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Performance, hematology, and immunology of pacu in response to dietary supplementation with fructooligosaccharides

Abstract – The objective of this work was to evaluate the effects of dietary supplementation with fructooligosaccharides (FOS) on the performance, hematology, and immunology of pacu (*Piaractus mesopotamicus*) juveniles. A total of 0 (control), 2.0, and 4.0 g kg^{-1} of the probiotic were added to the fish diets. Fish (49.3±4.3 g) were allotted into 12 tanks of 60 L, in a completely randomized design (n=4). After 60 days, final weight, weight gain, specific growth rate, and feed conversion ratio were improved in the fish fed with the prebiotic, although feed intake was not affected by the treatments. The number of red blood cells was higher only in fish fed with 2.0 g kg^{-1} FOS. The opposite was observed for the hematimetric indices mean corpuscular volume and mean corpuscular hemoglobin, which decreased in fish fed with 2.0 g kg^{-1} FOS. The number of defense cells, such as leukocytes and thrombocytes, also increased in the prebiotic treatments. The evaluated immunological parameters were not influenced by prebiotic supplementation. Dietary FOS supplementation improved pacu growth and health.

Index terms: *Piaractus mesopotamicus*, feed additives, fish nutrition, prebiotics.

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Desempenho, hematologia e imunologia do pacu em resposta à suplementação dietética com frutooligossacarídeos

Resumo – O objetivo deste trabalho foi avaliar o efeito da suplementação dietética com frutooligossacarídeos (FOS) sobre o desempenho, a hematologia e o sistema imune de juvenis de pacu (*Piaractus mesopotamicus*). Um total de 0 (controle), 2,0 e 4,0 g kg^{-1} do prebiótico foi adicionado à dieta dos peixes. Os peixes (49,3±4,3 g) foram alocados em 12 tanques de 60 L, em delineamento inteiramente casualizado (n=4). Após 60 dias, o peso final, o ganho de peso, a taxa de crescimento específico e a conversão alimentar melhorou nos peixes alimentados com o prebiótico, apesar do consumo de ração não ter sido afetado pelos tratamentos. O número de células vermelhas foi maior apenas nos peixes alimentados com 2,0 g kg^{-1} de FOS. O oposto foi observado para os índices hematimétricos de volume corpuscular médio e hemoglobina corpuscular média, os quais diminuíram nos peixes alimentados com 2,0 g kg^{-1} de FOS. O número de células de defesa, como leucócitos e trombócitos, também aumentou nos tratamentos com o prebiótico. Os parâmetros imunológicos avaliados não foram afetados pela suplementação do prebiótico. A suplementação dietética com FOS melhorou o crescimento e a saúde do pacu.

Termos para indexação: *Piaractus mesopotamicus*, aditivos alimentares, nutrição de peixes, prebióticos.

Introduction

Prebiotics are indigestible substances that promote beneficial effects on the growth, feed efficiency, and immune response of the host (Guerreiro et al., 2018a). Fructooligosaccharides (FOS) are a common type of prebiotic derived from plants, consisting of a nondigestible linear chain of fructose units (Guerreiro et al., 2018b). Their effects on aquatic organisms began to be studied in Atlantic salmon [*Salmo salar* (Linnaeus, 1758)] farms, aiming to reduce antibiotic use (Grisdale-Helland et al., 2008). In the past decade, the study of FOS in aquaculture has grown substantially, registering positive effects on the growth and immunological parameters of several fish species such as sturgeon [*Acipenser stellatus* (Pallas, 1771)] (Akrami et al., 2013), rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792)] (Ortiz et al., 2013; Cid García et al., 2019), blunt snout bream [*Megalobrama amblycephala* (Yih, 1955)] (Zhang et al., 2014), Japanese sea bass [*Lateolabrax japonicus* (Cuvier, 1828)] (Wang & Li, 2020), and hybrid catfish [*Pangasianodon gigas* (Chevey, 1931) x *Pangasianodon hypophthalmus* (Sauvage, 1878)] (Hahor et al., 2019).

