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Inulin dietary supplementation attenuates the stress induced by pursuit/capture/atmospheric exposure and improves innate immune response in hybrid catfish (*Pseudoplatystoma reticulatum*♀ × *Leiarius marmoratus*♂) after exposure to *Aeromonas hydrophila*

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Abstract

The aim of the study was to evaluate the effects of inulin on stress and innate immunity of hybrid catfish (*P. reticulatum* × *L. marmoratus*). A total of 208 juvenile surubim, with initial average weight and length 37.91 ± 5.58 g and 18.51 ± 0.69 cm, were randomly distributed in 16 tanks (100 L) in a 4 × 5 factorial scheme. Inulin was used at four inclusion levels (0% — control, 0.25%, 0.50%, and 0.75%) for 31 days. At the end of period, blood aliquots were collected to characterize time zero. Then, the fish were exposed to pursuit/capture/atmospheric exposure stress for a new blood collection after 0.5 h. Soon after, the fish were exposed to *A. hydrophila*, and new blood samples were obtained at 3, 6, and 24 h post-challenge. There was a significant interaction on plasma cortisol in 0.50% inulin group. There was a significant reduction in hemoglobin and hematocrit at 24 and 3 h, respectively, after stress management + bacterial challenge. For glucose, a significant increase was observed after stress management (0.5 h) as well as a significant decrease at 6 h after stress management + bacterial challenge. A significant increase in total leukocytes and lymphocytes was observed at 6 h, while thrombocytes increased significantly at 6 and 24 h. No significant interaction was observed in leukocyte respiratory activity. Fish supplemented with 0.50% inulin showed a significant increase in serum lysozyme. The inclusions of inulin at 0.25% and 0.75% provide greater hormone cortisol homeostasis, while inulin at 0.50% improved the immune system of hybrid surubim.

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Highlights

- Inulin at 0.50% significantly increases serum lysozyme in hybrid catfish *Pseudoplatystoma reticulatum*♀ × *Leiarius marmoratus*♂.
- Inulin supplementation reduced the impact of chase/capture/aerial exposure stress.
- The inulin dietary supplementation at 0.25 and 0.75% provides greater stability of the hormone cortisol.
- Supplementation at 0.50% inulin improves the immune system of surubim.

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Introduction

Brazil stands out as one of the largest aquaculture producers in the world (FAO 2022), producing approximately 860 thousand tons of farmed fish in 2022 (Peixe BR 2023). Although Tilapia production is dominant in Brazilian fish farming (63.93% of the total volume), other Brazilian native fish species stand out in the aquaculture scenario, such as catfish of the genus *Pseudoplatystoma* and its hybrids, popularly known as surubim (Peixe BR 2023). In recent years, joint actions for scientific and technological development have enabled improvements in the commercial production of Neotropical species (Valenti et al. 2021), promoting gains in productivity and competitiveness of native Brazilian species in the national and international market (Peixe BR 2023).

The surubim reached the third position in Brazilian fish farming exports in 2022, with a growth of 186% in the year, being the highest percentage of growth among the native species of Brazil (Peixe BR 2023). Surubim, which include fish of the genera *Pseudoplatystoma*, *Phractocephalus*, and *Leiarius* (Hashimoto et al. 2012), are commercially valued fish, with the absence of intramuscular spines, white meat with a mild flavor, low fat content, and high carcass yield (Silva et al. 2015). Its total production in Brazil was approximately 11,571 tons, in 2019 (IBGE 2020). The most farmed siluriformes in South American aquaculture are surubim Cachara *Pseudoplatystoma reticulatum*, Pintado *Pseudoplatystoma corruscans*, and their hybrids (Fantini-Hoag et al. 2022). Another cultivated and economically important hybrid surubim is generated by crossing *Pseudoplatystoma reticulatum*♀ × *Leiarius marmoratus*♂.

Fish farming, including the cultivation of hybrid surubim (*P. reticulatum* × *L. marmoratus*) (Matos and Meurer 2021), faces many obstacles related to sanitary problems that most often result in bacterial outbreaks that can cause considerable economic losses (Maldonado-Miranda et al. 2022), mainly due to bacterial infections caused by *Aeromonas* spp. (Tavares-Dias and Martins 2017). Sustainable practices such as the use of phytotherapics, prebiotics, and probiotics are becoming popular in the international community, as they are seen as alternatives to the indiscriminate use of antibiotics capable of reducing such economic losses and preventing the spread of bacterial resistance in aquaculture environments (Mouriño et al. 2012; Brum et al. 2017; Vanderzwalmen et al. 2019; Butt et al. 2021; Tadese et al. 2022; Yilmaz et al. 2022; Rohani et al. 2022).

