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ORIGINAL ARTICLE

Aeromonas hydrophila dose and post-immunostimulation period as immunomodulatory effect indicator on Nile tilapia (*Oreochromis niloticus*)

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Abstract - In fish challenge tests, there is no standardization in the concentration and analysis period of parameters. This study investigates the immunomodulatory and cytotoxic effects of *A. hydrophila* dose and post-immunostimulation periods on Nile tilapia. Four level of bacterial doses (½, ¼, ⅛, and 0 LD_{50}) were administered, and immune parameters, hematological data, and erythrocyte abnormalities were analyzed at 3, 7, 10, and 14 days post-infection (dpi). The highest respiratory burst, total globulins, and lysozyme activity were detected at 7 dpi. Considering the bacteria doses, the lysozyme activity was higher in the ⅛ LD_{50} and ¼ LD_{50} of *A. hydrophila*. Erythrocytes, hematocrit, hemoglobin, leukocytes, lymphocytes, neutrophils, and monocytes counts remained unchanged. Ten days post-injection, the control group exhibited reduced abnormalities, showing a notable distinction from the ⅛ LD_{50} and ¼ LD_{50} treatments, particularly in hooked and notched nuclei abnormalities. Mortality rates were higher in infected groups, peaking at different times with the highest in ½ LD_{50}. For evaluating Nile tilapia immunity post *A. hydrophila* infection, it is recommended ¼ LD_{50} dose and blood collection at 7 and 10 dpi for immunological parameters and abnormalities in Nile tilapia erythrocytes, respectively.

Keywords: Immune system. *Oreochromis niloticus*. Respiratory burst. Lysozyme. Globulin.

Dose e período pós-imunoestimulação por *Aeromonas hydrophila* como indicador imunomodulatório na tilápia do Nilo (*Oreochromis niloticus*)

Resumo - Nos testes de desafio com peixes não há padronização na concentração e período de análise dos parâmetros. Este estudo investiga os efeitos imunomoduladores e citotóxicos de diferentes dosagens de *Aeromonas hydrophila* e dos períodos de avaliação dos parâmetros pós-imunoestimulação na tilápia do Nilo. Quatro níveis de doses bacterianas (½, ¼, ⅛ and 0 LD_{50}) foram administrados e os parâmetros imunológicos, dados hematológicos e anormalidades eritrocitárias foram analisados aos 3, 7, 10 e 14 dias pós-infecção (dpi). A maior atividade respiratória dos leucócitos, globulinas totais e atividade de lisozima foram detectadas em 7 dpi. Considerando as doses bacterianas, a atividade da lisozima foi maior nas doses ⅛ LD_{50} e ¼ LD_{50} de *A. hydrophila*. As contagens de eritrócitos, hematócritos, hemoglobina, leucócitos, linfócitos, neutrófilos e monócitos permaneceram inalteradas. Após dez dias da injeção, o tratamento com solução salina exibiu anormalidades reduzidas, mostrando uma distinção notável dos tratamentos ⅛ LD_{50} e ¼ LD_{50}, particularmente nas anormalidades de núcleos em forma de gancho e entalhados. As taxas de mortalidade foram mais elevadas nos grupos infectados, atingindo o pico em momentos diferentes, sendo as mais elevadas em ½ LD_{50}. Para avaliação da imunidade da tilápia do Nilo após infecção por *A. hydrophila*, recomenda-se a dose de ¼ LD_{50} e coleta de sangue aos 7 e 10 dpi para parâmetros imunológicos e anormalidades nos eritrócitos da tilápia do Nilo, respectivamente.

Palavras-chave: Sistema imunológico. *Oreochromis niloticus*. Burst respiratório. Lisozima. Globulina.

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Introduction

The burgeoning aquaculture sector have been associated with increased disease problems (Vallejos-Vidal *et al.*, 2016; Ibrahim *et al.*, 2022). Among the diseases challenging fish, especially those subjected to various stressful situations, there is motile Aeromonas septicemia triggered by *Aeromonas hydrophila* (El-Magd *et al.*, 2019). This species, recognized as a Gram-negative opportunistic bacterial pathogen in freshwater fish, stands out as the most virulent within the *Aeromonas* complex, causing outbreaks (Rhodes *et al.*, 2000; Algamal *et al.*, 2020). Nile tilapia, a prominent aquaculture species, become particularly vulnerable to *Aeromonas* spp. infections, notably when subjected to demanding circumstances (Aboyadak *et al.*, 2015; Bavia *et al.*, 2022).

To safeguard fish health, extensive research has explored natural or chemical substances as potential additives for aquaculture. The focus of immunostimulant effects on fish has predominantly revolved around evaluating the consequences on the innate immune system (Vallejos-Vidal *et al.*, 2016), which reacts to signals stemming from the strategies pathogens employ to invade, manipulate, and replicate within their hosts (Vance; Isberg; Portnoy, 2009). To assess fish immune status, some authors collect blood at the end of experiments and evaluate immune system parameters without using immune system challenges (Sado; Bicudo; Cyrino, 2010; Tukmechi *et al.*, 2011; Amlashi *et al.*, 2011; Maldonado-Garcia *et al.*, 2019; Dias *et al.*, 2020). Others utilize thermal stress, management, or hypoxia as immunostimulating factors (Chen *et al.*, 2020; Abdel-Ghany; El-Sisy; Salem, 2023; Nascimento *et al.* 2023; Ni *et al.*, 2023). Some authors prefer to evaluate the treatment's effect on the immune system after immunostimulation with bacteria (Jaramillo Jr.; Gatlin III, 2004; Sherif; Mahfouz, 2019).