Pacu [*Piaractus mesopotamicus* (Holmberg, 1887)] is an omnivorous freshwater fish of the Serrasalmidae family important for aquaculture in South American countries, including Brazil (Valladão et al., 2018). *Piaractus* species, such as *P. mesopotamicus* and *Piaractus brachypomus* (Cuvier, 1818), as well as their respective hybrids with tambaqui [*Colossoma macropomum* (Cuvier, 1816)], i.e., tambacu and tambatinga, represented 9.8% of the Brazilian production of cultivated fish in 2019 (IBGE, 2020). Despite the economic importance to South American aquaculture, the knowledge about the effects of prebiotics in *Piaractus* species is still limited to previous studies in which the diets of pacu were supplemented with β-glucans (Biller-Takahashi et al., 2014) and mannanoligosaccharides (Sado et al., 2014a, 2014b). Besides Serrasalmidae species, the effect of dietary supplementation with FOS has only been evaluated in tambaqui (Paz et al., 2019), with the inclusion of 1.0 to 5.0 g kg^{-1} of the prebiotic being recommended.

Although the positive effects of prebiotics on the performance of terrestrial and aquatic animals have been reported (Nawaz et al., 2018), contrary results were also obtained, with no benefits to fish due to diet supplementation with prebiotics (Liu et al., 2017). Especially regarding aquatic organisms, the inconsistent results of prebiotics can be related to prebiotic type and source, dietary inclusion levels, environmental conditions (ectothermy), stage development, and interspecific physiological features (Guerreiro et al., 2018a).

The objective of this work was to evaluate the effects of dietary supplementation with fructooligosaccharides on the performance, hematology, and immunology of pacu juveniles.

Materials and Methods

All experimental procedures were approved by the ethics committee on animal use of Universidade Tecnológica Federal do Paraná (protocol number 2017-021). Juvenile pacu were obtained from the Piscicultura Daniela commercial farm (Francisco Beltrão, PR, Brazil). Fish were fed with the same extruded commercial mix powder, with minimum guarantee levels of 320 g kg^{-1} crude protein and 50 g kg^{-1} crude lipid (Anhambi Nutrição Animal, Itapejara d'Oeste, PR, Brazil), which was used as the control diet for 15 days prior to the trial for adaptation to the experimental conditions.

A total of 2.0 and 4.0 g kg^{-1} FOS from chicory (Sigma-Aldrich, Inc., St. Louis, MO, USA) were added to the commercial mix powder feed used in the adaptation period. A treatment without any supplementation (0 g kg^{-1} FOS), used as the control, was also evaluated. Both mixes were separately processed using the MX-40 laboratory extruder (Inbramaq Ind. Brasileira de Máquinas Ltda., Ribeirão Preto, SP, Brazil) and then compressed into 2.0 mm pellets. The extruded feeds were oven-dried, at 45°C, for 24 hours, and the pellets were packed in plastic bags and stored under refrigeration until needed.

The trial was conducted in an indoor (12 hours light:12 hours dark photoperiod) water recirculation system composed of 12 rectangular tanks of 60 L each, with a biological filtration system, supplementary aeration, and temperature control. During all the experimental period, water quality parameters were measured daily with the HI98193 portable probe (Hanna Brasil, Barueri, SP, Brazil) to assess dissolved oxygen (6.3±0.3 mg L^{-1}) and temperature (26.9±2.8°C).

The mPA-210 pH meter (MS Tecnopon, Piracicaba, SP, Brazil) was used to determine pH, held at 6.1±0.1.

At the beginning of the trial, juveniles were individually weighed (49.3±4.3g initial weight) and randomly assigned to 60 L tanks (10 fish per tank) in a completely randomized design with four replicates (fish groups) per treatment. Fish were fed with experimental diets until apparent satiety for 60 days, two times a day at 9:00 a.m. and 5:00 p.m. The tanks were cleaned every day after the last feeding.

At the end of the feeding trial, fish were fasted for 24 hours, anesthetized with 50 mg L^{-1} of an alcoholic solution of benzocaine, counted, weighed, and sampled for hematological and immunological analyses. Growth parameters were calculated according to Fracalossi et al. (2012).