Among the prebiotics commonly used in aquaculture are gluco-oligosaccharides, mannan-oligosaccharides, and fructooligosaccharides (Ringø et al. 2010). A fructooligosaccharide that has gained prominence in aquaculture is inulin (Cerezuela et al. 2012; Song et al. 2014; Carbone and Faggio 2016; Herrera et al. 2019; Campos et al. 2022; Li et al. 2023), which occurs naturally in foods of plant origin and some plant varieties (Cerezuela et al. 2008), such as onion, garlic, burdock, chicory, and wheat (Niness 1999).

Inulin is a polysaccharide with a prebiotic reputation. It is a non-digestible food component (fiber) that acts as a microbial substrate, stimulating the proliferation of beneficial gut bacteria, increasing intestinal absorption, and providing physiological improvements that help strengthen innate immunity in the host (Niness 1999; Kaur and Gupta 2002).

In humans, a diet enriched with inulin fructans combined with probiotics can increase the biodiversity and richness of the intestinal microbiota, reduce symptoms of infectious diseases, stimulate the intestinal immune system, and modulate the response of the respiratory tract's immune system (De Giani et al. 2022). In addition, in tests with human models, it

was verified that in the bacterial fermentative catabolism of inulin fructans, short-chain fatty acids, which are fundamental biological molecules, are produced; however, negative effects related to inulin consumption have also been described, such as gastrointestinal symptoms in humans and exacerbated intestinal inflammation in mice (Tawfick et al. 2022). In broiler chickens, administration of inulin has been shown to be effective in generating potent stimulation of gene expression in the spleen and cecal tonsils (Dunislawska et al. 2021).

In aquaculture, soybean meal supplemented with inulin and oligofructose was effective to partially replace fish meal (up to 50%) in the pikeperch (*Sander lucioperca*) diet without impairing the growth performance or the immune system (Dadras et al. 2022). In common carp (*Cyprinus carpio*), dietary inulin has been shown to be beneficial to improve growth performance, immune systems, and innate antioxidants and promote biochemical parameters and digestion (Ajdari et al. 2022), while in Nile tilapia (*Oreochromis niloticus*), inulin supplementation at 0.4% was considered a promising strategy to improve fish healthy under saline stress at 16‰ (Zhou et al. 2020). In recent years, inulin has gained prominence in Brazilian aquaculture research due to its positive effects on the physiology of native fish (Mouriño et al. 2015; Campos et al. 2022; Oliveira et al. 2022).

Research in the field of immunology of marine (Cerezuela et al. 2008, 2012; Ahmdifar et al. 2011) and freshwater fish (Mouriño et al. 2015; Tiengtam et al. 2015; Campos et al. 2022) has shown that inulin supplementation improves the immune system by using selective interruption of the growth of pathogenic microorganisms and stimulating the proliferation of macrophages. However, there are no research data related to the effects of inulin-dietary supplementation on fish production factors, such as fish capture followed by air exposure and pathogen exposure.

Here, in the present study, we evaluated in an unprecedented way the effects of inulin dietary supplementation on the innate immune system of the hybrid surubim (*P. reticulatum*×*L. marmoratus*) before and after the physiological stress caused by the management of pursuit, capture, and atmospheric exposure, followed by exposure to *Aeromonas hydrophila*.

Material and methods

Experimental design

All procedures that involved the use of fish in this study were performed according to ethical principles in animal experimentation and approved by the Ethics Committee on the Use of Animals (CEUA) of the State University of Mato Grosso do Sul — UEMS, in Aquidauana, MS, Brazil, under protocol nº 14/2013. In all procedures for obtaining biological samples, fish were anesthetized by immersion in a eugenol solution at 50 mg L^{-1} according to De Oliveira et al. (2019). The fish came from a local fish farm and remained in a 7-day acclimatization period.

The experiment was carried out in the fish farming sector of the State University of Mato Grosso do Sul, at the University Unit of Aquidauana — MS, for 31 days. Two hundred and eight juveniles surubim, with average initial weight of 37.91 ± 5.58 g and length 18.51 ± 0.69 cm, were randomly distributed in 16 tanks of 100 L (13 fish per tank) with continuous water flow. The fish were fed twice daily with the experimental diets until apparent satiation. A completely randomized design in a 4×5 factorial scheme was structured, corresponding to the levels of inclusion of prebiotics in the diet and the periods of sample collection.

