmaPlot 11.0 were used for the statistical tests.

3. Results

3.1. Growth performance and biometric parameters

Growth performance of juveniles of *C. macropomum* fed experimental diets supplemented with FOS for 45 days are presented in Table 2. Note that final body mass (FBW), final length (FL), mass gain (WG) and condition factor (CF) were significantly higher in the groups fed 0.1% and 0.5% FOS compared to animals fed base diet ($p < 0.05$) at the end of the experimental period. The feed efficiency ratio (EF) was also higher in animals fed 0.1% and 0.5% FOS compared to groups receiving the base, 1% and 2% FOS supplemented diets ($p = 0.006$). There was no mortality in any of the treatments. In addition, animals fed 0.1 and 0.5% FOS supplemented diets presented higher specific growth rates at 15, 30 and 45 days (15 days $p < 0.001$; 30 days $p = 0.030$ and 45 days $p = 0.014$) (Fig. 1).

3.2. Hematological parameters

The effects of FOS supplementation on hematological parameters in juveniles of *C. macropomum* are presented in Table 3. At the end of the experimental period, hematocrit (Ht) and mean corpuscular volume (MCV) decreased as FOS diet supplementation increased, with significant difference for Ht of animals fed 2% FOS compared to those fed base diet ($p = 0.047$), and for MCV of animals fed 1% and 2% FOS compared to those fed base, 0.1% and 0.5% FOS diets ($p = 0.038$). There was also an increase in the number of red cells (RBC) and mean corpuscular hemoglobin concentration (MCHC) as FOS inclusion in the diets increased. The RBC differences were significant ($p = 0.045$) for animals fed 2% FOS compared to those fed base diet; and MCHC differences were significant ($p = 0.005$) for animals fed 1 and 2% FOS compared to those fed base diet.

There was a considerable increase in white blood cell count (WBC) in animals fed 0.5% FOS ($p = 0.027$) and a reduction in the number of monocytes for animals fed FOS supplemented diets compared to base diet ($p = 0.002$). It was noticed an increase in granulocytic leukocyte ($PAS\text{-}GL^+$) in all treatments, in particular for animals fed 0.5% FOS supplemented diet.

3.3. Biochemical parameters

Glucose levels increased in animals fed 1% FOS supplemented diets compared to those fed base diet (Fig. 2). Reduction of triglyceride levels in animals fed 0.1; 0.5; 1 and 2% FOS supplemented diets and

Table 2
Growth parameters (mean ± SEM; n = 3) of *Colossoma macropomum* after 45 days feeding experimental diet supplemented with fructooligosaccharide.[a].

Variables	Diets[b]					P value
	Base diet	0.1%	0.5%	1%	2%	
FBW (g)[c]	62.1 ± 2.4[a]	72.8 ± 1.0[b]	73.4 ± 1.8[bc]	62.2 ± 2.3[a]	66.6 ± 1.6[abc]	0.003
FL (cm)[d]	12.8 ± 0.2[a]	13.6 ± 0.1[b]	13.6 ± 0.2[b]	13 ± 0.1[ab]	13.2 ± 0.1[ab]	0.023
WG (%)[e]	74.8 ± 6.5[a]	103 ± 4.0[b]	104 ± 5.4[bc]	76.0 ± 6.6[a]	86.9 ± 6.8[abc]	0.012
FE (%)[f]	66 ± 1.6[a]	72.3 ± 0.8[b]	74.6 ± 0.7[bc]	67.1 ± 1.0[a]	68.7 ± 1.5[abc]	0.006
CF[g]	1.8 ± 0.06[a]	2.2 ± 0.01[b]	2.2 ± 0.04[b]	1.9 ± 0.06[a]	2.0 ± 0.04[ab]	0.003
HSI (%)[h]	1.3 ± 0.04	1.5 ± 0.17	1.4 ± 0.06	1.3 ± 0.06	1.4 ± 0.11	0.658

[a] Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($p < 0.05$).
[b] Diets represent supplementation with increased levels of fructooligosaccharide.
[c] Final body weight (FBW).
[d] Final lenght (FL).
[e] Weight gain (WG. %) = [(final wt. - initial wt.) x 100]/initial wt.
[f] Feed efficiency ratio (FE. %) = (wet weight gain/dry feed intake) x 100.
[g] Condition factor (CF) = weight/length^3.
[h] Hepatosomatic index (HSI) = (liver wt. × 100)/body wt.

cholesterol in animals fed 1 and 2% FOS supplemented diets compared to those fed base diet ($p < 0.05$) were observed.