Blood samples of four fish per tank (16 fish per treatment) were drawn from their caudal vessel using sterilized needles and syringes, and two aliquots were transferred to Eppendorf tubes (Eppendorf do Brasil, São Paulo, SP, Brazil) with and without heparin. Total red blood cells were counted with a Neubauer chamber using formaldehyde citrate buffer as a diluent; the hematocrit was determined in microhematocrit tubes after centrifugation for 5.0 min, at 10,000 *g*, and the hemoglobin content was obtained by cyanmethemoglobin on a spectrophotometer at 540 nm. The hematimetric indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated. Blood smears from sampled fish were stained with the May-Grünwald-Giemsa stain for total leukocyte and thrombocyte count by the indirect method (Ishikawa et al., 2008).

The same fish sampled for the hematological analysis, were used for analyses of leukocyte respiratory burst, serum and mucus lysozyme concentration, and total serum protein concentration. The production of reactive oxygen species by leukocytes (respiratory burst) was determined by the nitroblue tetrazolium (NBT) colorimetric assay. An aliquot of 100 µL of total heparinized blood was mixed with 100 µL of 0.2% Nitrotetrazolium Blue Chloride solution (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min at 25°C. After the incubation period, 50 µL of this suspension were added to 1.0 mL N, N-dimethylformamide (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged, at 755 *g*, for 5.0 min. Finally, the absorbance of the supernatant was measured at 540 nm using a spectrophotometer.

Serum lysozyme concentration was determined based on the lysis of *Micrococcus lysodeikticus* (Sigma-Aldrich, St. Louis, MO, USA), used as a standard. Before the fish serum analysis, a calibration curve was prepared from the L6876 standard lysozyme (Sigma-Aldrich, St. Louis, MO, USA) according to Abreu et al. (2009). Fish serum samples were obtained from blood stored in a microtube without anticoagulant and heated, at 56°C, for 30 min to inactivate complement system proteins and certify that the lysis of *M. lysodeikticus* had occurred only by lysozyme action. Afterwards, 150 µL fish serum and 150 µL sodium phosphate buffer were added to a glass cuvette and incubated at 26°C for 2.0 min in a spectrophotometer, and 300 µL *M. lysodeikticus* suspension (0.2 mg mL^{-1} sodium phosphate buffer) were added to complete a 600 µL final volume. The difference between the initial and final optical density was measured between 0.5 and 5.0 min, at 450 nm, also using a spectrophotometer. The equation of the lysozyme calibration curve was used to determine the serum lysozyme levels.

Fish mucus was collected to obtain lysozyme concentrations. Another three fish per tank (12 fish per treatment) were anesthetized with 50 mg L^{-1} of an alcoholic solution of benzocaine and immediately transferred to plastic bags containing 5.0 mL of 100 mol NH_4HCO_3 (pH 7.8) for 60 s. After this, the mucus extract was centrifugated, at 2,700 *g*, for 15 min to remove fish scales and insoluble materials, frozen at -20°C, and lyophilized using the L108 equipment (Liobras, São Carlos, SP, Brazil) for further analysis of lysozyme concentrations, as previously described.

After total blood centrifugation and serum collection, total serum protein concentrations were determined using the RHC-200 ATC portable refractometer (YHEquipment CO., Limited, Shenzhen, China), at 0.0~12.0 g dL^{-1}.

Data normality and homoscedasticity were previously analyzed using the Cramer Von Mises and Brown-Forsythe tests, respectively. When assumptions were met, the one-way analysis of variance was performed. If significant differences were registered, the differences between means were compared by Tukey's test, at 5% probability.

Results and Discussion

Supplementing pacu juveniles with FOS for 60 days clearly improved fish growth (Table 1). Final weight, weight gain, specific growth rate, and feed conversion ratio were improved regardless of the inclusion level, although feed intake was not significantly affected by FOS levels.