Remarkably, certain studies investigating immune responses in *A. hydrophila* infected fish have unveiled the positive effect of alternative immunostimulants in Nile tilapia aquaculture (Saputra *et al.*, 2016; Abdel-Razek; Awad; Abdel-Tawwab, 2019; Moustafa *et al.*, 2020; Gupta *et al.*, 2021; Liu *et al.*, 2022; Medeiros *et al.*, 2023). However, in fish challenge tests, there is no standardization in the concentration and analysis period of immunity parameters (Bandeira Junior; Baldisserotto, 2021). Some studies examine immunity at seven days post-infection (Biller-Takahashi *et al.*, 2013; Biller *et al.*, 2019; Ibrahim *et al.*, 2021), while others opt for 10 days (Zahran; Abd El-Gawad; Risha, 2018; Moustafa *et al.*, 2020; Kiadaliri; Firouzbakhsh; Deldar, 2020). Some studies investigate the innate immunity responses on the last day of the feeding trial, before the bacterial challenge, which is performed to observe animal survival (Ibrahim *et al.*, 2022; Liu *et al.*, 2022; Sherif *et al.*, 2023).

The immune response of the fish to infection may reach its peak at a specific time after inoculation with bacteria. This peak can vary among different immunological parameters and is influenced by the bacterial load to which the fish is exposed. In a study developed by Amar *et al.* (2017), higher lysozyme levels were observed in the early innate immune response of orange-spotted grouper against *Vibrio harveyi* JML1. This was accompanied by inhibited phagocytic and respiratory burst responses, as well as depleted total globulins during the same period. This coincided with the peak bacterial loads, emphasizing the impact of the injected bacterial dose on fish immunity, in addition to the analysis period.

Currently, there is no standardized format for evaluating immune parameters. Addressing this gap, the present study is dedicated to establish a post-immunostimulation period with *A. hydrophila* and an

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injected bacterial dose that can effectively elicit responses in the immune and hematological systems. Additionally, this study aims to assess its impact on erythrocyte abnormalities in Nile tilapia. The efforts are poised to give valuable insights, allowing the development of protocols for assessing Nile tilapia immunity through challenge tests with *A. hydrophila*.

Materials and Methods

Experimental conditions

The present study was carried out at the Aquaculture Laboratory, located at Embrapa Agropecuária Oeste, and was approved by the ethics committee in animal experimentation of the Embrapa Pantanal, under the protocol CEUA nº 02/2021. The Nile tilapia were donated by a local commercial hatchery, and *Aeromonas hydrophila* was obtained from a laboratory maintenance culture stock. Fish (n = 300) were randomly stocked in 12 experimental tanks of 600 L and acclimated for two weeks in a recirculating freshwater system. After this period, each fish group (147.34 ± 21.75 g per fish) was immunostimulated by intraperitoneal injection with 0.5 mL of *A. hydrophila* suspension. The treatments for infection were four doses of *A. hydrophila*: 0 (as the control group, which was saline 0.65%), ⅛LD_{50}, ¼ LD_{50} and ½ LD_{50}. The suspensions were prepared with serial dilutions. First, mother solution was prepared to obtain ½ McFarland scale (3.75 x 10^7 CFU·mL^{-1}), which represents the 7-day LD_{50} of the bacterium, previously determined. Then, 50 mL mother solution was diluted with 50 mL saline (0.65%) to obtain ¼ LD_{50} and so on for the ⅛ dose.

The fish were fed a commercially formulated diet twice daily (8 and 16 h) for 14 days. Unconsumed feed and excreta were siphoned off the tank bottom daily, and two-thirds of the tank water was exchanged daily. During the acclimation and experimental periods, water temperature, pH and total ammonia were 27.6 ± 0.5 °C, 7.24 ± 0.25, and 1.74 ± 0.45 mg·L^{-1}, respectively. Oxygen was maintained at 6.96 ± 0.72 mg·L^{-1} throughout the experiment by blowers and diffusers that supplied air to each tank.

Immune parameters

Three fish per replicate were randomly sampled at 3-, 7-, 10- and 14-days post-injection (dpi). Fish were anesthetized with eugenol (1:50.000 v/v) and blood samples were collected from the caudal vein with sterilized syringes. Blood was aliquoted into two Eppendorf® microtubes, one without and another containing 10 µL of heparin. Heparinized blood was used in the respiratory burst study; the blood without anticoagulant was left to clot at room temperature for 2 h; serum was then separated by centrifugation (3,000 g, 5 min, 25 °C) for evaluation of serum protein, albumin and lysozyme.

To measure the reactive oxygen species (ROS) produced by leukocytes during the oxidative burst (reduction of nitroblue tetrazolium [NBT] into phagocytes producing formazan granules), aliquots (100 µL) of homogenized, heparinized whole blood were poured into Eppendorf microtubes. They were added equal amounts of 0.2% NBT solution in sodium phosphate buffer (pH 7.4) (Sigma-Aldrich) and incubated for 30 min at room temperature. After homogenization, a 50 µL aliquot of the incubated mix was added to glass tubes containing 1.0 mL of N,N-dimethyl-formamide (DMF, Sigma-Aldrich). Tubes were then centrifuged for 5 min at 3,000 g. The reagent DMF, a high molecular weight solvent, lyses leukocytes membrane releasing the reduced NBT pigment to the solution. The absorbance of the supernatant was determined using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 540 nm (Klein, 1990;

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Anderson; Siwicki, 1995).

Serum lysozyme activity was measured by turbidimetric assay (Ellis, 1990). Serum samples were heated (bath at 56 °C for 30 min) for denaturation and inactivation of complement system proteins; 150 µL aliquots of serum were homogenized with 150 µL of sodium phosphate buffer (0.05 M; pH 6.2), incubated for 2 min at 26 °C. Then, 300 µL suspension of *Micrococcus lysodeikticus* (0.2 $mg \cdot L^{-1}$ in phosphate buffer; pH 6.2; Sigma-Aldrich) was added. The optical density was measured after 0.5 and 5 min at 450 nm in a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 26 °C. One unit of LA was defined as the amount of enzyme producing a decrease in absorbance of 0.001 $U \cdot min^{-1} \cdot mL^{-1}$ of serum. The serum lysozyme concentration was determined using a calibration curve obtained from standard lysozyme suspension (L 6876, Sigma-Aldrich).

