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Hemato-biochemical and immunological responses of *Colossoma macropomum* (Serrasalmidae) induced by dietary supplementation with the clay mineral Attapulgite (Alphafeed®)

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ABSTRACT: Aquaculture has been consolidating itself as an essential alternative to meet the global demand for animal protein, highlighting the importance of nutrition in maintaining fish physiology and health. Among the feed additives studied, attapulgite has generated interest for its effects on hematological, immunological, and biochemical modulation, although its physiological impacts on tambaqui fish are not yet fully understood. This study evaluated the hematological and immunological effects of dietary supplementation with different concentrations of attapulgite on juvenile tambaqui (*Colossoma macropomum*). At 20 and 40 days, blood samples from five fish per replicate were analyzed. Changes in hematocrit, hemoglobin, erythrocytes, and hematometric indices were observed. The concentrations of glucose, total protein, cholesterol, triglycerides, and albumin varied significantly with the inclusion of the adsorbent, indicating adverse impacts on metabolism. The leukocyte profile showed immune stimulation, with increased thrombocytes, lymphocytes, monocytes, and LG-PAS. It is concluded that supplementation with 1% attapulgite is safer for juvenile tambaqui, as it positively modulated the

Keywords: Adsorbent; leukocytes; minerals; Tambaqui

Respostas hemato-bioquímicas e imunológicas de *Colossoma macropomum* (Serrasalmidae) induzidas pela suplementação dietética com argilomineral Atapulgita (Alphafeed®)

RESUMO: A aquicultura vem se consolidando como alternativa essencial para suprir a demanda global por proteína animal, destacando-se a importância da nutrição na manutenção da fisiologia e saúde dos peixes. Entre os aditivos alimentares estudados, a atapulgita tem despertado interesse por seus efeitos na modulação hematológica, imunológica e bioquímica, embora seus impactos fisiológicos em tambaquis ainda não estejam totalmente esclarecidos. Este estudo avaliou os efeitos hematológicos e imunológicos da suplementação dietética com diferentes concentrações de atapulgita em juvenis de tambaqui (*Colossoma macropomum*). Aos 20 e 40 dias, amostras de sangue de cinco peixes por repetição foram analisadas. Alterações em hematócrito, hemoglobina, eritrócitos e índices hematimétricos foram observadas. As concentrações de glicose, proteína total, colesterol, triglicerídeos e albumina variaram significativamente com a inclusão do adsorvente, indicando impactos adversos no metabolismo. O perfil leucocitário mostrou estimulação imunológica, com aumento de trombócitos, linfócitos, monócitos e LG-PAS. Conclui-se que a suplementação com 1% de atapulgita é mais segura para juvenis de tambaqui, pois modulou positivamente a resposta imune e reduziu impactos sobre o metabolismo.

Palavras-chave: Adsorvente; leucócitos; minerais; Tambaqui

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INTRODUCTION

Driven by global population growth, aquaculture has emerged as a key sector in addressing the rising demand for animal protein in human diets. Over the past five decades, global per capita fish consumption has nearly doubled, reaching levels comparable to those of poultry and pork based on edible weight (FAO, 2024; Naylor et al., 2021; Edwards et al., 2019). However, in intensive aquaculture systems, inadequate management practices, imbalanced nutrition, water quality degradation, and high stocking densities compromise animal homeostasis (Lembo et al., 2019), resulting in immunosuppression and increased susceptibility to pathogens.

In this context, recent studies have shown that proper nutrition plays a key role in maintaining fish health by modulating innate and adaptive immune responses, thereby enhancing their activity (Dias et al., 2019; Hoshino et al., 2020). Among the nutritional strategies used, feed additives such as clay minerals, which act as natural toxin adsorbents, are particularly notable. These compounds have been linked to improvements in immunity, growth performance, and feed efficiency (Arshad et al., 2021; Ucar et al., 2019; Farrag et al., 2009).

These positive effects are largely attributed to the absorption and ion exchange properties of clay minerals, which operate through physical and chemical mechanisms (Du et al., 2023). In general, clays can optimize nutrient absorption and utilization, primarily by modulating digestive functions (Du et al., 2023). Additionally, in the gastrointestinal tract, they can prolong the transit time of compounds due to their expansion by water absorption, while simultaneously adsorbing deleterious substances such as mycotoxins (Peng et al., 2018; Vila-Donat et al., 2018).