The inclusion of 2.0 g kg^{-1} FOS increased growth and feed efficiency, which, however, did not show a significant improvement in fish fed with 4.0 g kg^{-1}, as also reported for hybrid tilapia [*Oreochromis niloticus* (Linnaeus, 1758) × *Oreochromis aureus* (Steindachner, 1864] (Poolsawat et al., 2020). Similar responses were found for tambaqui juveniles fed with commercial diets supplemented with 1.0 to 5.0 g kg^{-1} FOS, with decreases in growth and feed efficiency after that (Paz et al., 2019). These authors suggest that levels of dietary FOS higher than 5.0 g kg^{-1} increase the expenditure of the basal metabolism, especially due to an improvement in the immune system, decreasing the energy available for fish growth. In fact, when compared with diets that were not supplemented, the dietary supplementation with FOS starting at 5.0 g kg^{-1} did not benefit the productive performance of several other fish species, such as rainbow trout (Ortiz et al., 2013), turbot [*Scophthalmus maximus* (Linnaeus, 1758)] (Guerreiro et al., 2016), and Japanese sea bass (Wang & Li, 2020). Therefore, growth promotion due to FOS supply depends on complex and multifactorial factors, such as feeding levels and inter- and intraspecific features (Guerreiro et al., 2018a).

Fish hematology was also affected by dietary FOS supplementation (Table 2). The red blood cells of fish fed with 2.0 g kg^{-1} FOS significantly increased when compared with the other treatments. However, hematimetric indices such as MCV and MHC decreased in fish fed with 2.0 g kg^{-1} FOS. The obtained results were compatible with those previously reported for the species (Ranzani-Paiva et al., 1999; Tavares-Dias et al., 1999; Tavares-Dias, 2015), and no clinical signs of anemia were observed.

The dietary prebiotic stimulated the production of fish defense cells, and the number of both leukocytes and thrombocytes were higher at 2.0 g kg^{-1} FOS, followed by 4.0 g kg^{-1} and the control treatment (Table 2). These results indicate a better health status, since those cells participate in and are responsible for fish inflammatory and hemostatic responses against foreign substances such as pathogenic microorganisms (Rieger & Barreda, 2011; Fink et al., 2015). Despite the increase in the number of defense cells, serum and mucus lysozyme concentration, leukocyte respiratory burst, and total serum protein were not affected (Table 3). Similar results were observed for rainbow trout fed with 5.0 g kg^{-1} dietary FOS for 70 days (Cid García et al., 2019).

The immunomodulation of prebiotics in fish has been previously reported, and the permanence and benefits of this modulation seem to be time dependent. In fact, FOS affect immune responses indirectly because the microbial fermentation of the prebiotic leads to the production of short-chain fatty acids, which improves gut probiotic microbiota, resulting in immune responses (Guerreiro et al., 2018a).

Table 1. Growth performance parameters of juvenile pacu (*Piaractus mesopotamicus*) fed with diets with increasing levels of fructooligosaccharides (FOS) for 60 days[(1)].

Parameters	Dietary FOS supplementation levels (g kg^{-1})			One-way Anova[(2)]
	0.0	2.0	4.0	(p-value)
Initial weight (g per fish)	50.36±0.82	49.12±2.77	48.34±3.86	0.686
Final weight (g per fish)	94.72±7.06b	120.50±6.60a	110.40±7.72a	0.012
Weight gain (g per fish)	44.35±7.98b	71.37±7.38a	62.05±4.03a	0.006
Daily feed intake (g per fish)	2.09±0.08	2.11±0.15	2.01±0.14	0.664
SGR (% body weight per day)	1.04±0.14b	1.49±0.13a	1.37±0.03a	0.008
Feed conversion ratio (FCR)	2.88±0.40a	1.78±0.06b	1.96±0.20b	0.005

[(1)]Means followed by equal letters, in the rows, do not differ by Tukey's test, at 5% probability. [(2)]Analysis of variance. SGR, specific growth rate.

In the present study, there was a trend of reduction in the beneficial effects of the defense blood cells (leukocytes and thrombocytes) in pacu fed with 4.0 g kg^{-1} FOS for 60 days. Therefore, it is plausible to suppose that the absence of effects on the immunological parameters of pacu fed with FOS diets may be due to a long time of supplementation.