Serum total proteins and serum albumin were both determined by colorimetric assays using commercial kits (Bioclin®, Belo Horizonte, Brazil). The determination of serum globulins was performed by indirect method through the subtraction of the albumin concentration from the concentration of total protein.

Hematological parameters

Hematological analyzes were performed on the seventh day after infection evaluating the changes caused by the pathogens. For this, nine fish from each treatment were sampled with a heparinized syringe, collecting about 1.0 mL of blood through caudal venipuncture. Hematological analyzes were performed at the time of collection to check for possible changes in each treatment. Glucose level was determined by spectrophotometric assay using commercial kits based on the GOD-PAP (glucose oxidase/peroxidase-4-aminophenazone-phenol) method (Trinder, 1969). The number of erythrocytes was determined in a Neubauer chamber using saline solution (0.7%) as a diluent, hematocrit, hemoglobin level by the cyanomethemoglobin method and reading in a spectrophotometer. Blood extension slides were prepared using the Rosenfeld method for the differential and total leukocyte count, by the indirect method (Ranzani-Paiva *et al.*, 2013).

Micronucleus test, binuclei, and nuclear abnormalities

Blood samples were collected from the caudal vein of one fish randomly selected from each replicate for micronucleus analysis in each analysis period. A drop of blood was immediately placed on duplicate clean glass microscope slides, drawn across the surface (blood smear technique) and allowed to air-dry. The dried slides were stained with a modified Rosenfeld staining method (Tavares-Dias; Moraes, 2003). From each fish, 2,000 cells were scored under 1000X magnification (Pinheiro-Sousa *et al.*, 2019) to determine the frequencies of micronucleus (MN), binuclei (BN) and nuclear abnormalities (NAs), as described by Cavaş, Garanko and Arkhipchuk (2005) and Ota *et al.* (2022). Coded slides were scored using a blind review by a single observer. The total frequency of abnormalities was calculated by summing the frequency of all abnormalities.

Clinical signs and mortality

After the fish had been injected with bacteria, the cumulative mortality was monitored for 14 days simultaneously with the collection of blood samples. Dead fish were removed from the tanks daily. Percent mortality was computed as the total number of dead fish divided by the total number of stocked fish and multiplied by 100. Fish were observed for clinical

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symptoms throughout the experimental period.

Statistical analysis

Plasma total globulins, lysozyme activity, and oxidative burst activity between treatments were analyzed by two-way ANOVA to test the effect of *A. hydrophila* infection doses and period post-infection (3-, 7-, 10-, and 14-days post-injection). The differences were compared by Duncan's multiple range test. Data are presented as means ± standard error of the mean. The leukocyte count was performed by ANOVA, normality was verified by the Shapiro-Wilk test and homogeneity by the Bartllett test. The frequency of abnormalities in erythrocytes data was analyzed with Kruskal-Wallis test, and pairwise comparisons were done with Dunn's test. The frequencies of micronuclei, binuclei, and nuclear abnormalities were measured and expressed per 2,000 cells. Mortality data were square root transformed before analysis, normality was verified by the Shapiro-Wilk test, homogeneity by the Bartllett test and the Tukey test was used to identify significant differences. All statistical analyses were performed in RStudio for Windows (R Inc., 1.1.423 - © 2009-2018), with a cut-off of $\alpha = 0.05$ for statistical significance.

Results

Immune parameters

Total globulins before injection was 2.72 ± 0.41 mg ml^{-1} and decreased 3 days after infection. At 7 dpi, total globulins increased coinciding with the peaks of oxidative activity of leucocyte and lysozyme activity (Table 1). After 10 dpi, the total globulins started to decrease. Total globulins and respiratory burst activity in fish did not present a difference among bacteria doses. In addition, the treatments ¼ LD_{50} and ⅛ LD_{50} showed significantly higher lysozyme levels than fish at ½ LD_{50} and control group ($p < 0.05$; Table 1).

Infection with any tested concentration of *A. hydrophila* resulted in improved oxidative activity of leukocytes than in control group. The highest respiratory burst activity of leukocytes was observed between 3-7 dpi (Table 1). Plasma lysozyme activity was different ($p < 0.05$) among treatments at 3 and 14 days post-infection, and there weren't differences at 7 and 10 days (Table 1). In control and ¼ LD_{50} treatment, lysozyme activity was higher on day 7 post-infection ($p < 0.05$). The plasmatic glucose concentrations were equal for all treatments.

Hematological parameters

In the study, there were no significant changes in the hematological parameters of the red blood cell counts (erythrocytes, hematocrit and hemoglobin), and leukocyte response on the seventh day after the injection of *A. hydrophila* in Nile tilapia (Table 2). The plasmatic glucose concentrations were equal for all treatments (Table 2).

Abnormalities in Nile tilapia erythrocytes

Micronucleus test data indicates that the frequency of abnormalities in fish erythrocytes infected with *A. hydrophila*, at 3 days post-infection, decreased in all treatments when compared to the value before infection, with the exception of the control group (Fig.1). The control group presented the highest kidney-shaped nuclei, binucleated nuclei, and hooked nuclei abnormalities at 3-days post infection (Table 3). Ten days after injection, the control group were significantly different from the ⅛ LD_{50} and ¼ LD_{50} in hooked abnormality (Table 3).

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Table 1. Blood immune responses of Nile tilapia after 3, 7, 10 and 14-days post-infection in different doses with *Aeromonas hydrophila*.