Some studies have reported positive effects of mineral use in aquaculture (Arshad et al., 2021; Ucar et al., 2019; Kanyilmaz & Tekellioğlu, 2016); however, critical qualitative and quantitative questions remain regarding the optimal mineral type, appropriate dose-response relationship, and targeted cultured species. Nevertheless, the efficacy of attapulgite as a feed additive in captive tambaquis (*Colossoma macropomum*) remains unexplored. To address this gap, the present study investigated the potential effects of dietary attapulgite inclusion 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) with an initial mean weight of 45.05 ± 6.46g were randomly assigned to 12 experimental tanks (100 L polypropylene water containers). The Attapulgite 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 attapulgite); b) 5 g of attapulgite/kg of feed (0.5%); c) 10 g of attapulgite/kg of feed (1%), and d) 20 g of attapulgite/kg of feed (2%). We collected 100 g of feed from each group, and the centesimal composition of the experimental diets was analyzed in triplicate. Table 1 shows the chemical composition of the experimental diets.

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

Parameters	0%	0.5%	1%	2%
Dry matter (%)	92,43 ± 0,85 [a]	92,09 ± 0,19 [a]	91,90 ± 0,12 [a]	91,64 ± 0,14 [a]
Crude protein (%)	38,61 ± 1,54 [a]	39,71 ± 1,79 [a]	40,54 ± 1,49 [a]	36,10 ± 1,22 [b]
Ether extract (%)	7,09 ± 0,23 [a]	7,01 ± 0,26 [a]	7,00 ± 0,01 [a]	7,10 ± 0,16 [a]
Ash (%)	12,23 ± 0,03b [c]	12,62 ± 0,21 [bc]	13,09 ± 0,09 [b]	13,86 ± 0,19 [a]
Calcium (%)	0,45 ± 0,01 [a]	0,26 ± 0,04 [b]	0,26 ± 0,04 [b]	0,23 ± 0,05 [b]
Phosphorus (%)	1,51 ± 0,20 [a]	1,49 ± 0,09 [a]	1,43 ± 0,00 [a]	1,44 ± 0,01 [a]
Copper (mg kg^{-1})	384,21 ± 17,59 [b]	537,40 ± 7,14 [b]	778,96 ± 58,74 [b]	997,20 ± 46,95 [a]
Zinc (mg kg^{-1})	276,73 ± 4,53 [a]	279,53 ± 3,39 [a]	275,73 ± 3,02 [a]	282,52 ± 3,70 [a]
Iron (mg kg^{-1})	13,89 ± 0,33 [a]	13,63 ± 0,75 [a]	14,23 ± 0,19 [a]	14,69 ± 1,21 [a]
Manganese (mg kg^{-1})	42,56 ± 0,52 [b]	49,79 ± 0,76 [b]	63,77 ± 4,98 [b]	74,2,12 [a]

Data expressed as mean±standard deviation. Letters indicate significant differences according to Tukey's test (p<0.05)

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. Attapulgite was incorporated into the feed by spraying with distilled water for fixation. 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, 6.95 ± 0.57 mg L^{-1}; temperature, 29.45 ± 0.15°C, and pH, 7.18 ± 0.93, showing that water quality in experimental tanks was adequate for tambaqui.

Growth parameters

After the feeding period (20 and 40 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 106.μL^{-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 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). The eosinophil count showed a non-normal distribution; therefore, the Kruskal-Wallis test was applied, followed by Dunn's post-hoc test. 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

After 20 and 40 days of feeding *C. macropomum* diets supplemented with varying concentrations of attapulgite, no significant differences ($p > 0.05$) were found in growth parameters or the hepatosomatic index (HSI) (Table 2).

Table 2 - Growth performance, survival, and hepatosomatic index (HSI) de *Colossoma macropomum* fed with diets containing different concentrations of attapulgite (Alphafeed®) after 40 days.

