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Original article

Hemato-immunological responses of juvenile tambaqui (*Colossoma macropomum*) after commercial clay dietary supplementation

Respuestas hematoinmunológicas de juveniles de tambaquí (*Colossoma macropomum)* después de la suplementación dietética con arcilla comercial

Yuri Ian Carvalho Furtado[1,*], Fernanda Layza Souza de Souza[2], Mosa Valdina Ferreira Moreira[1], Amanda Mendes Pacheco[2], Clara Brito Salomão[2], Eliane Tie Oba Yoshioka[1,3]

[1] Programa de Pós-Graduação em Biodiversidade Tropical, Universidade Federal do Amapá – UNIFAP, Brasil
[2] Universidade do Estado do Amapá – UEAP, Colegiado de Engenharia de Pesca, Brasil
[3] Empresa Brasileira de Pesquisa Agropecuária, Embrapa Amapá, Brasil

Abstract

The present study evaluated the physiological and biochemical responses of tambaqui (*Colossoma macropomum*) to different dietary supplementation levels of bentonite. We evaluated four treatments with three replicates (10 fish *per* tank). The sodium bentonite adsorbent (Buntech) was incorporated into the commercial feed at three levels in addition to the control group (0%, 0.5%, 1.0%, and 2%). After 10 and 20 days of feeding, we collected blood samples from five fish in each replicate for analysis. We observed the changes in hematocrit, hemoglobin, erythrocytes, and hematimetric indices of the tambaqui. Their glucose concentration, total proteins, total cholesterol, triglycerides, and albumin differed significantly due to the bentonite inclusion. We also recorded reductions in *C. macropomum* defense cells due to physiological damage caused by the adsorbent agent, resulting in thrombocytopenia, leukopenia, lymphocytopenia, and monocytopenia. Such reductions indicated a reaction and migration to inflammatory foci; the addition of this product did not reflect any improvement in the hematological-biochemical profile of this fish species. Our results suggest that sodium bentonite dietary supplementation has deleterious effects on *C. macropomum* and is not indicated as a food additive.

Keywords: *Colossoma macropomum*; Hematology; Immunostimulant; Freshwater fish.

Resumen

El presente estudio evaluó las respuestas fisiológicas y bioquímicas del tambaquí (*Colossoma macropomum*) a diferentes niveles de suplementación dietética con bentonita. Se evaluaron cuatro tratamientos con tres réplicas (10 peces por tanque). El adsorbente de bentonita sódica (Buntech) se incorporó al alimento comercial en tres niveles además del grupo control (0 %, 0,5 %, 1,0 % y 2 %). A los 10 y los 20 días de alimentación, se recolectaron muestras de sangre de cinco peces de cada réplica para su análisis. Se observaron cambios en el hematocrito, la hemoglobina, los eritrocitos y los índices hematimétricos del tambaquí. La concentración de glucosa, las proteínas totales, el colesterol total, los triglicéridos y la albúmina difirió significativamente debido a la inclusión de la bentonita. Hubo reducciones en el número de las células de defensa de *C. macropomum* debido al daño fisiológico causado por el agente adsorbente, lo que resultó en trombocitopenia, leucopenia, linfocitopenia y monocitopenia. Dichas reducciones sugerían una reacción y la migración a focos inflamatorios; la adición del producto no reflejó una mejoría en el perfil hematológico-bioquímico de esta especie de pez. Nuestros resultados indican que la suplementación dietética con bentonita sódica tiene efectos nocivos sobre *C. macropomum*, por lo cual no está indicada como aditivo alimentario.

Palabras clave: *Colossoma macropomum*; Hematología; Inmunoestimulante; Peces de agua dulce.