		Burst (absorption 630 nm)	**Immunoglobulin** ($mg \cdot dL^{-1}$)	**Lysozyme** ($\mu \cdot mL^{-1}$)
A. hidrophyla doses				
0 (D0, control group)		0.495 ± 0.094	2.075 ± 0.265	0.382 ± 0.253^{b}
⅛ LD_{50} (D1)		0.488 ± 0.056	2.197 ± 0.463	0.600 ± 0.197^{a}
¼ LD_{50} (D2)		0.487 ± 0.075	2.448 ± 0.794	0.690 ± 0.216^{a}
½ LD_{50} (D3)		0.472 ± 0.077	2.618 ± 0.830	0.495 ± 0.258^{b}
	p-value	0.857	0.100	0.002
Period (days-post infection)				
3 (3 dpi)		0.491 ± 0.088^{b}	1.978 ± 0.663^{c}	0.518 ± 0.294^{ab}
7 (7 dpi)		0.542 ± 0.066^{a}	2.838 ± 0.785^{a}	0.722 ± 0.216^{a}
10 (10 dpi)		0.460 ± 0.065^{b}	2.269 ± 0.471^{b}	0.567 ± 0.145^{ab}
14 (14 dpi)		0.449 ± 0.041^{b}	2.183 ± 0.302^{bc}	0.360 ± 0.216^{b}
	p-value	0.007	0.006	0.005
Interaction				
D0 x 3 dpi		0.528 ± 0.152	2.120 ± 0.524^{ab}	0.198 ± 0.134^{b}
D0 x 7 dpi		0.537 ± 0.076	2.187 ± 0.180^{ab}	0.552 ± 0.315^{ab}
D0 x 10 dpi		0.474 ± 0.098	2.013 ± 0.161^{ab}	0.596 ± 0.065^{ab}
D0 x 14 dpi		0.440 ± 0.031	1.980 ± 0.111^{ab}	0.183 ± 0.090^{b}
D1 x 3 dpi		0.458 ± 0.045	1.465 ± 0.346^{b}	0.532 ± 0.244^{ab}
D1 x 7 dpi		0.525 ± 0.082	2.657 ± 0.116^{ab}	0.798 ± 0.161^{a}
D1 x 10 dpi		0.480 ± 0.048	2.377 ± 0.116^{ab}	0.489 ± 0.192^{ab}
D1 x 14 dpi		0.488 ± 0.052	2.047 ± 0.225^{ab}	0.581 ± 0.079^{ab}
D2 x 3 dpi		0.430 ± 0.026	1.753 ± 0.264^{b}	0.825 ± 0.039^{a}
D2 x 7 dpi		0.592 ± 0.040	3.050 ± 1.012^{ab}	0.856 ± 0.177^{a}
D2 x 10 dpi		0.492 ± 0.054	2.563 ± 0.942^{ab}	0.634 ± 0.060^{ab}
D2 x 14 dpi		0.435 ± 0.005	2.427 ± 0.447^{ab}	0.442 ± 0.236^{ab}
D3 x 3 dpi		0.547 ± 0.057	2.615 ± 1.266^{ab}	0.518 ± 0.319^{ab}
D3 x 7 dpi		0.514 ± 0.068	3.457 ± 0.994^{a}	0.680 ± 0.138^{ab}
D3 x 10 dpi		0.395 ± 0.008	2.123 ± 0.121^{ab}	0.549 ± 0.231^{ab}
D3 x 14 dpi		0.431 ± 0.051	2.277 ± 0.214^{ab}	0.233 ± 0.178^{b}
	p-value	0.007	0.004	0.004

*Values expressed as means ± SE (n = 3). Small letters indicate significant differences between treatments for each parameter or sampling periods (days post infection) ($p < 0.05$, two-way ANOVA followed by the Duncan's multiple range test).

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Table 2. Hematological parameters of Nile tilapia after infection with different doses of *Aeromonas hydrophila*, after 7-days post infection.

Hematological parameters	Treatments			
	Control	⅛ LD_{50}	¼ LD_{50}	½ LD_{50}
Glucose	62.1 ± 11.3	52.4 ± 4.9	21.5 ± 14.8	60.3 ± 16.1
Erythrocytes (x10^6/µL)	222.0 ± 4.9	199.8 ± 40.7	191.6 ± 17.3	221.2 ± 10.4
Hematocrit (%)	30.3 ± 1.1	29.2 ± 4.2	30.0 ± 4.8	30.7 ± 6.0
Hemoglobin (g/dL)	0.23 ± 0.02	0.25 ± 0.03	0.25 ± 0.03	0.26 ± 0.03
Leukocytes (%)	225.5 ± 7.09	202.8 ± 14.8	203.2 ± 16.4	219.0 ± 1.5
Lymphocytes (%)	95.1 ± 1.8	88.9 ± 3.3	89.1 ± 4.2	94.2 ± 1.2
Neutrophils (%)	4.44 ± 1.49	7.66 ± 2.35	6.99 ± 2.37	4.55 ± 1.50
Monocytes (%)	0.44 ± 0.41	2.88 ± 0.87	3.88 ± 2.93	1.22 ± 0.68

*Values expressed as means ± SE (n = 3). Capital superscript letters indicate significant differences between groups by Tukey's test ($p < 0.05$).

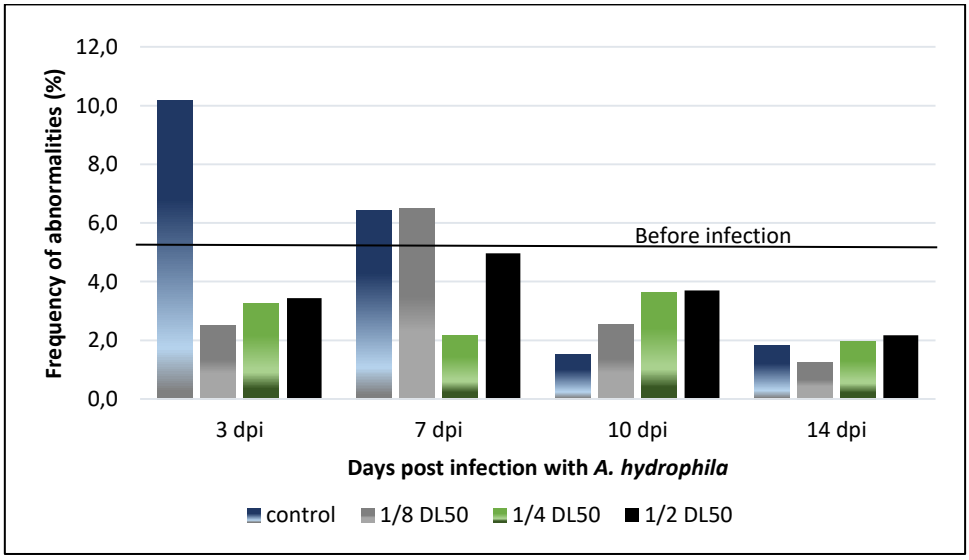

Figure 1. Total frequency of abnormalities in erythrocytes of Nile tilapia challenged by *A. hydrophila*.