Parameters	0 %	0.5%	1%	2%
Initial weight (g)	45,05 ± 6,46^a	46,01 ± 6,73^a	44,18 ± 4,88^a	47,08 ± 6,86^a
Final weight (g)	68,93 ± 9,01^a	66,70 ± 9,11^a	69,48 ± 8,36^a	66,62 ± 7,56^a
Initial length (cm)	11,16 ± 0,61^a	11,42 ± 0,56^a	11,03 ± 0,63^a	11,18 ± 0,64^a
Final length (cm)	12,58 ± 0,73^a	12,59 ± 1,16^a	12,66 ± 0,67^a	12,67 ± 0,58^a
Fish survival (%)	100	100	100	100
HSI (%)	1,73 ± 0,40^a	1,70 ± 0,24^a	1,54 ± 0,17^a	1,59 ± 0,07^a

Data expressed as mean±standard deviation. Letters indicate significant differences according to Tukey's test (p<0.05)

Dietary inclusion of attapulgite for 20 and 40 days induced several physiological changes in *C. macropomum*, particularly in hematological and biochemical responses; however, it did not enhance growth performance or affect liver function. These findings suggest that incorporating up to 2% attapulgite in the diet does not directly impact the growth of juvenile *C. macropomum* over 40 days, nor does it interfere with hepatic energy reserve allocation. This outcome may be related to the use of high-quality dietary ingredients and controlled rearing conditions.

Moreover, the use of attapulgite as a dietary additive in fish warrants careful consideration, as this is the first study to evaluate its inclusion in diets for tambaqui, a native Brazilian species. To date, most research on attapulgite has focused on its application in poultry nutrition, particularly for reducing aflatoxin levels in feed due to its adsorptive properties (Sánchez et al., 2012; Plasencia, 2004; Márquez & De Hernandez, 1995).

On the other hand, blood parameters were affected either by the inclusion of attapulgite in the diets or by its interaction with time throughout the experimental period (Table 3). Hematocrit (Ht) levels decreased significantly in fish fed diets supplemented with 1% attapulgite at 20 days, and with 0.5%, 1%, and 2% inclusion levels at 40 days. Fish fed diets containing 1% and 2% attapulgite showed a reduction in hemoglobin (Hb) concentration compared to the other groups. The erythrocyte count was significantly higher ($p < 0.05$) in the 0.5% attapulgite group at both 20 and 40 days. Mean corpuscular volume (MCV) values were lower ($p < 0.05$) at 40 days in groups fed 0.5%, 1%, and 2% attapulgite, with only the 0.5% group showing a reduction at 20 days. A decrease in mean corpuscular hemoglobin (MCH) was noted in fish fed 2% attapulgite after 20 days and in those fed 0.5% after 40 days. Time also had a significant effect ($p < 0.05$), causing an increase in MCH at 40 days. Mean corpuscular hemoglobin concentration (MCHC) increased significantly ($p < 0.05$) in the 0.5% group at 20 days and in the 0.5%, 1%, and 2% groups at 40 days.

Table 3 - Hematological variables and hematimetric indices (mean ± standard deviation) of *Colossoma macropomum* fed with diets containing different concentrations of attapulgite (Alphafeed®) after 20 and 40 days.

Parameters	20 days			
	0 %	0.5%	1%	2%
Ht (%)	27.67 ± 2.78 [a]	25.23 ± 3.59 [a]	23.53 ± 4.50 [b]	27.30 ± 3.61 [a]
Hb (g dL^{-1})	7.15 ± 0.77 [ab]	7.96 ± 1.46 [a]	6.33 ± 0.93 [b]	6.23 ± 0.91 [b]
RBC (x 10^6 μL^{-1})	1.22 ± 0.18 [b]	1.36 ± 0.12 [a]	1.23 ± 0.13 [b]	1.30 ± 0.15 [ab]
MCV (fL)	231.51 ± 46.45 [a]	187.49 ± 37.94 [b]	194.03 ± 45.90 [a]	210.95 ± 26.28 [a]
MCH (g dL^{-1})	59.72 ± 11.64 [a]	59.28 ±14.32 [a]	52.09 ± 9.51 [a]	48.52 ± 8.80 [b]
MCHC (g dL^{-1})	25.31 ± 2.47 [b]	31.67 ± 4.71 [a]	24.43 ± 1.76 [b]	23.12 ± 3.83 [b]
Parameters	**40 days**			
	0%	**0.5%**	**1%**	**2%**
Ht (%)	34,00 ± 2,23 [a]*	24,21 ± 5,02 [b]	24,90 ± 2,59 [b]	23,60 ± 3,68 [b]*
Hb (g dL^{-1})	8,50 ± 0,95 [a]*	8,36 ± 0,94 [a]	7,79 ± 1,00 [a]*	7,81 ± 0,92 [a]*
RBC (x 10^6 μL^{-1})	1,29 ± 0,13 [bc]	1,50 ± 0,11 [a]*	1,13 ± 0,12 [bc]	1,24 ± 0,11 [b]
MCV (fL)	265,14 ± 36,14 [a]*	162,39 ± 38,09 [bc]	220,88 ± 31,54 [bc]	191,74 ± 36,30 [b]
MCH (g dL^{-1})	66,35 ± 11,06 [a]	57,54 ± 9,65 [b]	69,17 ± 10,85 [a]*	63,22 ± 8,55 [a]*
MCHC (g dL^{-1})	25,16 ± 3,63 [b]	33,34 ± 3,58 [a]	31,59 ± 4,98 [a]*	32,50 ± 3,26 [a]*