The experimental diet consisted of a commercial diet (maximum moisture content of 12%, digestible energy 3500 kcal kg^{-1}, vitamin C 350 mg kg^{-1}, crude fat 8%, and minimum percentages of 40% crude protein, 3.5% calcium, and 1.5% phosphorus) with four inclusion levels of inulin (INUFLORA®) 0.0% (control), 0.25%, 0.5%, and 0.75%, with four replications for each treatment. The different levels of inulin were added and homogenized with the aid of a mixer (concrete mixer type) to the commercial feed and stored under refrigeration at 5 °C until use.

Water quality was evaluated daily (7:00 am and 6:00 pm) before feeding, with multiparameter HANNA. During the experimental period, the variables remained in dissolved oxygen 5.21 ± 0.59 mg L^{-1}, temperature 25.90 ± 0.91 °C, and pH 7.18 ± 0.32. Total ammonia and nitrite were not detectable.

Hemato-immunological analysis, bacterial challenge, and stress parameters

At the end of the dietary supplementation period, blood aliquots from 2 fish per experimental unit ($n = 8$ per treatment) were collected by puncture of the caudal vessel with syringes emulsified with anticoagulant and antiglycolytic (3% EDTA, Heparin, and Glistab). Initially, a baseline blood collection was performed, characterizing moment zero (initial sampling). Then, the fish were subjected to physiological stress management (pursuit/capture/aerial exposure) using a net for pursuit and capture (for 1 min), followed by atmospheric exposure (for 1 min). Thirty minutes after the physiological stress, the blood was collected again, and then the fish were exposed to *A. hydrophila*.

The bacterial challenge consisted of intraperitoneal inoculation of *A. hydrophila* (0.8×10^6 colony-forming unit — CFU) according to Rodrigues et al. (2021) after stress induction by pursuit/capture and aerial exposure. The bacterial suspension was predetermined with a subclinical dose to stimulate the immune system of the fish. Blood samples from 2 fish per experimental unit ($n = 8$ per treatment per collection time) were collected at 3, 6, and 24 h after exposure to the pathogen. With the blood samples, hematological, physiological, and immunological indicators were determined.

An aliquot of blood collected with EDTA (3%) was used to determine hematocrit (Ht) using the microhematocrit method by Goldenfarb et al. (1971), hemoglobin by cyanmethemoglobin method according to Collier (1944), and counting the number of erythrocytes in a Neubauer chamber. With the values of hematocrit, hemoglobin, and number of erythrocytes according to Wintrobe (1934), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated. Blood smears were made in duplicate and stained with May Grünwald-Giemsa-Wright (Tavares-Dias and Moraes 2004) for differential leukocyte count and total thrombocyte and leukocyte count.

The analysis of the respiratory burst activity of blood leukocytes was performed by means of reactive oxygen species (ROS) using the nitroblue tetrazolium dye (NBT). For this purpose, 100 µL of heparinized blood was added to 100 µL of nitroblue tetrazolium (NBT), homogenized, and incubated at 25 °C for 30 min. After the incubation period, 50 µL of the mixture was diluted in 1000 µL of N, N-Dimethylformamide (DMF) and centrifuged at 3000 *g* for 5 min, with the supernatant reading at optical density by spectrophotometry at a wavelength of 540 nm.

Serum lysozyme analysis was performed with serum obtained from blood samples collected without anticoagulants, left at room temperature for about 2 h for clotting. To obtain the serum, the samples were centrifuged at 3000 *g* for 5 min and stored in a −70 °C freezer

until the moment of the analysis, which was carried out by means of a turbidimetric test, according to Ellis (1990) and adapted by Abreu et al. (2009). Survival was calculated based on the following formula (100 × final number of animals) / initial number of animals).

An aliquot of blood was collected with EDTA anticoagulant (3%) and used for the determination of plasma glucose by the endpoint method using a commercial colorimetric determination kit (Glistab). Plasma cortisol was performed using stored heparinized plasma (− 70 °C), using the Diagnostics Biochem Canada Inc. (DBC) Kit.

Statistical analysis

For the physiological, immunological, and hematological data, a completely randomized design in a 4 × 5 factorial scheme (levels of the prebiotic inulin × sampling times) was adopted. All data were submitted to normality (Shapiro–Wilk), the means were subjected to an analysis of variance (ANOVA), and when significant to the means, they were compared using the Tukey test ($p < 0.05$).