Clinical signs and mortality

External hemorrhage was observed on the head (Fig. 2A) and caudal (Fig. 2B) regions in the moderate and high-dose infection groups 24 hours post-infection. Later, some individuals showed exophthalmos (Fig. 2C).

The mortality of the ¼ and ½ LD_{50} groups started following the 1st day post-exposure to *A. hydrophila* while in the ⅛ LD_{50} group started after six days (Fig. 3A). The highest mortality was recorded six, seven, and nine days before injection in ½ LD_{50}, ¼ LD_{50}, and ⅛ LD_{50} groups, respectively (Fig. 3A). Survival in control group was 100%, and was significantly different from the ¼ LD_{50} and ½ LD_{50} groups ($p < 0.05$, Fig. 3B).

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Table 3- Frequency of abnormalities (%) in erythrocytes of Nile tilapia over time (0, 3, 7, 10, and 14-days after inoculation), with different doses *A. hydrophyla* infection (0, ⅛ LD_{50}, ¼ LD_{50}, and ½ LD_{50}).

Abnormalities in erythrocytes	***A. hydrophila* dose**			
	0	**⅛**	**¼**	**½**
	3 days post-infection			
Notched	0.925 ± 0.247	0.767 ± 1.001	0.917 ± 0.464	0
Lobed	0.025 ± 0.035	0	0.183 ± 0.160	0
Blebed	0.575 ± 0.530[a]	0.333 ± 0.493[ab]	0.717 ± 0.453[a]	0[b]
Kidney-shaped	1.700 ± 0.919[a]	0.467 ± 0.534[ab]	0.500 ± 0.507[ab]	0.067 ± 0.057[b]
Micronucleus	0.150 ± 0.210	0.033 ± 0.577	0.050 ± 0.050	0
Binucleated	1.700 ± 0.919[a]	0.467 ± 0.310[ab]	0.500 ± 0.260[ab]	0.067 ± 0.057[b]
Vacuolated	3.900 ± 5.090	0.167 ± 0.288	0	3.250 ± 5.629
Bilobed	0.425 ± 0.389	0.083 ± 0.076	0.117 ± 0.104	0.033 ± 0.029
Hook	0.775 ± 0.247[a]	0.183 ± 0.120[ab]	0.267 ± 0.175[ab]	0.017 ± 0.028[b]
	7 days post-infection			
Notched	2.333 ± 2.139	0.467 ± 0.333	0.567 ± 0.493	2.200 ± 3.031
Lobed	0.900 ± 1.153	0.083 ± 0.104	0.050 ± 0.050	0.100 ± 0.100
Blebed	0.767 ± 0.680	0.267 ± 0.236	0.450 ± 0.482	0.400 ± 0.444
Kidney-shaped	0.250 ± 0.050	0.633 ± 0.850	0.317 ± 0.236	0.217 ± 0.246
Micronucleus	0.200 ± 0.173	0.033 ± 0.028	0	0.167 ± 0.288
Binucleated	0.267 ± 0.057	0.633 ± 0.850	0.283 ± 0.202	1.483 ± 1.404
Vacuolated	0.933 ± 1.616	3.867 ± 5.133	0.200 ± 0.304	0.100 ± 0.173
Bilobed	0.050 ± 0.086	0.133 ± 0.152	0.083 ± 0.104	0.050 ± 0.050
Hook	0.717 ± 0.982	0.367 ± 0.058	0.217 ± 0.175	0.250 ± 0.250
	10 days post-infection			
Notched	0.933 ± 0.534	0.700 ± 0.655	1.825 ± 1.156	2.250 ± 0.482
Lobed	0.033 ± 0.028	0.350 ± 0.200	0.325 ± 0.318	0.067 ± 0.057
Blebed	0.350 ± 0.050	0.750 ± 0.500	0.575 ± 0.176	0.750 ± 0.260
Kidney-shaped	0.133 ± 0.076	0.383 ± 0.189	0.350 ± 0.353	0.283 ± 0.029
Micronucleus	0	0.017 ± 0.029	0.025 ± 0.035	0.033 ± 0.029
Binucleated	0.017 ± 0.028	0	0.075 ± 0.035	0.083 ± 0.076
Vacuolated	0.033 ± 0.057	0	0	0
Bilobed	0	0.050 ± 0.086	0.025 ± 0.035	0.067 ± 0.076
Hook	0.017 ± 0.028[b]	0.300 ± 0.056[a]	0.425 ± 0.176[a]	0.167 ± 0.075[ab]
	14 days post-infection			
Notched	0.375 ± 0.530	0.567 ± 0.678	1.100 ± 1.299	0.783 ± 0.175
Lobed	0.050 ± 0.070	0.050 ± 0.086	0.167 ± 0.152	0.183 ± 0.057
Blebed	0.350 ± 0.282	0.317 ± 0.464	0.350 ± 0.150	0.400 ± 0.304
Kidney-shaped	0.125 ± 0.035	0.067 ± 0.076	0.200 ± 0.259	0.150 ± 0.086
Micronucleus	0	0	0.017 ± 0.029	0.083 ± 0.104
Binucleated	0	0.100 ± 0.173	0.017 ± 0.029	0.250 ± 0.433
Vacuolated	0	0	0	0
Bilobed	0.750 ± 0.848	0.033 ± 0.058	0.033 ± 0.058	0.233 ± 0.321
Hook	0.175 ± 0.106	0.117 ± 0.160	0.083 ± 0.076	0.083 ± 0.076

Values are means ± SD (n = 3). Mean values in the same column with different lower-case letters differ significantly by Dunn's test ($p < 0.05$).