Ht: Hematocrit; Hb: Hemoglobin concentration; RBC: Red blood cells count; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration. Letters indicate significant differences in two-way ANOVA followed by post -hoc Tukey ($p<0.05$); *: significant difference ($p<0.05$) when comparing 20- and 40-day-period groups using the same treatment and for the same parameter.

Hematological analysis showed that dietary inclusion of attapulgite in *C. macropomum* affects oxygen transport and blood cell integrity in a dose-dependent manner (Aride et al., 2017). The lowest Hb values were observed in fish fed diets containing attapulgite for 20 days, and reduced Ht values were recorded after 40 days of feeding with the clay, indicating a less favorable physiological condition compared to fish not receiving the adsorbent (Costa et al., 2022; Centeno et al., 2007). These reductions may be associated with the lower crude protein and calcium content in the 2% attapulgite diet, which could have impaired erythropoiesis, since hemoglobin and erythrocyte synthesis depend on the availability of amino acids and minerals such as iron and calcium (Kulkeaw & Sugiyama, 2012; De Souza & Bonilla-Rodriguez, 2007).

An increased erythrocyte count was observed in fish fed diets supplemented with 0.5% attapulgite after 20 and 40 days. This may represent a physiological adaptation aimed at maintaining adequate oxygen levels, possibly through stimulation of hematopoietic organs, thereby increasing circulating erythrocyte numbers and enhancing oxygen and metabolic transport (Kordrostami et al., 2023; Dias et al., 2018). Conversely, Palm et al. (2022) reported that the African catfish (*Clarias gariepinus*) did not exhibit increased erythrocyte or leukocyte counts when fed diets containing clay and a mineral mix (montmorillonite, muscovite, kaolinite, quartz, and others) with properties similar to attapulgite.

The hematological indices MCV, MCHC, and MCH indicated that the inclusion of attapulgite influenced erythrocyte size and hemoglobin concentration within these cells, suggesting that the adsorbent may interfere with the absorption of essential nutrients and trigger compensatory physiological and morphological adjustments to maintain oxygen levels (Ranzani-Paiva et al. 2013; Tavares-Dias & Moraes 2010).

El-Dahhar et al. (2024) observed increased MCHC and MCH values with higher concentrations of bentonite for the European seabass (*Dicentrarchus labrax*). Conversely, Furtado et al. (2025) and Ucar et al. (2019) reported reductions in MCHC values in *C. macropomum* and the rainbow trout (*Oncorhynchus mykiss*), respectively, suggesting that clays interact with cellular metabolism under different conditions (Ranzani-Paiva et al. 2013; Tavares-Dias & Moraes 2010).

In the present study, fish fed diets containing 2% attapulgite for 20 days and 0.5% for 40 days showed a significant reduction in MCH values, which may reflect a lower mean hemoglobin content per erythrocyte. This alteration may be associated with an adaptation to the dietary challenge, potentially compromising oxygen transport efficiency, although without apparent impairment of erythrocyte respiratory function.