Results

There was a significant interaction ($p < 0.001$) on plasma cortisol in surubim fed a diet with 0.50% inulin and in the control group. An increase in circulating cortisol levels was observed after 3 h of interaction between the factors (stress management + bacterial challenge + 0.50% inulin) and 0.5 h after stress management in the control group. Inulin supplementation reduced the impact of stressors at levels of 0.25% and 0.75%, maintaining circulating cortisol levels (Table 1).

For the hematological variables of the red series, there was no significant interaction ($p > 0.05$) between the different levels of inulin and the other factors.

However, a significant difference ($p < 0.05$) was observed in the blood variables in relation to the collection period, with a significant increase in the number of erythrocytes after stress management (0.5 h). There was a significant reduction in hemoglobin 24 h after stress management + bacterial challenge, and a significant reduction

Table 1 Plasma cortisol (ug dL^{-1}) of hybrid surubim (*Pseudoplatystoma reticulatum* × *Leiarius marmoratus*) after 31 days of inulin-dietary supplementation at different inclusion levels. The table shows the amount of plasma cortisol (mean ± standard error) at different collection times, with or without the influence of capture stress-atmospheric exposure + bacterial infection. Means followed by lowercase letters on the same line indicate a significant difference by Tukey's test ($p < 0.05$) between the treatments. Means followed by capital letters in the same column indicate an interaction effect ($p < 0.001$) between the treatments and sampling times. *PE*, 30 min after capture-exposure to air; *AH*, after *Aeromonas hydrophila* challenge

Sampling time	Treatments			
	0%	0.25%	0.50%	0.75%
Basal (0 h)	3.53 ± 0.16^{aC}	3.81 ± 0.16^{aB}	3.44 ± 0.17^{aB}	3.79 ± 0.16^{aB}
PE (0.5 h)	4.21 ± 0.16^{aB}	4.11 ± 0.17^{aB}	3.98 ± 0.16^{aB}	4.08 ± 0.16^{aB}
PE + AH (3 h)	4.57 ± 0.16^{bB}	4.22 ± 0.16^{bB}	5.44 ± 0.16^{aA}	4.41 ± 0.16^{bB}
PE + AH (6 h)	5.65 ± 0.16^{aA}	5.84 ± 0.16^{aA}	5.95 ± 0.16^{aA}	5.65 ± 0.16^{aA}
PE + AH (24 h)	5.71 ± 0.16^{aA}	5.69 ± 0.16^{aA}	5.97 ± 0.16^{aA}	5.89 ± 0.16^{aA}

Table 2 Erythrogram (mean ± standard error) of hybrid surubim (*Pseudoplatystoma reticulatum* × *Leiarius marmoratus*) after 31 days of inulin-dietary supplementation at different inclusion levels. The table shows the erythrogram (mean ± standard error) at different collection times, with or without the influence of capture stress-atmospheric exposure + bacterial infection. Means with different letters in the column indicate a significant difference by Tukey's test ($p < 0.05$) between the sampling time. *PE*, 30 min after capture-exposure to air; *AH*, after *Aeromonas hydrophila* challenge; *RBC*, total erythrocytes; *MCV*, mean corpuscular volume; *MCHC*, mean corpuscular hemoglobin concentration

Sampling time	Hematocrit (%)	Hemoglobin (g dL^{-1})	RBC ($\times 10^6$ μL^{-1})	MCV (fL)	MCHC (g dL^{-1})
Basal $^{(0\ h)}$	40.18 ± 1.56^a	4.59 ± 0.51^a	1.60 ± 0.17^b	271.88 ± 14.32^a	11.94 ± 1.62^b
PE $^{(0.5\ h)}$	36.50 ± 1.56^a	5.11 ± 0.51^a	2.72 ± 0.17^a	165.08 ± 14.32^b	14.81 ± 1.62^b
PE + AH $^{(3\ h)}$	26.00 ± 1.56^b	5.31 ± 0.52^a	3.15 ± 0.17^a	96.19 ± 14.32^c	20.71 ± 1.64^a
PE + AH $^{(6\ h)}$	27.46 ± 1.56^b	5.08 ± 0.53^a	3.56 ± 0.17^a	87.45 ± 14.32^c	19.16 ± 1.68^a
PE + AH $^{(24\ h)}$	29.09 ± 1.56^b	3.46 ± 0.51^b	2.23 ± 0.17^b	139.72 ± 14.32^b	12.04 ± 1.62^b

in hematocrit from 3 h after stress management + bacterial challenge. Mean corpuscular volume significantly reduced after stress management and was more significant between 3 and 6 h (stress management + bacterial challenge) (Table 2).