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***Correspondencia:**
Yuri Ian Carvalho Furtado;
yuri_furtado@hotmail.com

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Introduction

Colossoma macropomum, commonly known as tambaqui, belongs to the Serrasalminae subfamily and is native to the Amazon and Orinoco rivers and their tributaries (**Goulding & Carvalho**, 1982; **Hilsdorf *et al.***, 2022; **Mirande**, 2010). The tambaqui has several desirable traits for aquaculture, including hardiness, ease of adaptation to captive management, excellent meat, and high commercial value (**Garcez *et al.***, 2021). The global demand for high-quality and economically sustainable aquaculture feeds is increasing, driven by the rapid development of the aquaculture sector (**Prabhu *et al.***, 2019). More than 70% of Brazil's fish production comes from intensive farming (**Instituto Brasileiro de Geografia e Estatística - IBGE**, 2014).

Although there is much uncertainty about the complex interplay between immune function and resistance to disease in fish, it is widely recognized that adequate nutrition plays a crucial role in strengthening the immune system (**Hadiuzzaman *et al.***, 2022; **Ucar *et al.***, 2019; **Martin and Król**, 2017). In fish, the immune system is composed of innate and adaptive components, including leukocytes such as the lymphocytes, neutrophils, and macrophages, which act against pathogens (**Ranzani-Paiva *et al.***, 2013).

The innate immune response, acting as the first line of defense, is mediated by physical and chemical barriers, in addition to cells such as neutrophils and macrophages (**Uribe *et al.***, 2011), whereas the adaptive response, which is more specific, involves lymphocytes that recognize specific antigens and have a complex interaction between the two systems to protect efficiently the organism (**Secombes *et al.***, 2012). Efforts to modulate the immune response through dietary supplements have increased significantly (**Ribeiro *et al.***, 2016; **Dias *et al.***, 2019; **Hoshino *et al.***, 2020).

Bentonite, a type of clay with sorption/absorption properties that also acts as a mycotoxin adsorbent, has the potential to modulate the immune response in fish through different mechanisms. One of the hypotheses proposes that, by adsorbing toxins and other harmful compounds in the digestive tract, bentonite reduces the burden on the immune system and allows it to function more efficiently, for which there have been studies to demonstrate its potential as immunomodulatory agent in aquaculture (**Kiron**, 2012; **Fazio**, 2019). Another proposal is that it interacts directly with immune cells, influencing the production of immunoglobulins, kidney function, and liver enzymes, and improving immunological and antioxidant parameters. Besides, bentonite may affect the intestinal microbiota, which plays a key role in modulating the immune response (**El-Dahhar *et al.***, 2024; **Abdel-Rahim *et al.***, 2023).

Previous studies have demonstrated the positive effects of bentonite in the diet of hybrid groupers (*Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*) (**Arshad *et al.***, 2021), the European seabass (*Dicentrarchus labrax*), the rainbow trout (*Oncorhynchus mykiss*) (**Ucar *et al.***, 2019), the gilthead seabream (*Sparus aurata*) (**Kanyilmaz & Tekellioğlu**, 2016), and the Nile tilapia (*Oreochromis niloticus*) (**Farrag *et al.***, 2009), leading to improvements in innate immunity, growth performance, and feed intake, as well as reducing the toxic effects of lead oxide. However, the beneficial effects of bentonite as a feed additive for captive-raised tambaqui have not yet been explored. In this context, we studied the potential use of natural bentonite clay on the hematological and biochemical responses of *C. macropomum*.

Materials and methods

The present study was authorized by the Embrapa Amapá Ethics Committee for the Use of Animals in Experiments (CEUA) (Number 019-CEUA/CPAFAP) and registered in the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the identification number A0D6DC0.

Experimental design and diets

The experiments were conducted at the Embrapa Amapá, Aquaculture and Fisheries Laboratory, Macapá, Amapá State, Brazil. Tambaqui (*C. macropomum*) specimens (n = 120)

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with an initial mean weight of 125.82±21.67g were randomly assigned to 12 experimental tanks (100 L polypropylene water containers). The sodium bentonite adsorbent (Buntech Tecnologia em Insumos, Indaiatuba, SP, Brazil) was incorporated into the commercial feed at three levels. The experimental groups were run in triplicate as follows: a) control (0%, with no bentonite); b) 5 g of bentonite/kg of feed (0.5%); c) 10 g of bentonite/kg of feed (1%), and d) 20 g of bentonite/kg of feed (2%). We collected 100 g of feed from each to analyze the centesimal composition of the experimental diets in triplicate. **Table 1** shows the chemical composition of the experimental diets.