Moreover, the composition of the intestinal microbiota may change with time. In European sea bass, for instance, the supply of dietary FOS improved the richness and number of gut lactic acid bacteria after one to four weeks (Guerreiro et al., 2018b). However, in mirror carp [*Cyprinus carpio* (Linnaeus, 1758)] juveniles, diets supplemented with β-glucan caused positive effects on bacterial numbers and richness after only two weeks, disappearing after four weeks in the case of allochthonous microbiota (Kühlwein et al., 2013).

The time dependence of prebiotic modulation on the hematological and defense blood cells of pacu fed with mannan oligosaccharides was previously reported by Sado et al. (2014b). Additionally, Paz et al. (2019) highlighted an improvement in the growth and immunological parameters of tambaqui juveniles fed with 1.0 and 5.0 g kg^{-1} FOS diets from 15 to 45 days, but, when FOS levels were increased up to 20 g kg^{-1}, these parameters were similar to those of diets that were not supplemented. The use of FOS as a prebiotic for South American neotropical fish is still incipient and studies on time, rate, and feeding protocols, as well as on pacu physiology and immunology, are still scarce and needed for the safe use of FOS in aquaculture.

Table 2. Hematological parameters of juvenile pacu (*Piaractus mesopotamicus*) fed with diets with increasing levels of fructooligosaccharides (FOS) for 60 days[(1)].

Parameters	Dietary FOS supplementation levels (g kg^{-1})			One-way Anova[(2)]
	0.0	2.0	4.0	(p-value)
RBC (10^6 cells μL^{-1})	1.48±0.24b	2.33±0.29a	1.58±0.14b	<0.0001
Hematocrit (%)	33.1±4.7	31.9±3.7	32.9±3.0	0.6439
Hemoglobin (g dL^{-1})	5.24±0.69	5.58±0.67	5.30±0.56	0.3992
MCV (fL)	224.1±22.4a	138.0±19.9b	202.3±21.7a	<0.0001
MHC (ρg $cell^{-1}$)	36.3±7.5a	24.3±4.6b	33.8±5.6a	<0.0001
MCHC (g dL^{-1})	17.07±3.78	17.77±3.30	16.21±1.88	0.4660
TLC (10^3 cells μL^{-1})	11.56±3.36c	39.60±11.6a	27.49±3.33b	<0.0001
TC (10^3 cells μL^{-1})	6.97±2.17c	20.46±3.59a	14.42±3.64b	<0.0001

[(1)]Means followed by equal letters, in the rows, do not differ by Tukey's test, at 5% probability. [(2)]Analysis of variance. RBC, red blood cells; MCV, mean corpuscular volume; MHC, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; TLC, total leukocyte count; and TC, thrombocyte count.

Table 3. Immunological parameters of juvenile pacu (*Piaractus mesopotamicus*) fed with diets with increasing levels of fructooligosaccharides (FOS) for 60 days[(1)].

Parameters	Dietary FOS supplementation levels (g kg^{-1})			One-way Anova[(2)]
	0.0	2.0	4.0	(p-value)
LRB (optical density)	0.274±0.04	0.269±0.04	0.293±0.05	0.3743
SLC (μg mL^{-1})	1.39±0.33	1.08±0.37	1.17±0.40	0.1490
MLC (μg mL^{-1})	1.05±0.48	0.97±0.42	1.12±0.42	0.7441
TSP (g dL^{-1})	5.31±0.31	5.23±0.26	5.10±0.26	0.2197

[(1)]Means followed by equal letters, in the rows, do not differ by Tukey's test, at 5% probability. [(2)]Analysis of variance. LRB, leukocyte respiratory burst; SLC, serum lysozyme concentration; MLC, mucus lysozyme concentration; and TSP, total serum protein concentration.

Conclusion

The dietary supplementation with 2.0 g kg^{-1} fructooligosaccharides improves the growth parameters, modulates the immune system, and increases the circulating organic defense cells of juvenile pacu (*Piaractus mesopotamicus*).

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