The interaction between the factors was not significant ($p > 0.05$) and did not change the blood glucose levels of the fish between treatments. However, a significant difference ($p < 0.05$) was observed between the different sampling times. There was a significant increase ($p < 0.001$) in fish blood glucose levels after stress management (0.5 h) as well as a significant decrease at 6 h after stress management + bacterial challenge (Fig. 1).

No significant interaction ($p > 0.05$) was observed between supplementation with different levels of inulin and the other factors in the blood variables leukocytes and thrombocytes of surubim. However, there was an acute innate immune response related to stress management + bacterial challenge. A significant increase ($p < 0.05$) in the number of total leukocytes and lymphocytes was observed at 6 h, while the number of thrombocytes increased significantly ($p < 0.05$) at 6 and 24 h (Table 3).

No significant interaction ($p > 0.05$) was observed between supplementation with different levels of inulin and the other factors in leukocyte respiratory activity. However, it was possible to verify a significant increase ($p < 0.001$) in leukocyte respiratory activity related to time after stress management (0.5 h), as well as after stress management + bacterial challenge, when compared to the baseline sample (Fig. 2).

A significant interaction ($p < 0.001$) in serum lysozyme levels was evidenced between treatments. Fish supplemented with 0.50% inulin showed a significant increase ($p < 0.05$) in serum lysozyme at baseline sampling, with a subsequent reduction after capture stress and aerial exposure; returning to baseline levels after 24 h with the joint action of the factors (stress management + bacterial challenge) (Table 4).

Discussion

According to Sopinka et al. (2016), the different adaptive physiological responses to stress that impact the immune system, such as changes in circulating cortisol levels, may vary according to the species and their different lineages, the age and size of the animals, and

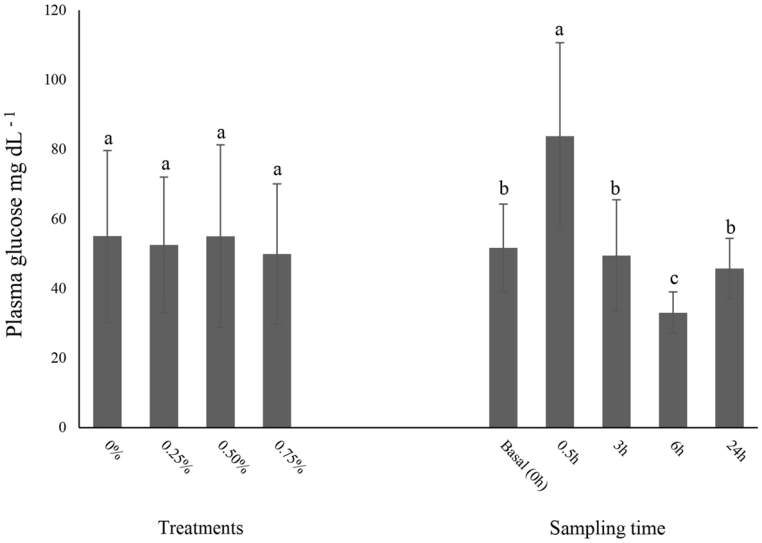

Fig. 1 Plasma glucose of hybrid surubim (*P. reticulatum*×*L. marmoratus*) after 31 days of inulin-dietary supplementation at different inclusion levels. The figure shows the amount of plasma glucose (mean ± standard error) under the effect of different concentrations of inulin (0%, 0.25%, 0.50%, and 0.75%) in the diet of surubim, and at different sampling times. Basal (0 h) — no influence of pursuit/capture/atmospheric exposure stress and bacterial infection. (0.5 h) — with influence of pursuit/capture/atmospheric exposure stress. (3 h) (6 h), and (24 h) — with influence of chase/capture/atmospheric exposure stress + bacterial challenge with *A. hydrophila*. Different letters indicate a significant difference by Tukey's test ($p < 0.05$) between treatments

Table 3 Thrombogram and leukogram of hybrid surubim (*Pseudoplatystoma reticulatum*×*Leiarius marmoratus*) after 31 days of inulin-dietary supplementation at different inclusion levels. The table shows the thrombogram and leukogram (mean ± standard error) at different collection times, with or without the influence of capture stress-atmospheric exposure + bacterial infection. Means with different letters in the column indicate a significant difference by Tukey's test ($p < 0.05$) between the sampling time. *IL*, immature leukocytes; *TL*, total leukocytes; *Thrb*, thrombocytes; *Lf*, lymphocytes; *Nt*, neutrophils; *PE*, 30 min after capture-exposure to air; *AH*, after *Aeromonas hydrophila* challenge