Fish were fed with commercial rations (grain size 3.00 mm) containing 32% crude protein (Acqua Line, Rações Supra, Alisul Alimentos S.A., São Leopoldo, RS, Brazil) four times a day (at 08:00, 11:00, 14:00, and 17:00) during the acclimatization and all the experiment periods. The feed offered per day was equivalent to 5% of the total biomass of each experimental tank. During the experiment period, oxygen levels, temperature, and pH of the water were monitored using a multiparametric probe (Horiba, model AK88) and we obtained the following means and standard deviations: dissolved oxygen, 6.01 ± 0.38 mg/L; temperature, 29.47 ± 0.12ºC, and pH, 5.12 ± 0.68, showing that water quality in experimental tanks was adequate for tambaqui (**Aride *et al.***, 2007).

Growth parameters

After the feeding period (10 and 20 days), five tambaqui specimens from each replicate (n=120) were captured for blood collection, and then the total length and total weight of the specimens were registered.

Hematological analysis

We collected a blood sample (1,0 mL) per specimen by puncturing the caudal vein with a 3 mL syringe containing anticoagulant (EDTA 5%). We determined the following hematological parameters: hematocrit (Ht), i.e., the percentage of erythrocytes in the blood obtained through centrifugation of capillary tubes in a microhematocrit centrifuge for 5 minutes (Micro Spin, model CE120, Hangzhou, China) and read the results using a reading card (**Goldenfarb *et al.***, 1971); we determined the hemoglobin concentration (Hb) using the cyanomethemoglobin method expressing the values in g dL^{-1} (**Collier**, 1944); finally, red blood cell count (RBC) was done by diluting blood samples in a formol-citrate solution and counting in erythrocytes x $10^6.\mu L^{-1}$ in a Neubauer chamber under an optical microscope (Boeco, model BOE-01, Hamburg, Germany). We calculated the following hematimetric indices based on these results: mean corpuscular volume (MCV, in fL), mean corpuscular hemoglobin (MCH, in g dL^{-1}), and mean corpuscular hemoglobin concentration (MCHC, in g dL^{-1}) (**Ranzani-Paiva *et al.***, 2013). The leukocyte respiratory activity (respiratory burst) was determined as described by **Sahoo *et al.*** (2005) and **Biller-Takahashi *et al.*** (2013) with absorbance readings at

Table 1. Proximate chemical composition (%) of the experimental diets: a) Control (0%, with no bentonite); b) 5 g of bentonite/kg of feed (0.5%); c) 10 g of bentonite/kg of feed (1%); d) 20 g of bentonite/kg of feed (2%)

Parameters	0%	0.5%	1%	2%
Dry matter (%)	90.78 ± 0.11[a]	91.11 ± 0.01[a]	92.08 ± 1.62[a]	90.09 ± 1.32[a]
Crude protein (%)	32.67 ± 0.17[a]	31.83 ± 0.78[a]	32.50 ± 0.85[a]	31.62 ± 1.09[a]
Ether extract (%)	5.31 ± 0.33[a]	4.64 ± 0.54[a]	4.27 ± 0.19[b]	4.45 ± 0.23[a]
Ash (%)	11.93 ± 0.03[a]	13.64 ± 1.6[a]	12.62 ± 0.18[a]	12.37 ± 1.64[a]
Calcium (%)	1.62 ± 0.37[a]	0.99 ± 0.12[b]	1.02 ± 0.15[b]	1.18 ± 0.10[a]
Phosphorous (%)	1.61 ± 0.11[a]	1.60 ± 0.10[a]	1.50 ± 0.04[a]	1.70 ± 0.14[a]

Data expressed as mean±standard deviation. Letters indicate significant differences ($p<0.05$).