3.4. Phagocytic activity

Phagocytosis was higher ($p < 0.05$) in animals fed 0.5; 1 and 2% FOS supplemented diets compared to those fed based diet (Fig. 3). The regression ($F = 36.87 + 2.30*x - 0.06*x^2$; $R^2 = 0.70$) indicates an ideal value of 1.34% FOS diet supplementation for juveniles of *C. macropomum*.

3.5. Resistance test against Aeromonas hydrophila

After the experimental manipulation, most of the fish presented clinical signs typical of *A. hydrophila* infection, such as flank corrosion, lethargy, ascites, pale gills and hemorrhagic septicemia mainly in the liver, spleen and gills. After 18 days, there was no survival of the animals fed base diet, but there was 33.3% survival of animals fed 0.1% FOS, 50% survival of animals fed 0.5% FOS, 25% survival of animals fed 1% FOS- and 16.6% survival of animals fed 2% FOS supplemented diet (Fig. 4).

4. Discussion

It was verified several beneficial effects of FOS for juveniles of *C. macropomum*, mainly for animals fed 0.1 and 0.5% FOS supplemented diets. These positive effects are related to growth, mainly mass and length gain, specific growth rate and food efficiency; and to immunity, mainly white cell number, phagocytic capacity, accumulated survival increase in the bacterial test and condition factor.

FOS is selectively fermented by intestinal bacteria of the genus *Bifidobacterium* and *Lactobacilli*, which grow and increase their metabolic activities in the presence of this oligosaccharide. This contributes to the host's well-being and reinforces the prebiotic property of FOS (Akter et al., 2015; Huynh et al., 2017). Grisdale-helland et al. (2008) recommended supplementation of FOS and Mannanoligosaccharide (MOS) based mainly on positive growth rates observed for Atlantic salmon (*Salmo salar*). These authors also pointed to the need for further studies to determine the best level of inclusion and use duration.

It was observed higher SGR ($p < 0.001$) for animals fed 0.1 and 0.5% FOS supplemented diets after 15 days. Because of this, and also because of the effects related to increased immunity, the FOS supplementation can open doors to new studies focusing on immediate issues in fish farming, such as the preparation of fish for transportation,

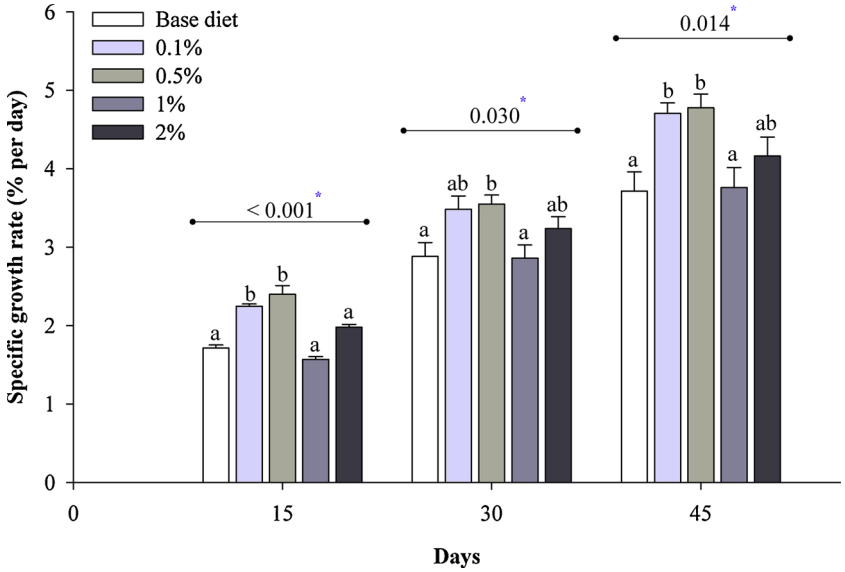

Fig. 1. Specific growth rate of *Colossoma macropomum* fed diets supplemented with frutooligosaccharide (0; 0.1; 0.5; 1 and 2%) for 45 days. Each value represents mean ± SEM (n = 3). Different letters indicate significant differences ($p < 0.05$). (*) Represents the p-value analyzed by Tukey test.