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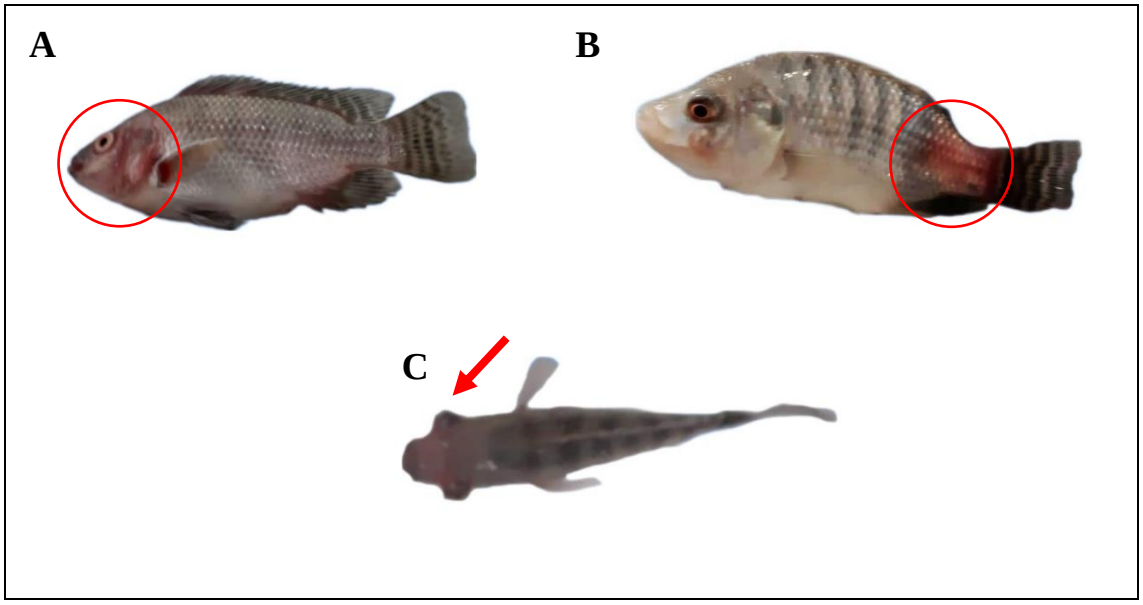

Figure 2. Clinical signs of *Oreochromis niloticus* infected by *A. hydrophila* showing (A) hemorrhage on the head, (B) hemorrhage on the caudal region, and (C) exophthalmos.

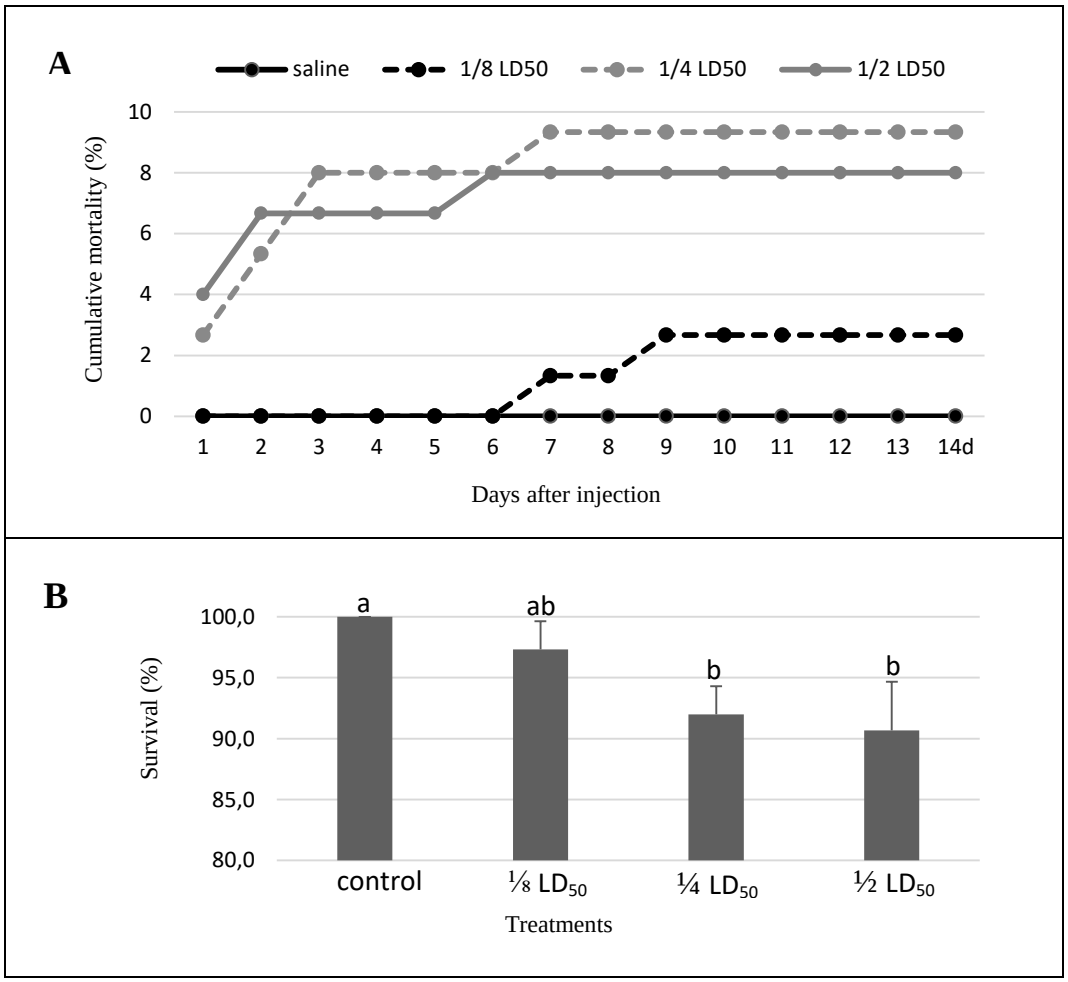

Figure 3. Cumulative mortality (A) and survival (B) of Nile tilapia infected with saline (control group) or *A. hydrophila* doses.