540 nm in a spectrophotometer (Biospectro, SP-220, Curitiba, Paraná, Brazil). Blood smears were prepared in duplicate and stained with the May-Grünwald-Giemsa-Wright staining to obtain total leukocytes, total thrombocytes, and differential leukocytes counts by the indirect method (**Ishikawa *et al.***, 2008). After centrifugation of the blood (at 75 G, for 10 minutes) (Centrifuge 5424, Eppendorf, Hamburg, Germany), the plasma obtained was used to determine the total proteins, albumin, glucose, total cholesterol, and triglyceride concentrations (Labtest Diagnóstica S.A., Lagoa Santa, Minas Gerais, Brazil). The samples were read using a spectrophotometer (Biospectro SP-220, Curitiba, Paraná, Brazil) at specific wavelengths for each metabolite.

Statistical analysis

The data were subjected to normality and homoscedasticity tests using the Shapiro-Wilk and Levene methods, respectively, and, when necessary, they were transformed (total erythrocyte count). We used one-way and two-way variance analyses (ANOVA) and Tukey's *a posteriori* tests to compare the means. Diet and time were used as the main factors. Differences were considered significant at 5% probability (**Zar**, 2010). Tests were performed using the statistical software Sigmaplot 12.0.

Results and discussion

Adsorbents in aquaculture are increasingly used to prevent and mitigate animal health issues (**Palm *et al.***, 2022; **Ucar *et al.***, 2019). Prevention can be achieved by enhancing immunity and suppressing pathogens (**El-Dahhar *et al.***, 2024). **Table 2** shows the growth performance and survival rates of animals fed with different concentrations of the adsorbent for 20 days. Bentonite in the diet of *C. macropomum* did not significantly increase the total length or weight gain. Growth is a multifactorial process, and bentonite, although important, does not by itself explain growth performance. However, the species showed adequate development at this stage compared to the control group, and no mortality was recorded.

Hematological parameters provide valuable information about the health status of fish and are fundamental indicators for assessing animal well-being and optimal farming conditions (**Ranzani-Paiva *et al.***, 2013; **Fazio**, 2019; **Hoshino *et al.***, 2020). The innate immune system of fish is the primary line of defense against a wide range of pathogens, and it has a more significant role in fish than mammals (**Saurabh & Sahoo**, 2008). In our study, hematocrit (Ht) in animals fed with 1% and 2% bentonite for 10 days significantly increased compared to the 0% and 0.5% groups (**Table 3**), but these values remained within the expected range for the species and were consistent with specimens' health (**Aride *et al.***, 2017; **Tavares-Dias *et al.,*** 2009).

Tambaqui receiving a diet including 0.5% bentonite adsorbent for 10 days showed a decrease in hemoglobin (Hb) concentration compared to the other groups. However, after 20 days of 0.5%, 1%, and 2% bentonite feeding, Hb and red blood cell count (RBC)

Table 2. Initial and final weight of juvenile tambaqui (Colossoma macropomum) fed with the following experimental diets: Control (0%, no bentonite); b) 5 g of bentonite/kg of feed (0.5%); c) 10 g of bentonite/kg of feed (1%); d) 20 g of bentonite/kg of feed (2%)

Parameters	0%	0.5%	1%	2%
Initial weight (g)	128.23±21.71[a]	123.40±21.22[a]	123.06±22.87[a]	128.60± 21.31[a]
Final weight (g)	247.66±18.64[a]	234.66±26.36[a]	255.50± 44.23[a]	263.07± 32.96[a]
Initial length (cm)	15.39±1.21[a]	15.69 ± 0.94[a]	15.76 ± 1.08[a]	15.70 ± 0.96[a]
Final length (cm)	19.11 ± 1.15[a]	19.08 ± 0.85[a]	19.40± 1.53[a]	19.98 ± 1.14[a]
Fish survival (%)	100	100	100	100

Data expressed as mean±standard deviation. Letters indicate significant differences ($p<0.05$).