Table 3
Hematological parameters (mean ± SEM) of *Colossoma macropomum* after 45 days feeding experimental diet supplemented with fructooligosaccharide.[a].

Parameters	Diets					p value
	Base diet	0.1% FOS	0.5% FOS	1% FOS	2% FOS	
RBC (x10^6/mm^3)[b]	1.68 ± 0.04[a]	1.75 ± 0.03[ab]	1.78 ± 0.07[ab]	1.85 ± 0.06[ab]	1.87 ± 0.05[b]	0.045
MCV (fl)[b]	178.41 ± 5.67[a]	169.90 ± 7.47[ab]	171.47 ± 17.12[ab]	151.99 ± 3.25[b]	147.37 ± 2.60[b]	0.038
MCH (pg)[d]	39.65 ± 1.13	46.81 ± 0.27	47.40 ± 6.81	42.16 ± 3.23	42.39 ± 1.71	0.500
MCHC (%)[e]	23.72 ± 0.48[a]	26.02 ± 0.26[ab]	26.37 ± 0.07[ab]	27.83 ± 0.64[b]	28.63 ± 1.30[b]	0.005
Ht (%)[f]	31.17 ± 0.58[a]	29.39 ± 0.82[ab]	29.83 ± 1.34[ab]	28.33 ± 0.93[ab]	26.86 ± 0.07[b]	0.047
Hb (g.dL^{-1})[g]	7.53 ± 0.47	7.65 ± 0.21	8.04 ± 0.53	7.66 ± 0.33	7.73 ± 0.51	0.937
WBC (x 10^3. µL^{-1})[h]	32.49 ± 3.16[a]	32.88 ± 4.66[a]	49.88 ± 4.96[b]	47.78 ± 7.90[ab]	37.68 ± 3.21[ab]	0.027
Thrombocytes (x 10^3. µL^{-1})	39.19 ± 3.22	37.14 ± 1.59	31.04 ± 2.46	41.50 ± 1.48	35.07 ± 2.83	0.196
Lymphocytes (%)	87.83 ± 1.45	86.33 ± 1.17	87.33 ± 2.42	91.00 ± 2.34	84.73 ± 1.42	0.207
Monocyte (%)	8.0 ± 1.13[a]	3.33 ± 0.62[b]	4.17 ± 0.75b	2.50 ± 0.62[b]	3.65 ± 1.18[b]	0.002
Neutrophil (%)	4.17 ± 0.79	7.0 ± 0.87	6.77 ± 1.57	4.75 ± 1.78	8.25 ± 1.89	0.242
PAS - GL$^+$ (%)[i]	0.32 ± 0.20[a]	2.88 ± 0.59[bc]	4.37 ± 0.69[c]	1.33 ± 0.42[b]	2.67 ± 0.68[b]	< 0.001

[a] Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($p < 0.05$).
[b] RBC: Red blood cell.
[c] MCV: Mean corpuscular volume.
[d] MHC: Mean corpuscular hemoglobin.
[e] MCHC: Mean corpuscular hemoglobin concentration.
[f] Ht: Hematocrit.
[g] Hb: Hemoglobin.
[h] WBC: White blood cell.
[i] SGC: Special granulocytic cell.

reproduction, among other management routines that cause stress. This aspect is also endorsed by Ganguly et al. (2013) and Guerreiro et al. (2017).