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A deeper understanding of Nile tilapia's response *to A. hydrophila* is essential for optimizing evaluation periods post-infection and determining bacterial doses in immunological and cytotoxic experiments. This study tracks changes in innate immune parameters and the frequency of erythrocyte abnormalities in Nile tilapia infected with varying *A. hydrophila* doses, assessed at 0, 3-, 7-, 10-, and 14-days post-infection (dpi). Clinical symptoms, such as external hemorrhagic and exophthalmos signs, observed in our study, align with previous reports (Khalil; Emeash, 2018; Abdel-Magid *et al.*, 2018; Shameena *et al.*, 2021). In stressful conditions, some hormones, such as cortisol, produce a secondary response increasing glucose in fish blood. Thus, plasma glucose can be used as a stress indicator in fish (Barton, 2000; Nakano *et al.*, 2014). Plasma glucose was equal in this study; which means the stressful condition was the same for all treatments.

The respiratory burst response, crucial for combating fish pathogens (Neumann *et al.*, 2001), displayed higher oxidative burst activity at the infection onset (3 dpi) in the ⅛ LD_{50} group compared to control in the present study. This result of this assay partially corroborated with the kinetics of ROS production in Nile tilapia, in which respiratory burst activity was higher in the *A. hydrophila* group in all periods evaluated, including at 1, 3, and 7 days of post-infection (Elbahnaswy; Elshopakey, 2020). Other studies also indicated macrophages stimulated in *Piaractus mesopotamicus* (Claudiano *et al.*, 2019) and *Sparus aurata* (Reyes-Becerril *et al.*, 2011), but only the first cited investigated the time ROS maximum release, which occurred at 3 days post-inoculation with *A. hydrophila*. On the contrary, tilapia infected with *A. hydrophila* and diet without fenugreek showed reduced respiratory burst activity compared with that of the other groups (without challenge and fenugreek, fenugreek treatment with challenge, and fenugreek treatment without challenge) on 6 h and 1-week post-challenge (Moustafa *et al.*, 2020). In this case, the blood NBT was influenced by the challenge and dietary fenugreek, being appreciably increased in fish fed with fenugreek without *A. hydrophila* inoculation on 24 h post-challenge (Moustafa *et al.*, 2020).

In this study, lysozyme activity was influenced by dose and period. The intermediate dose (¼ LD_{50}) presented the highest lysozyme activity at day 3 dpi activity while the oxidative burst was not the most elevated parameter. After ten days of infection lysozyme activity decreased. Lysozyme is an important defense of the innate immune system mediating protection against microbial invasion (Saurabh; Sahoo, 2008) and have lysis activities against bacteria (Uribe *et al.*, 2011). Similarly, significantly elevated circulating levels of lysozyme post-pathogen exposure were observed on days 1 and 3 in Nile tilapia infected with *A. hydrophila* (Elbahnaswy; Elshopakey, 2020). Plasma lysozyme mainly comes from degraded leukocytes (granulocytes and monocytes) during infection or inflammation (Amar *et al.*, 2017), while oxidative bursts are predominantly from neutrophils (Abdel-Magid *et al.*, 2018), explaining the inverse relation between these immunological parameters. The lysozyme activity decreased possibly due to the reduction of bacterial load, concomitant with the action of other defense mechanisms (Amar *et al.*, 2017).

The total globulin is frequently evaluated in immunostimulant-supplemented diets (Vallejos-Vidal *et al.*, 2016; Ibrahim *et al.*, 2022). As results showed, the Ig did not significantly differ between bacterial doses. However, an inverse relation between dose and response at 7 dpi can be noted, with descending order levels of globulins presented by ½ LD_{50}, ¼ LD_{50}, ⅛ LD_{50} and control. The high variation among fish in the

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same group likely hampered the detection of the bacteria's effect on this parameter. Other studies showed significantly higher IgM levels in tilapia infected with *A. hydrophila* at days 1 and 7 of infection (Elbahnaswy; Elshopakey, 2020, Tellez-Bañuelos *et al.*, 2010). From another point of view, if were assumed that globulins were suppressed in an early stage of infection, as observed by Amar *et al.* (2017), the first fish reaction (suppression) against bacteria for this parameter may have occurred before 3 dpi. Binding (opsonizing) bacteria for phagocytosis could deplete natural antibodies before specific antibodies start to be produced by B cells of infected fish (Coeurdacier *et al.*, 1997; Evenberg *et al.*, 1986; Latinne *et al.*, 1994). Therefore, fish immunoglobulin needs a longer time to react after suppression (Ellis, 1989; Zanon *et al.*, 2014), explaining the increase of globulin levels later (7 to 10 dpi) in our study.