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Table 3. Hematological variables and hematimetric indices (mean ± standard deviation) of *Colossoma macropomum* fed with these experimental diets: Control (0%, no bentonite); b) 5 g of bentonite/kg of feed (0.5%); c) 10 g of bentonite/kg of feed (1%); d) 20 g of bentonite/kg of feed (2%) for 10 and 20 days, respectively

Parameters	Factors			
	0%	**0.5%**	**1%**	**2%**
	10 days			
Ht (%)	27.00±2.61[b]	26.76±3.58[b]	28.70±2.96[a]	30.40±3.85[a]
Hb (g dL^{-1})	7.60±0.95[a]	6.88±1.37[b]	8.06±0.76[a]	7.52±0.91[a]
RBC (x 10^6 μL^{-1})	1.57±0.30[a]	1.47±0.22[a]	1.65±0.40[a]	1.34±0.18[a]
VCM (fL)	185.65±45.82[a]	178.30±26.62[b]	184.21±46.88[b]	220.35±31.01[a]
HCM (g dL^{-1})	46.92±14.95[a]	47.63±11.69[a]	52.35±16.09[a]	56.88±10.47[a]
CHCM (g dL^{-1})	28.18±2.97[a]	25.85±4.12[a]	28.07±1.34[a]	25.10±3.47[a]
Parameters	**Factors**			
	0%	**0.5%**	**1%**	**2%**
	20 days			
Ht (%)	27.39±3.13[a]	27.50±2.46[a]	27.85±1.02[a]	28.66±1.15[a]
Hb (g dL^{-1})	8.16±0.98[a]	6.61±0.97[b]	6.87±0.82[b]	7.10±0.60[b]
RBC (x 10^6 μL^{-1})	1.46±0.34[a]	1.08±0.20[b]	1.01±0.12[b*]	1.11±0.17[b*]
VCM (fL)	177.27±21.93[b]	249.65±40.35[a*]	276.30±28.37[a*]	268.86±39.74[a*]
HCM (g dL^{-1})	56.03±11.18[a]	58.15±12.99[a]	64.37±13.76[a]	64.05±11.15[a]
CHCM (g dL^{-1})	30.70±4.89[a*]	23.97±2.00[b]	24.42±3.25[b*]	24.69±2.15[b]

Letters indicate significant differences in two-way ANOVA followed by post-hoc Tukey ($p<0.05$); *: significant difference ($p<0.05$) when comparing 10- and 20-day-period groups using the same treatment and for the same parameter. Ht: Hematocrit; Hb: Hemoglobin concentration; RBC: Red blood cells count; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration

reduced significantly compared to the control group, which indicates an interaction between the diet and the feeding period, particularly in the 1% group, and suggests normochromic anemia due to the extended period of adsorbent consumption (**Ranzani-Paiva *et al.***, 2013; **Kanyilmaz & Tekellioğlu**, 2016), despite that, according to **Tavares-Dias *et al.*** (2009), Hb concentration of *C. macropomum* ranged from 6.3 to 13.7 g dL^{-1}, showing similar values to those reported in the present study. This was also the case in the study by **Costa *et al.*** (2022) for tambaqui diets containing silage made from fish and vegetable residues. Hb, Ht, and RBC are the main hematological parameters reflecting the initial response of the erythrocytic series, which indicates blood oxygen transport capacity and its subsequent utilization by the body. The RBC, Hb, and Ht values found in the tambaqui in the present study were similar to those described by **Tavares-Dias *et al***. (2009). However, RBC reduced after 20 days of feeding, particularly in the groups receiving bentonite, suggesting decreased oxygen transport capacity possibly leading to viral, bacterial, and parasitic infections, exposure to toxins, nutritional deficiencies, and blood loss (**Currie *et al***., 2022; **Witeska *et al***., 2015).

The mean corpuscular volume (MCV) was higher at 10 days in the 0% and 2% groups compared to the 0.5% and 1% groups (**Table 3**). At 20 days, MCV was similar in the groups with bentonite supplementation and statistically higher than the control group (0%). Besides the additive, there was a strong positive interaction with time, with an MCV significant increase in the 0.5%, 1%, and 2% groups. The mean RBC, MCV, and MCHC values in fish fed with bentonite indicated morphological adjustments where RBC reduction was compensated by the increase in the volume of this cell, which resulted in