The immune response varied according to the concentration of the clay mineral after 20 and 40 days of feeding (Table 4). A significant increase ($p < 0.05$) in thrombocyte count was observed at 20 days in fish fed the diet containing 2% attapulgite, and at 40 days in those receiving diets with 0.5% and 1% attapulgite. Total leukocyte counts were significantly higher ($p < 0.05$) in fish fed diets containing 0.5% attapulgite after 40 days. Lymphocyte counts were significantly elevated ($p < 0.05$) in all attapulgite-supplemented groups (0.5%, 1.0%, and 2%) after 20 days of feeding. However, after 40 days, only the group receiving 0.5% attapulgite maintained significantly higher lymphocyte number. Monocyte counts were significantly higher ($p < 0.05$) in all attapulgite-treated groups at both 20 and 40 days. Neutrophil counts were significantly reduced ($p < 0.05$) only at 40 days in fish fed the 2% attapulgite diet, while eosinophil counts were significantly lower ($p < 0.05$) in the group fed 1% attapulgite. Leukocyte respiratory burst activity increased ($p < 0.05$) at 20 days in all groups receiving the adsorbent. Although elevated levels persisted at 40 days, no significant differences were observed among the groups at that time.

Table 4 - Total thrombocytes and total and differential leukocytes count in juvenile tambaqui *C. macropomum* fed with diets containing different concentrations of attapulgite (Alphafeed®) after 20 and 40 days.

Parameters	20 days			
	0 %	0.5%	1%	2%
Thrombocytes (x10^3 µL)	50.40 ± 7.50^{bc}	56.42 ± 5.24^{ac}	41.66 ± 4.81^{b}	61.46 ± 7.19^{a}
Leukocytes (x10^3 µL)	125.08 ± 19.41^{a}	139.88 ± 11.85^{a}	128.19 ± 13.90^{a}	136.98 ± 16.16^{a}
Lymphocytes (x10^3 µL)	51.77 ± 8.91^{b}	65.44 ± 10.46^{a}	63.84 ± 6.94^{a}	69.52 ± 9.55^{a}
Monocytes (x10^3 µL)	37.48 ± 6.87^{b}	44.50 ± 8.62^{a}	41.19 ± 7.98^{a}	45.99 ± 5.07^{a}
Neutrophils (x10^3 µL)	22.25 ± 5.24^{a}	23.66 ± 6.61^{a}	19.20 ± 3.02^{a}	21.40 ± 2.15^{a}
Eosinophils (x10^{3}µL)	2.43 ± 0.65^{a}	2.73 ± 0.62^{a}	2.47 ± 0.68 ^{a}	2.61 ± 0.73^{a}
LG- PAS (x10^{3}µL)	9.93 ± 1.45^{a}	10.79 ± 1.50^{a}	10.07 ± 1.16^{a}	10.82 ± 1.64^{a}
Burst (OD)	0,56 ± 0.08^{b}	0.77 ± 0.06^{a}	0.73 ± 0.08^{a}	0.72 ± 0.12^{a}
Parameters	**40 days**			
	0 %	**0.5%**	**1%**	**2%**
Thrombocytes (x10^3 µL)	50,49 ± 10,47^{c}	85,68 ± 10,69^{a*}	81,22 ± 16,26ª*	53,74 ± 6,50^{b*}
Leukocytes (x10^3 µL)	132,32 ± 13,89^{d}	153,27 ± 11,92ª	115,73 ± 12,27^{b*}	125,35 ± 12,00^{c*}
Lymphocytes (x10^3 µL)	51,84 ± 8,22^{b}	64,36 ± 12,60^{a}	52,19 ± 10,94^{b*}	53,58 ± 8,16^{b*}
Monocytes (x10^3 µL)	40,01 ± 10,66^{a}	34,20 ± 11,43ª*	27,73 ± 5,96^{b*}	35,58 ± 11,52ª
Neutrophils (x10^3 µL)	11,16 ± 3,23^{a}	14,19 ± 4,27^{a}	11,40 ± 3,74^{a}	10,07 ± 3,01^{b}
Eosinophils (x10^{3}µL)	1,73 ± 0,65^{a}	3,06 ± 2,53^{a}	1,27 ± 0,38^{b}	3,07 ± 1,90^{a}
LG- PAS (x10^{3}µL)	9,85 ± 3,1^{b}	18,26 ± 7,69^{a}	10,54 ± 4,59^{b}	11,33 ± 3,95^{b}
Burst (OD)	0,76 ± 0,10^{a}	0,77 ± 0,12^{a}	0,83 ± 0,11^{a}	0,82 ± 0,14^{a}