Sampling time	IL ($\times 10^3$ μL^{-1})	TL ($\times 10^3$ μL^{-1})	Thrb ($\times 10^3$ μL^{-1})	Lf ($\times 10^3$ μL^{-1})	Nt ($\times 10^3$ μL^{-1})
Basal $^{(0\ h)}$	1.16 ± 0.20^{a}	23.97 ± 3.59^{b}	27.13 ± 5.10^{b}	19.36 ± 3.17^{b}	3.42 ± 0.57
PE $^{(0.5\ h)}$	1.35 ± 0.20^{a}	33.29 ± 3.59^{b}	22.85 ± 5.10^{b}	27.60 ± 3.17^{b}	4.14 ± 0.57
PE + AH $^{(3\ h)}$	0.35 ± 0.35^{b}	23.07 ± 4.35^{b}	20.77 ± 6.17^{b}	20.30 ± 3.840^{b}	2.28 ± 0.69
PE + AH $^{(6\ h)}$	0.23 ± 0.46^{b}	54.12 ± 5.15^{a}	52.05 ± 7.49^{a}	50.62 ± 4.55^{a}	3.35 ± 0.81
PE + AH $^{(24\ h)}$	1.86 ± 0.19^{a}	25.65 ± 3.59^{b}	58.69 ± 5.01^{a}	19.40 ± 3.11^{b}	4.29 ± 0.56

according to the duration and the nature of the stressor stimulus and experimental procedures adopted.

Brycon amazonicus and *Brycon cephalus* require a long recovery time for circulating cortisol, approximately 24 h and 96 h. In adult and juvenile matrinxãs, the return of cortisol levels to baseline values occurred in about 24 h (Urbinati et al. 2004; Montoya et al. 2017, 2018), while in Salmon (*Salmo salar*), this return of cortisol to baseline levels did not occur after 48 h (Culbert et al. 2022). Acerete et al. (2004) stated the return to

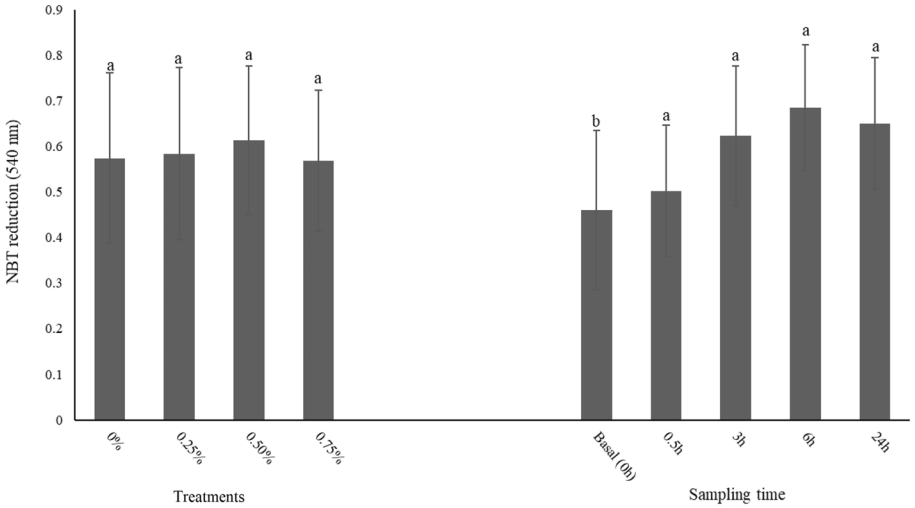

Fig. 2 Leukocyte respiratory activity of hybrid surubim (*P. reticulatum* × *L. marmoratus*) after 31 days of inulin-dietary supplementation at different inclusion levels. The figure shows the results (mean ± standard error) of the analysis of the respiratory burst activity of blood leukocytes performed using reactive oxygen species (ROS) using nitroblue tetrazolium (NBT) under the effect of different concentrations of inulin (0%, 0.25%, 0.50%, and 0.75%) in the diet of surubim, and at different sampling times. Basal (0 h) — no influence of pursuit/capture/atmospheric exposure stress and bacterial infection. (0.5 h) — with influence of pursuit/capture/atmospheric exposure stress. (3 h) (6 h), and (24 h) — with influence of chase/capture/atmospheric exposure stress + bacterial challenge with *A. hydrophila*. Different letters indicate a significant difference by Tukey's test ($p < 0.05$) between treatments