more space for hemoglobin. Consequently, it did not harm the exchange of respiratory gases, a basic function of erythrocytes in the blood (**Tavares-Dias & Moraes**, 2010; **Ranzani-Paiva *et al.***, 2013). However, erythrocyte indices (MCV, MCH, MCHC) help in the differential diagnosis of anemia and indicated here a hypochromic macrocytic anemic process (**Ranzani-Paiva *et al.***, 2013). This also suggests cellular impairment as a result of RBC reduction after 20 days with bentonite in *C. macropomum* diets. No differences in MCH were observed ($p>0.05$) among the groups or between different feeding times. After 20 days of testing, the groups with bentonite inclusion in the diet showed the lowest MCHC values ($p<0.05$), resulting from the lower Hb concentration in these groups. This supports the findings for rainbow trout (*O. mykiss*) using bentonite and copper (Cu) in experimental diets (**Ucar *et al.***, 2019).

The addition of 1% and 2% bentonite in diets for tambaqui significantly increased the respiratory burst activity of leukocytes after 20 days (**Table 4**), which has been used as a marker of innate immunity in fish (**Biller-Takahashi *et al.***, 2013) and might be a mechanism for restoring homeostasis. During burst, pathogens are destroyed through phagocytosis, increasing intracellular oxygen consumption by leukocytes and producing reactive oxygen species (ROS), which result in free radicals and cellular metabolism byproducts (**Biller & Takahashi**, 2018; **Biller-Takahashi *et al.***, 2015; **Dong *et al.***, 2017). These byproducts play a crucial role in destroying invading agents. Thus, burst can act as defense against pathogens, but it also has a reactive effect on the animal's immune system, triggered in tambaqui by the higher bentonite levels in the diet.

Additionally, *C. macropomum* fed for 20 days with diets containing different levels of adsorbents showed a decrease in the total number of leukocytes, thrombocytes, lymphocytes, monocytes, and neutrophils in circulation, alongside an increase in respiratory burst activity (**Table 4**). These leukocyte count alterations may be related to stress or physiological damage caused by the use of clay supplementation in the diets compared to the control. It is important to note that leukocytes are essential for evaluating the fish immune system (**Witeska *et al.***, 2023; **Fazio**, 2019; **Tavares-Dias *et al.***, 2007), as they work together to maintain tissue integrity against infectious agents and are responsible for the fish immune balance migrating through the bloodstream to sites of injury or tissue infection during adverse inflammatory processes (**Ranzani-Paiva *et al.***, 2013; **Kiron**, 2012; **Fazio**, 2019).

The stress response in fish can be assessed through indicators such as serum or plasma glucose and cortisol and lysozyme activity, which change in response to stressful stimuli (**Urbinati *et al.***, 2020). Our results showed a significant decrease in plasma glucose levels in the fish after 20 days of cultivation compared to 10 days (**Table 5**). Such reduction is

Table 4. Total thrombocytes and total and differential leukocytes count in juvenile tambaqui (*Colossoma macropomum*) fed with experimental diets: Control (0%, no bentonite); b) 5 g of bentonite/kg of feed (0.5%); c) 10 g of bentonite/kg of feed (1%); d) 20 g of bentonite/kg of feed (2%) after 20 days

Parameters	0%	0.5%	1%	2%
Burst (OD)	0.25 ± 0.03^{b}	0.26 ± 0.03^{b}	0.29 ± 0.04^{a}	0.29 ± 0.03^{a}
Thrombocytes ($x10^3$ µL)	28.55 ± 12.80^{a}	19.02 ± 19.02^{b}	20.76 ± 6.56^{b}	20.61 ± 5.91^{b}
Leukocytes ($x10^3$ µL)	143.43 ± 41.55^{a}	108.14 ± 24.14^{b}	99.35 ± 14.11^{b}	112.95 ± 23.30^{b}
Lymphocytes ($x10^3$ µL)	63.68 ± 21.02^{a}	51.66 ± 14.69^{a}	53.64 ± 22.10^{a}	55.77 ± 13.34^{a}
Monocytes ($x10^3$ µL)	55.47 ± 16.23^{a}	39.73 ± 8.96^{b}	34.40 ± 9.31^{b}	42.215 ± 9.90^{b}
Neutrophils ($x10^3$ µL)	20.73 ± 7.74^{a}	13.57 ± 4.99^{b}	13.44 ± 3.11^{b}	13.25 ± 6.12^{b}
Eosinophils ($x10^3$µL)	0.53 ± 1.17^{a}	0.07 ± 0.01^{a}	0.17 ± 0.25^{a}	0.21 ± 0.32^{a}
LG-PAS ($x10^3$ µL)	3.05 ± 1.81^{a}	3.13 ± 1.41^{a}	2.35 ± 1.57^{a}	1.69 ± 1.32^{a}