As observed in the present study for *C. macropomum*, Zhang et al. (2015) observed increased growth variables using 0.6% inclusion of FOS in diets for *Megalobrama terminals*, Wu et al. (2013) for *Megalobrama amblycephala* fed 0.4% FOS supplemented diet and Akter et al. (2015) also found better growth rates for *Pangasionodon hypophytalmus* fed diets containing 0.6% MOS.

On the other hand, Ai et al. (2011) did not observe improvements in the growth of *Larimichthys crocea* fed FOS. However, when added in diets containing *B. subtilis*, these authors did observe improvements in growth performance, feed efficiency, lysozyme activity and superoxide dismutase activity. In fact, the combination of prebiotics and probiotics (symbiotics) has shown more effective effects on growth and improvement of fish immunity (Huynh et al., 2017; Hoseinifar et al., 2016). However, it should be considered that few studies (Guerreiro et al., 2017; Mo et al., 2015) have been designed to determine the optimal proportion of each component in the diet and, therefore, the maximum potential of these additives is yet to be explored.

The better absorption of carbohydrates, lipids and proteins, related to the higher growth rates observed in this study for animals feeding FOS supplemented diets, explain the increase in plasma glucose, also verified in other studies using prebiotics (Amirkolaie and Rostami, 2015; Tremaroli, 2012). However, further studies on the metabolism of beneficial microbes in the presence of prebiotics, and their metabolic

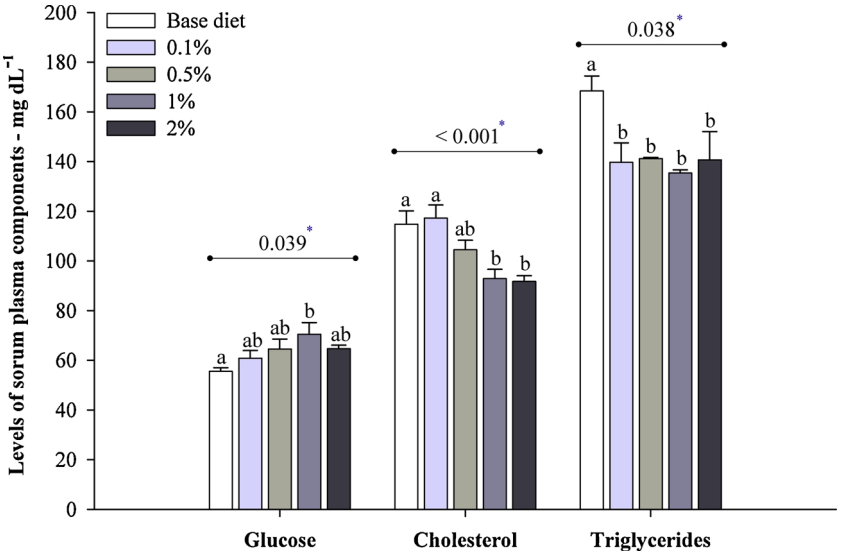

Fig. 2. Plasma levels of cholesterol, triglycerides and glucose (mean ± SEM) of *Colossoma macropomum* fed diets supplemented with fructooligosaccharide (0; 0.1; 0.5; 1 and 2%) for 45 days. Different letters indicate significant differences ($p < 0.05$) (n = 3). (*) Represents the p-value analyzed by Tukey test.