Data expressed as mean±standard deviation. Letters mean significant differences (p<0.05). LG-PAS: Leukocyte granular-PAS positive. Letters indicate significant differences in two-way ANOVA followed by post-hoc Tukey (p<0.05); *: significant difference (p<0.05) when comparing 20- and 40-day-period groups using the same treatment and for the same parameter

The leukocyte profile suggests that fish fed with atapulgite experienced immune stimulation triggered by the adsorbent. This response aligns with the findings of El-Dahhar et al. (2024), who reported that dietary supplementation with bentonite clay led to a moderate increase in leukocyte number in *D. labrax*. Clay minerals have the ability to interact with dietary and gastrointestinal compounds, potentially modulating the microbiota or interacting with antigenic elements (Damato et al., 2022). Moreover, the increase in leukocyte levels may reflect a mild inflammatory response

(Ranzani-Paiva et al., 2013), possibly due to greater exposure to the adsorbent. This hypothesis is further supported by the observed increase in the number of thrombocytes, lymphocytes, monocytes, and LG-PAS cells in *C. macropomum* fed diets supplemented with attapulgite. The burst activity, increased significantly at 20 days and remained high for an additional 20 days, indicating activation of phagocytic leukocytes, while hematological parameters and phagocytic capacity showed no significant differences between groups with or without adsorbent supplementation (Costa et al., 2022; Aride et al., 2017; Centeno et al., 2007).

Biochemical markers were affected by the inclusion of attapulgite at different dietary concentrations in fish (Table 4). Plasma glucose levels increased significantly ($p < 0.05$) in the 0.5% and 1% groups after 20 days, and in the 2% group after 40 days, which showed the highest mean among all treatments. In contrast, total protein concentrations were significantly lower in fish fed diets containing 0% and 2% attapulgite at both time points. Total cholesterol levels decreased ($p < 0.05$) in all groups receiving dietary attapulgite after 20 days, regardless of the inclusion level. However, at 40 days, cholesterol levels were similar across all groups but significantly higher than those observed at 20 days, with elevated values in all attapulgite-supplemented groups. Triglyceride concentrations were significantly reduced in the 2% group at 20 days, while at 40 days, significant increases ($p < 0.05$) were observed in the 0.5%, 1%, and 2% groups. The control group had the lowest plasma albumin levels ($p < 0.05$) at 20 days. In contrast, after 40 days, this pattern was reversed: the control group had significantly higher albumin levels compared to all attapulgite-fed groups, whose values were also lower than those recorded at 20 days .

Table 5 – Plasma metabolite profile of *C. macropomum* fed with diets containing different concentrations of attapulgite (Alphafeed®) after 20 and 40 days

Parameters	20 days			
	0 %	0.5%	1%	2%
Glucose (mg dL^{-1})	71.03 ± 14.16^{ab}	81.67 ± 16.86 ^{a}	87.72 ± 11.29 ^{a}	51.20 ± 9.39 ^{b}
Total protein (g dL^{-1})	1.50 ± 0.60^{b}	2.79 ± 0.39 ^{a}	2.62 ± 0.39 ^{a}	1.34 ± 0.45 ^{b}
Total Cholesterol (mg dL^{-1})	128.82 ± 33.36^{a}	72.13 ± 11.77 ^{b}	65.75 ± 13.08 ^{b}	93.33 ± 25.17 ^{b}
Triglycerides (mg dL^{-1})	175.95 ± 26.73^{a}	159.73 ± 33.33 ^{a}	149.85 ± 33.52 ^{a}	69.63 ± 24.56 ^{b}
Albumin (g dL^{-1})	0.96 ± 0.19^{b}	1.67 ± 0.16 ^{a}	1.59 ± 0.22 ^{a}	1.46 ± 0.27 ^{a}
Parameters	**40 days**			
	0 %	**0.5%**	**1%**	**2%**
Glucose (mg dL^{-1})	72,04 ± 7,85 ^{b}	80,13 ± 11,28 ^{b}	77,42 ± 8,08 ^{b}	102,15 ± 10,99 ^{a*}
Total protein (g dL^{-1})	1,64 ± 0,18 ^{b}	2,39 ± 0,26 ^{a*}	2,21 ± 0,22 ^{a*}	1,39 ± 0,09 ^{b}
Total Cholesterol (mg dL^{-1})	227,30 ± 11,33 ^{a*}	233,63 ± 24,28 ^{a*}	232,16 ± 21,29 ^{a*}	230,83 ± 29,92 ^{a*}
Triglycerides (mg dL^{-1})	191,28 ± 20,40 ^{b}	237,68 ± 40,11 ^{a*}	217,72 ± 31,86 ^{a*}	251,69 ± 55,02 ^{a*}
Albumin (g dL^{-1})	1,52 ± 0,14 ^{a*}	0,78 ± 0,07 ^{b*}	0,61 ± 0,03 ^{b*}	0,89 ± 0,08 ^{b*}