Data expressed as mean±standard deviation. Letters mean significant differences ($p<0.05$). LG-PAS: Leukocyte granular-PAS positive

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Table 5. Plasma metabolite profile (n=15) of *Colossoma macropomum* fed with experimental diets: Control (0%, no bentonite); b) 5 g of bentonite/kg of feed (0.5%); c) 10 g of bentonite/kg of feed (1%); d) 20 g of bentonite/kg of feed (2%) for 10 and 20 days

Parameters	**Factors**			
	0%	**0.5%**	**1%**	**2%**
	10 days			
Glucose (mg dL^{-1})	71.48±15.48^{a}	82.55±14.53^{a}	77.25±17.63^{a}	78.00±19.76^{a}
Total protein (g dL^{-1})	3.10±0.37^{a}	3.26±0.21^{a}	3.29±0.23^{a}	3.24±0.26^{a}
Total Cholesterol (mg dL^{-1})	96.63±9.73^{b}	118.93±23.26^{a}	88.60±14.28^{b}	78.31±16.26^{b}
Triglycerides (mg dL^{-1})	147.52±24.59^{a}	151.57±16.36^{a}	136.63±22.05^{ab}	126.01±15.85^{b}
Albumin (g dL^{-1})	0.82±0.07^{cd}	0.44±0.13^{b}	1.04±0.11^{a}	0.93±0.10^{ad}
Globulin (g dL^{-1})	2.16 ± 0.36^{a}	2.38 ± 0.34^{a}	2.18 ± 0.34^{a}	2.30 ± 0.22^{a}
Parameters	**Factors**			
	0%	**0.5%**	**1%**	**2%**
	20 days			
Glucose (mg dL^{-1})	44.15 ± 11.34^{b*}	56.34 ± 7.57^{a*}	62.27 ± 7.80^{a*}	63.13 ± 10.88^{a*}
Total protein (g dL^{-1})	3.69 ± 0.30^{a*}	3.27 ± 0.25^{b}	3.40 ± 0.29^{b}	3.55 ± 0.41^{a*}
Total Cholesterol (mg dL^{-1})	85.65 ± 7.21^{b}	116.39 ± 18.06^{a}	128.13 ± 17.00^{a*}	100.29 ± 15.07^{b*}
Triglycerides (mg dL^{-1})	132.18 ± 19.81^{b}	158.88 ± 22.61^{a}	176.29 ± 17.64^{a*}	163.71 ± 32.68^{a*}
Albumin (g dL^{-1})	0.66 ± 0.06^{c*}	0.96 ± 0.20^{d*}	0.49 ± 0.08^{b*}	1.51 ± 0.18^{a*}
Globulin (g dL^{-1})	3.03 ± 0.30^{a*}	2,06 ± 0,61^{b*}	2,91 ± 0.33^{a*}	2.08 ± 0.40^{b}

Data expressed as mean±standard deviation. Letters mean significant differences in the ANOVA two-way test followed by post-hoc Tukey ($p<0.05$). *: significant difference ($p<0.05$) when comparing 10- and 20-day groups with the same treatment and parameter

often observed with immunostimulatory diets due to the reduction in stress effects (**Hoshino *et al.***, 2017; **Dias *et al.***, 2019). A likely explanation is that the diet may have stimulated insulin activity, which serves as a secondary stress (**Brandão *et al.***, 2006) indicator and results in reduced glucose levels. Furthermore, it may be related to energy metabolism regulation under the influence of clay (**Schell *et al.***, 1993; **Shannon *et al.***, 2017), which is known for its adsorbent and stabilizing properties (**Fazio**, 2019; **Kiron**, 2012).