Table 4 Serum lysozyme (ng μL^{-1}) of hybrid surubim (*Pseudoplatystoma reticulatum* × *Leiarius marmoratus*) after 31 days of inulin-dietary supplementation at different inclusion levels. The table shows the amount of serum lysozyme (mean ± standard error) at different collection times, with or without the influence of capture stress-atmospheric exposure + bacterial infection. Means followed by lowercase letters on the same line indicate a significant difference by Tukey's test ($p < 0.05$) between the treatments. Means followed by capital letters in the same column indicate an interaction effect ($p < 0.001$) between the treatments and sampling times. *PE*, 30 min after capture-exposure to air; *AH*, after *Aeromonas hydrophila* challenge

Sampling time	Treatments			
	0%	0.25%	0.50%	0.75%
Basal $^{(0\ h)}$	7.93 ± 1.36^{bB}	5.46 ± 1.36^{bC}	10.33 ± 1.15^{aA}	5.84 ± 1.24^{bB}
PE $^{(0.5\ h)}$	8.26 ± 1.75^{aB}	10.02 ± 1.15^{aB}	5.74 ± 1.75^{bB}	9.42 ± 1.75^{aA}
PE + AH $^{(3\ h)}$	4.81 ± 1.24^{aC}	5.53 ± 1.24^{aC}	6.23 ± 1.24^{aB}	6.32 ± 1.52^{aB}
PE + AH $^{(6\ h)}$	8.27 ± 1.15^{aB}	9.90 ± 1.24^{aB}	7.42 ± 1.07^{aB}	8.83 ± 1.24^{aA}
PE + AH $^{(24\ h)}$	13.19 ± 1.15^{aA}	14.41 ± 1.07^{aA}	11.17 ± 1.15^{aA}	11.18 ± 1.15^{aA}

baseline levels of cortisol after transport only between 7 and 14 days after handling. Here, we present similar results, whose plasma cortisol levels remained elevated after 24 h from the stressful handling of the capture followed by the bacterial challenge. Research such as these demonstrate that the normalization of cortisol levels after stressors tends to take more than 24 h, both for Brazilian teleost species and for exotic species. Thus, studies that assess cortisol levels over longer periods are necessary.

The concentration of erythrocytes is related to the nutritional status of the fish, tending to be lower in anemic individuals (Fazio 2019). This blood cell is related to the transport of oxygen in the respiratory process, and its concentration is a relevant parameter related to the animal adaptive response to stress conditions (Sopinka et al. 2016). Resembling the results found in the present study, supplementation with the prebiotic inulin did not improve the hematological variables of *Oreochromis niloticus*, *Huso huso*, and *Pseudoplatystoma* spp. (Ibrahem et al. 2010; Ahmdifar et al. 2011; Mouriño et al. 2012), while satisfactory results were reported by Tiengtam et al. (2015) and Mouriño et al. (2015), with increased erythropoiesis in supplemented groups compared to the control group. The different responses found may be related to the concentrations used, longer or shorter supplementation, species and intestinal morphology, fermentation, and intestinal microbiota (Hoseinifar et al. 2010; Ringø et al. 2010).

In routine fish farming activities, animals are exposed to biotic and abiotic stressors such as transport, overpopulation, different rearing systems, temperature changes, and low dissolved oxygen in water, which can alter the physiological state and disrupt homeostasis (Sampaio and Freire 2016; Vanderzwalmen et al. 2018; Herrera et al. 2019).

Here, in the present study, an increase in hemoglobin (3 h after stress management + bacterial challenge) and in the number of erythrocytes (0.5 h after stress management) suggests a greater requirement for oxygen transport as a mechanism for supplying the energy demand and maintaining homeostasis for survival (Tavares-Dias and Moraes. 2004), while the reduction in hematocrit and corpuscular volume indicates hemodilution of cells, due to osmoregulatory disorders (Burgos-Aceves et al. 2019).

The significant increase in glycemia observed in surubim after stress-inducing management indicates glucose mobilization with the purpose of replacing energy demands, through the action of catecholamines in the degradation of hepatic glycogen (Mommsen et al. 1999; Salbego et al. 2017). According to Tort (2011), still as part of an acute response to stress and due to the release of catecholamines, erythrocytes and leukocytes can be mobilized by the autonomic nervous system, as happened in surubins after handling capture-exposure to air and bacterial infection, increasing circulation of total leukocytes and lymphocytes (6 h) and thrombocytes (6 and 24 h) (Table 3).