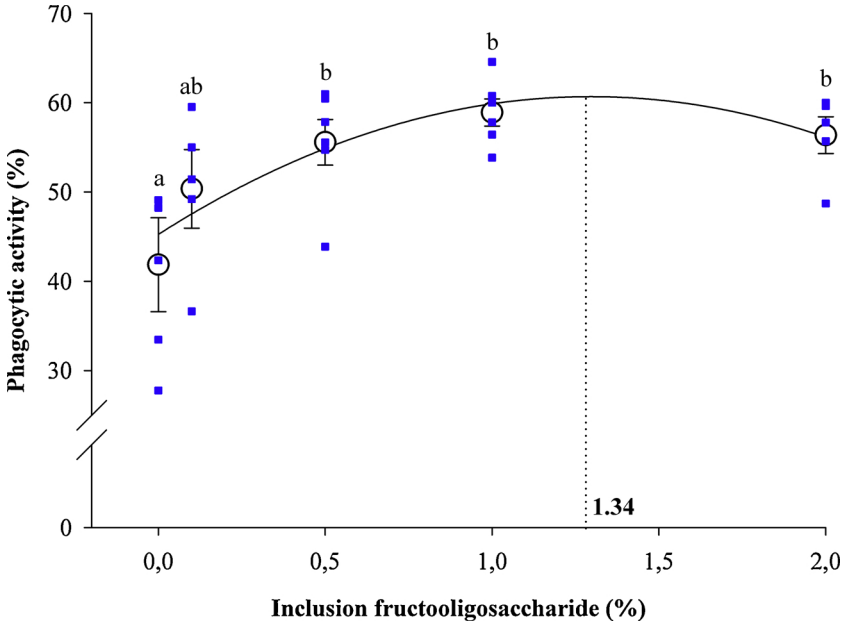

Fig. 3. Percentage of phagocytosis (mean ± SEM) in the blood of *Colossoma macropomum* fed diets supplemented with fructooligosaccharide (0; 0.1; 0.5; 1 and 2%) for 45 days (n = 6). Different letters indicate significant differences ($p < 0.05$). Equation ($F = 3687 + 230*x - 006*x^2$; $R^2 = 072$).

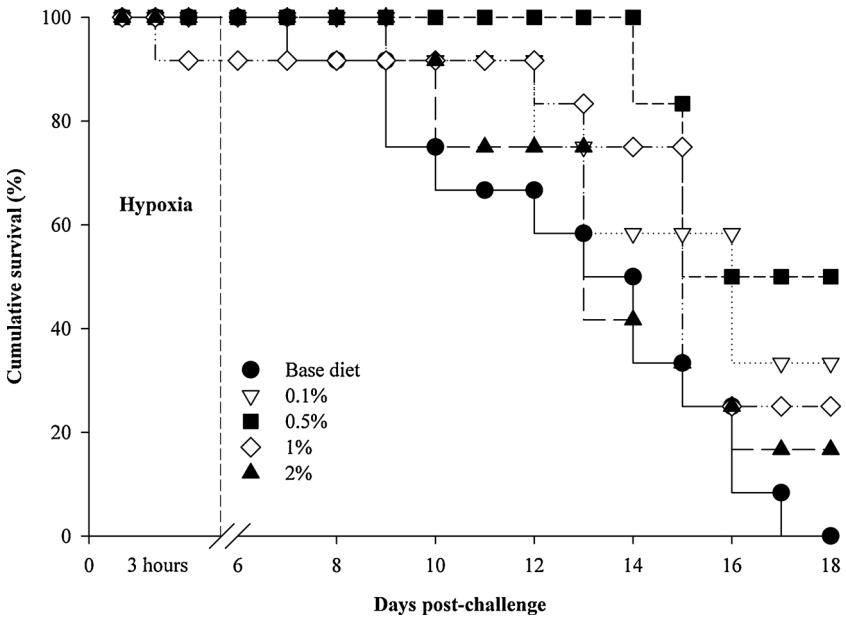

Fig. 4. Cumulative survival rate of *Colossoma macropomum* fed diets supplemented with fructooligosaccharide (0; 0.1; 0.5; 1 and 2%) for 45 days and subsequently challenged by *Aeromonas hydrophila* (see text for details).

influences, are needed. Additionally, Yong et al. (2007) and Goh and Klaenhammer (2015) indicated that FOS is hydrolyzed extracellularly in an exo-type, followed by the uptake generated by the hydrolysis of fructose through the sucrose-dependent phosphotransferase system. Therefore, short-chain fatty acids (SCFAs) are generated and assimilated rapidly in the intestine; are metabolized in different tissues and exert a direct influence on host metabolism. Propionate inhibits cholesterol formation and lipogenesis, while acetate stimulates them. It seems then that during fermentation of FOS, the proportion of acetate and propionate reaching the liver can act reducing triglycerides and cholesterol (Sabater-Molina et al., 2009), explaining the present result. In addition, cholesterol levels may be reduced due to precipitation and excretion with bile salts. The liver uses cholesterol for synthesis of bile salts. The reduction of serum cholesterol, in turn, decreases the serum concentration of triglycerides, since it acts in the transport of tri-esters (Tremaroli, 2012; Sabater-Molina et al., 2009).