Total protein concentration in fish reflects aspects of their physiology, including nutritional status, general health, stress level, humoral defense capacity, and well-being (**Abdel-Rahim *et al.***, 2023). After 20 days of feeding, the 0.5% and 1% bentonite groups presented lower total protein levels compared to the other treatments. According to **Chagas *et al.*** (2007), tambaqui specimens are considered well-nourished when plasma total protein levels are above 2.0 g dL^{-1}, which aligns with the present study and the findings of **Costa *et al***. (2022) and **Silva *et al.*** (2020).

Interactions between diet and time were observed with 1% and 2% bentonite, resulting in a significant increase in plasma lipid profile levels ($p<0.05$), which could be a crucial benefit in membrane structure; besides, lipids are precursors to all steroid hormones. Such interactions may be triggered by liver and kidney dysfunction, leading to elevated cholesterol levels in the bloodstream (**Öner *et al.***, 2008) due to the stress induced by bentonite in their diet. Another possible explanation for the increase in total cholesterol and triglycerides levels is related to the increase in feed viscosity, endogenous feed losses, and fermentation in fish intestines, among other causes (**Rosas *et al.***, 2008). **Jiang *et al.*** (2010) presented similar results with the inclusion of clays in piglets' diets with and without the mycotoxin zearalenone, which significantly increased plasma cholesterol levels.

Stress in fish triggers an increase in energy demand and mobilization of plasma proteins used to meet energy needs, maintain osmotic balance, and improve the immune response, besides reflecting liver and kidney health (**Javed & Usmani**, 2015; **Costa *et al.***, 2019). At 10 days, there was a variation in albumin levels between groups, with the 1% group presenting the highest value and the 0.5% group the lowest. At 20 days, the variation was even more evident. The 2% group had the highest albumin value, while the 1% group had the lowest. Regarding globulin, significant differences were observed only at 20 days. The 0% and 1% groups exhibited the highest globulin values, while the 0.5% and 2% groups had the lowest. Albumin and globulin are important proteins in the blood of fish: the first contributes to osmotic pressure and the transport of substances (**Mobarhan**, 1988), while globulin is related to the immune response (**De Souza *et al.***, 2020). The relationship between albumin and globulin can be a health indicator. In our study, there appeared to be a change in this relationship over time, especially in the groups treated with bentonite, suggesting a possible negative influence of bentonite on the fish protein metabolism. The albumin increase in the 1% (10 days) and 2% (20 days) groups can be attributed to the synthesis of proteins needed to meet the high energy demand since the synthesis occurs in the liver, besides indicating an increase in the tambaqui immune response (**Morante *et al.***, 2021).

Conclusion

Our study is the first to provide hemato-immunological analyses of tambaqui fed with sodium bentonite-supplemented diets. The findings highlight that the adsorbent supplementation did not improve the hematological-biochemical profile of this fish species. Physiological damage caused by the adsorbent, such as thrombocytopenia, leukopenia, lymphocytopenia, and monocytopenia, pointed to the response and migration of the cells to inflammatory sites. In conclusion, our results indicate that dietary supplementation with sodium bentonite had deleterious effects on the organism of the *C. macropomum* evaluated and, therefore, it is not recommended as a food additive for this fish species.

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Author contributions

Conceptualization, E.T.O.Y. and Y.I.C.F.; methodology, E.T.O.Y., Y.I.C.F., F.L.S.S., M.V.F.M., A.M.P., C.B.S.; software Y.I.C.F; validation Y.I.C.F. and E.T.O.Y; investigation Y.I.C.F., F.L.S.S. and E.T.O.Y.; resources Y.I.C.F., F.L.S.S., M.V.F.M., A.M.P., C.B.S., and E.T.O.Y.; original draft preparation Y.I.C.F. and E.T.O.Y.; manuscript review and editing Y.I.C.F., F.L.S.S., M.V.F.M., A.M.P., C.B.S., and E.T.O.Y. All authors read and agreed on the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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