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

Attapulgite is a fibrous clay composed of aluminum and magnesium silicates, known for its adsorptive and stabilizing properties. The hyperglycemia observed in fish fed diets containing 0.5% and 1% attapulgite after 20 days, and 2% after 40 days, may be related to stress levels likely induced by the presence of the adsorbent in the feed. Due to its high adsorption capacity, attapulgite may interfere with the absorption of nutrients such as amino acids and minerals, triggering a stress response characterized by cortisol release and elevated plasma glucose levels via hepatic gluconeogenesis (Diniz & Honorato, 2012).

The increase in glucose levels, possibly induced by the stress response, may activate compensatory metabolic pathways such as glycogenesis to store the excess glucose as hepatic glycogen, or direct this glucose toward lipogenesis, resulting in triglyceride formation (Wang et al., 2017). The presence of elevated copper and manganese levels in the diet containing 2% attapulgite may have contributed to the triglyceride reduction observed at 20 days, possibly due to oxidative stress and lipogenesis inhibition, as suggested by Zhao et al. (2022). However, at 40 days, triglyceride levels increased significantly, surpassing control values, indicating not a metabolic balance but rather a possible lipid dysfunction induced by prolonged exposure to the adsorbent. These findings are consistent with those of Kayiira et al. (2024), who reported lipid accumulation and hepatic alterations in fish fed diets supplemented with copper.

The increase in glucose and decrease in albumin levels observed in *C. macropomum* after 40 days of feeding attapulgite-supplemented diets indicate systemic metabolic alterations that may be associated with elevated stress levels. This stress response can trigger the release of catecholamines and cortisol, modulating energy metabolism by mobilizing lipid reserves into the bloodstream, resulting in higher cholesterol and triglyceride levels (Silva et al., 2012; Barton & Iwama, 1991).

The reduction in total protein levels in fish fed diets containing 2% attapulgite suggests that high concentrations of the adsorbent may impair the absorption of essential nutrients. A similar finding was reported by El-Naby et al. (2025) in tilapia (*Oreochromis niloticus*) supplemented with biocide clay, attributing this effect to the adsorptive capacity of the clay mineral, which interferes with amino acid availability. These findings support the hypothesis that excessive use of adsorbents can negatively impact fish protein metabolism.

CONCLUSIONS

This study is the first to provide hematoimmunological analyses in *C. macropomum* fed diets supplemented with attapulgite. The results show that supplementation with 0.5% of the clay mineral for up to 40 days stimulates the immune system, increases plasma protein levels and MCHC values, and does not compromise biochemical homeostasis. However, concentrations above 1% over prolonged periods may cause adverse hematological and metabolic alterations, such as hypoproteinemia and hypoalbuminemia. Further research is needed to evaluate the effects of 0.5% inclusion over longer cultivation periods and under stress conditions, such as exposure to mycotoxins in the diet.

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COMPLIANCE WITH ETHICAL STANDARDS

Authors’ contributions: Conceptualization: ETOY, YICF; Data Curation: YICF; Formal Analysis: YICF; Funding Acquisition: ETOY; Investigation: ETOY, YICF, MVFM, AMP, GSA; Methodology: ETOY, YICF, MVFM., AMP, GSA.; Project Administration: ETOY, YICF; Resources: ETOY; Software: YICF; Supervision: ETOY; Validation: ETOY, YICF; Visualization: YICF; Writing – original draft: YICF; ETOY; Writing – review & editing: ETOY, YICF, MVFM, AMP, GSA.

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