Leukocytes are related to the immune response of fish to infections caused by invasive agents (Fazio 2019). Pickering (1993) reported that the decline in lymphocytes may be related to a reduction in the fish's ability to defend itself against pathogens. However, dietary supplementation with different levels of inulin did not influence the leukogram or the thrombogram of the surubim. These results are similar to those found in species such as *Oncorhynchus mykiss*, supplemented with prebiotics and probiotics, with no effect on such blood elements (Hoseinifar et al. 2015).

In short-term stress events, the immune system may remain unaffected, with cardiovascular responses quickly returning to balance. While some essential immune mechanisms such as phagocytosis remain active, others may not be significantly affected (Tort 2011).

Cerezuela et al. (2008) did not observe significant effects on leukocyte respiratory activity when supplementing the *Sparus aurata* diet with different concentrations of inulin. However, Cerezuela et al. (2012) obtained satisfactory results with *S. aurata*, while Gupta et al. (2008) observed an increase in the levels of oxidative activity, hemoglobin, total leukocyte count, serum lysozyme activity, and greater survival of *Labeo rohita* post-challenge with *A. hydrophila*.

Acute stress can become adaptive because the fish reacts to the challenge and the successful outcome can result in an adaptability to new stressful episodes (Awad and Awaad 2017). This was demonstrated in the 6-h sample time, with an increase in blood leukocytes

(thrombocytes, lymphocytes, and total leukocytes), as well as the activation of leukocyte respiratory activity after stress, in which the acute phase of stress favored the mobilization of defense cells and the distribution of different cell types, according to the biological demand (Barcellos et al. 2000). However, maintaining its long-term effects can lead to chronic stress.

Resembling results to those found here in the present research were described by Buentello et al. (2010) in *Sciaenops ocellatus*, Mouriño et al. (2015) in *Pseudoplatystoma* spp., and Soleimani et al. (2012) in *Rutilus Rutilus*, who found an increase in serum lysozyme after supplementation with prebiotic sources.

Lysozyme — which is mainly produced by leukocytes — is an important bacteriolytic component of the innate immune system of several species of marine and freshwater fish (Lie et al. 1989). Lysozyme acts as a primary reaction of the fish's innate defense. Serum lysozyme levels naturally rise in the hours following the infectious process and correlate with the intensity of the leukocyte response to the infectious process (Jeney et al 1997; Roberts 2012; Levinson 2016). This variation described above for lysozyme concentration was found in the present study, with its levels decreasing in the first hours after capture stress and aerial exposure and returning to baseline values after 24 h of exposure to stressors (capture stress and aerial exposure + infection with *A. hydrophila*).

The immune response of fish to *A. hydrophila* depends on several factors, such as strain virulence, concentration used, water temperature, and age of the fish (Nayak 2020). Under stressful conditions such as those simulated in the present study (persecution/capture/exposure to air), pathogenic bacteria such as *A. hydrophila* have their development and pathogenicity enhanced (Barcellos et al. 2000), a fact that was not evidenced in the present research. Therefore, it is possible to state that the dose of *A. hydrophila* used was not sufficient to cause generalized septicemia, and only stimulated the innate immune system of the fish as planned.

Conclusion

In common situations in aquaculture activity, such as high densities and environmental variations, the inclusions of inulin at 0.25% and 0.75% provide greater hormone cortisol homeostasis, which at high levels can negatively affect the immune system of the animals and consequently the growth performance. To improve the immune system of hybrid surubim, supplementation of 0.50% inulin is indicated, as it provided an increase in serum lysozyme.

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Author contribution PTNV: experimental execution, writing — original draft, methodology. TARR: experimental execution. LEF: experimental execution. RAR: experimental execution; FP: methodology, responsible for the bacterial challenge. MSO: data curation, final writing. MLM: writing — original draft. CMC: conceptualization, methodology, project administration, resources, and supervision.

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Data availability The data related to this research are available upon prior request.

Declarations

Ethical approval All procedures that involved the use of fish in this study were performed according to ethical principles in animal experimentation and approved by the Ethics Committee on the Use of Animals (CEUA) of the State University of Mato Grosso do Sul — UEMS, in Aquidauana, MS, Brazil, under protocol nº 14/2013.

Competing interests The authors declare no competing interests.

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