Lymphocytes play a role in generating immunoglobulins and regulating the immune defense (Scapigliati, 2013). Similarly, neutrophils act as key phagocytic leukocytes, while monocytes are durable phagocytic cells that multiply in circulation in reaction to infections, inflammation, and stress (Rieger; Barreda, 2011; Sivagurunathan *et al.*, 2012). Our study showed unchanged leukocytes, lymphocytes, neutrophils, and monocytes counts in *A. hydrophila* infected groups, in contradiction with other studies (De Chavez; Encinares, 2018; Charlie-Silva *et al.*, 2019; Claudiano *et al.*, 2019; Elbahnaswy; Elshopakey, 2020; Neamat-Allah; Mahmoud; Mahsoub, 2021; Oliveira *et al.*, 2024). The unchanged hematological parameters can be explained by both the bacteria dose and the post-immunostimulation analysis period. In 1st week post-infection with *A. hydrophila*, higher values of white blood cell counts were noticed in *O. niloticus*, but the bacteria dose used was not reported (De Chavez; Encinares, 2018). At 11 dpi and in higher *A. hydrophila* dose (the lethal dose 50.0%), tilapia showed an increase in the number of total leukocytes, with a significant increase in lymphocyte counts, compared to fish in the physiological standard group (Oliveira *et al.*, 2024). Tilapias challenged by *A. hydrophila* (0.5 ml of 10^8 CFU mL^{-1}) displayed leukocytosis, lymphocytosis and neutrophilia and revealed anemia (Neamat-Allah; Mahmoud; Mahsoub, 2021). Higher values of white blood cell counts were noticed in *O. niloticus* in 1st week post-infection with *A. hydrophila* (De Chavez; Encinares, 2018). Tilapias challenged by 0.5 ml of 10^8 CFU mL^{-1} *A. hydrophila* revealed anemia and displayed leukocytosis, lymphocytosis and neutrophilia (Neamat-Allah; Mahmoud; Mahsoub, 2021). However, tilapia inoculated with the LD_{50} of *A. hydrophila* showed an increase in the number of total leukocytes compared to fish in the physiological standard group at 11 dpi.

Depending on the severity of the infection, fish display diverse pathological changes, including those related to cytotoxic and genotoxic effects. The negative effect of *A. hydrophila* has been observed in some studies (Bagdonas; Lazutka, 2007; Bischofberger; Iacovache; Van Der Goot, 2012; Patel *et al.*, 2016; Deepika *et al.*, 2019; Sooksawat *et al.*, 2022). Soluble products from active immune cells, like reactive oxygen species from the oxidative burst, despite being efficient antimicrobial agents, have a mode of action generally non-specific (Morel; Doussiere; Vignais, 1991; Nathan; Hibbs, 1991; Stuart; Ezekowitz, 2005). As such, they can be highly toxic to both microorganisms and host cells (Rieger; Barreda, 2011). Moreover, exotoxins reported from *A. hydrophila* (Tomás, 2012) were found to lyse red blood cells (Mancheño *et al.*, 2005), and caused toxicity to peripheral blood leukocytes of *O. niloticus* (Subramani

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et al., 2016). In the present study, it was observed decreased levels of abnormalities in erythrocytes in all infected groups at 3 dpi and higher frequency in notched nuclei and hook nuclei at 10 dpi in the ½ LD_{50} and ¼ LD_{50}. As the highest level of oxidative burst activity was at 7 dpi, we can assume that the free radicals from active immune cells were not toxic to erythrocytes in Nile tilapia infected with *A. hydrophila*. These abnormalities may be due to the cytotoxin released by bacteria, which probably was not significant in the lowest dose of infected groups. Thereby, the antioxidants present in fish feed in this study probably played an important role in the frequency of abnormalities. Change in antioxidant status following infection indicates the important role played by enzyme activities not only in immunity but also in the removal of excessive reactive oxygen species (Abdel-Magid *et al.*, 2018). In this sense, the fish´s antioxidant status can be promoted by feed antioxidants, such as vitamin C, and dramatically reduced the micronuclei frequency (Abo-Al-Ela *et al.*, 2017).

Fish health and immunity are closely connected to the antioxidant defense system. Subsequently, the greater the defense system, the greater antibacterial activity against the challenging *A. hydrophila,* and the lower cumulative mortality rates (Ibrahim *et al.*, 2022). In the present study, the control group showed the lowest mortality rate, but not the highest lysozyme activity or oxidative burst activity. This finding is probably due to the absence of stimulus to the immune response, that is, the bacteria (i.e., the absence of the pathogen *A. hydrophila*) probably did not activate the immune response of the host. Additionally, all the *A. hydrophila* infected groups presented higher mortality than control in this study, indicating that the activated immune system of fish was not enough to prevent death. It was also observed that a higher dose of injected bacteria led to elevated mortality, and the peak of mortality varied according to the bacteria doses. These facts can be explained by the depletion of the fish immune system in higher bacterial loads and the short-lived advantage of bacteria in subverting the host immune response. This agrees with results presented by Sun *et al.* (2024) who observed that the infected grass carp (*Ctenopharyngodon idella*) with *A. hydrophila* gained an advantage resulting in susceptible individuals' death and showing a survival rate decrease with the increase of bacterial concentration injected.

Conclusions

When evaluating the innate immune response, it is necessary to challenge the fish's immune system with a pathogen. In the case of *A. hydrophila* for tilapia, an intraperitoneal dose of bacteria corresponding to ¼ LD_{50} is recommended for this purpose, considering mortality and the immunological parameters analyzed. This study reveals that *A. hydrophila* infection induces erythrocyte abnormalities in Nile tilapia, particularly at higher infection doses. Furthermore, after bacterial immunostimulation, the best blood collection time to evaluate immunological parameters and mortality is 7 dpi, while for analysis of abnormalities in Nile tilapia erythrocytes would be at 10 dpi.

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Conflict of Interests

The authors declare that the research was conducted in the absence of any potential conflicts of interest.

Ethical Statements

The authors confirm that the ethical guidelines adopted by the journal were followed by this work, and all authors agree with the submission, content and transfer of the publication rights of the article to the journal. They also declare that the work has not been previously published nor is it being considered for publication in another journal.

The experiment was approved by the ethics committee in animal experimentation of the Embrapa Pantanal, under the protocol CEUA (Ethics Committee on the Use of Animals) no 02/2021.

The authors assume full responsibility for the originality of the article and may incur on them any charges arising from claims, by third parties, in relation to the authorship of the article.

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