Hematology is important to monitor the physiological and immunological status of fish affected by various stressors (Firouzbakhsh et al., 2014; Martins et al., 2009). A reduction of MCV and monocytes was observed in animals fed FOS supplemented diets. The same was observed by Yarahmadi et al. (2016) and Azimirad et al. (2016). Despite the changes, it is noted that the organisms were able to adapt to the effects of FOS inclusion without metabolic impairment. The same was observed for *Oreochromis niloticus* fed 0.5% inulin (Ibrahem et al., 2010) and *Oncorhynchus mykiss* fed a symbiotic composed of a probiotic (*Enterococcus faecium* IMB52 5×10^{11} CFU g^{-1}) and fructooligosaccharide (Firouzbakhsh et al., 2014).

In addition to the positive effects on growth, the consumption of prebiotics has been strongly associated with beneficial effects related to immunomodulation (Guerreiro et al., 2017). The mechanisms involve the selective increase of the population of bacterial groups that inhabit the intestine, which, in turn, modulate the production of cytokines and

local antibodies. Substances produced via fermentation of FOS penetrate the intestinal epithelium and activate the intestinal-associated lymphoid tissue (GALT). The increase of bacterial colonies stimulated by the presence of prebiotics also increases the immune response through the processes of infection control. However, this led to considerably increased energy expenditure for these processes (Tremaroli, 2012). For this reason, it was found that animals fed diets supplemented with FOS levels above 0.5% did not show better growth performance. This fact was also observed in other studies using prebiotics (Akrami et al., 2013; Yarahmadi et al., 2016).

In this study, special granulocytic cells (PAS-GL^{+}) appear to be active in the innate defense mechanism, which is corroborated by other studies with *C. macropomum* (Ribeiro et al., 2018; Pinheiro et al., 2015). We also observed an increase in the total number of white cells. In addition, it is important to consider that innate immunity is the main defense mechanism in fish, and bacterial outbreaks are more common when there is immune suppression (Uribe et al., 2011).

Macrophages, granulocytes, and monocytes are the first line of defense because they migrate to the site of inflammation and are responsible for phagocytosis and damage of pathogens (Hoseinifar et al., 2016). Based on this, it was found increased *in vitro* phagocytosis of *A. hydrophila* strains in the animals that received FOS in their diets. Similarly, other studies have reported increased phagocytosis of fish fed prebiotics (Guerreiro et al., 2017; Carbone and Faggio, 2016) and probiotics (Martins et al., 2009; Hai, 2015). Thus, the present results show the increase in indicators related to immunity and cleanness supported the higher survival rate during the bacterial challenge of juveniles of *C. macropomum* fed diets containing FOS. Increased animal survival was also reported in other studies using prebiotics (Ibrahem et al., 2010; Yarahmadi et al., 2016; Soleimani et al., 2012).

Our results indicate that the beneficial effects of FOS range from growing performance to immunological responses. Specifically, diets supplemented with 0.1 and 0.5% FOS had the greatest benefits for *C. macropomum*. Therefore, the FOS can be used to promote growth and to improve fish cleanness in breeding systems, confirming our hypothesis and can be quite useful in Aquaculture. However, further studies are needed to elucidate the immunomodulatory mechanisms of this oligosaccharide.

5. Conclusion

In conclusion, this study reinforces FOS functionality as a prebiotic for farmed fish. We recommend the use of FOS in fish diets because this practice contributes to the reduction of the use of antibiotics in aquaculture. This study also suggests the use of FOS in the proportion of 0.1% or 0.5% in diets for *C. macropomum* in view of the positive effects on growth performance, hematological parameters, phagocytic activity and resistance against *A. hydrophila* infection.

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