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Levamisole enhances the innate immune response and prevents increased cortisol levels in stressed pacu (*Piaractus mesopotamicus*)

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ABSTRACT

We analyzed the effects of levamisole on stress and the innate immune responses of pacu (*Piaractus mesopotamicus*). A total of 300 fish (180 ± 1.27 g) were fed a diet containing levamisole hydrochloride (LHC) for 15 days, then distributed into the following groups: T0 (control group); T1 (100), T2 (150), T3 (300) and T4 (500) mg kg^{-1} LHC (15 fish per group and four replicates per treatment). After this, fish (n = 8 per treatment) were exposed to air for three minutes to simulate stress conditions and were then challenged with the bacterium *Aeromonas hydrophila* to stimulate the immune system. Fish were sampled at 1, 3 and 24 h after bacterial inoculation to measure plasma cortisol and glucose concentrations, the leukocyte respiratory burst (LRB), hemolytic activity of the complement system (HAC_{50}) and serum lysozyme activity (SLA). LHC attenuated the increase in plasma cortisol at 1 h (500 mg kg^{-1}) and 3 h (300 mg kg^{-1}) after air exposure and bacterial inoculation compared to control fish. The highest glucose concentrations were observed at 1 and 3 h after stress, which then returned to initial levels after 24 h, without any effect of LHC. The LHC 100 mg kg^{-1} dose increased LRB 1 h after inoculation and activated the HAC_{50} 3 h later. At 24 h, all LHC concentrations increased the HAC_{50}. SLA was reduced after inoculation, throughout the experimental period, without an effect of levamisole. Our results indicate that the oral administration of levamisole for 15 days modulated circulating cortisol levels during the stress response and improved the innate immune response against *A. hydrophila* infection in pacu.

1. Introduction

Stress is associated with several kinds of practices in aquaculture, such as handling, capture, confinement and transport [1,2]. Stress causes increased release of catecholamines by chromaffin cells and cortisol by interrenal tissue [2] in the cephalic kidney. The release of these hormones drive adaptive metabolic, ionic and immunological responses favoring fish survival under stress conditions and can cause at long-term damage of reproduction, growth, and immune responses due to the loss of adaptive capacity [2,3]. Under immunosuppressive conditions, fish become more susceptible to pathogens, which can cause several losses in the production cycle [4].

Most of the research on stress physiology in fish has focused on aquaculture because of the interest in reducing stress caused by the routine practices while maximizing production. In this way, the prophylactic management of fish using immunostimulants can prevent or reduce the deleterious effects of stress. The inclusion of an immunostimulant in the feed is currently used in while farming fish [5–8], increasing the probability of fish survival through the modulation of the immune system by improving resistance against pathogenic infections [9–11].

Levamisol is a substance of the imidotiazol group, and on the market it can be found in the form of levamisole hydrochloride (LHC). It has antiparasitic effect in production animals such as sheep [12], bovine [13], and also in the human species [14], paralyzing the muscles of the parasites in the host gastrointestinal tract [15]. Its antimicrobial effect on fish has been proven through the control of protozoa [16], nematodes [17,18], and bacteria [19]. In farmed fish, several studies demonstrate its immunostimulating

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effect, improving leukocyte production [20], respiratory burst activity [21], phagocytic activity [22,23], cytotoxic cell activity [24], oxidative radical production and serum myeloperoxidase [25], lysozyme [19,26,27], and complement system activity [19,21], although its mechanism of action is not known. Furthermore, a study in rats suggested that immunostimulatory effect of levamisole could be mediated by a reduction in circulating corticosterone levels [28]. Thus, the immunomodulatory capacity of LHC can be used to reduce the harmful effects of stress in fish farming, in addition to its known efficacy in controlling fish pathogenic infections [8].

The accelerated growth of aquaculture in Brazil is a known fact [29]; however, research on native species is still needed and should be intensified. The pacu (*Piaractus mesopotamicus*) is a farmed fish of economic importance in South America [30,31], which qualifies this species as an experimental model. Previous studies have focused the physiology of stress [32,33] and its relationship with immune responses in this fish [6,8,34]. In this line, the current study analyzed the effectiveness of levamisole as a modulator of stress and as an immunostimulant in pacu after air exposure and challenge with *Aeromonas hydrophila*, a bacterium that causes diseases in several farmed fish species in Brazil [35].

2. Material and methods

2.1. Fish and experimental protocol

A total of 300 juvenile pacu (180 ± 1.2 g; 16 ± 0.4 cm), supplied by the Centro de Aquicultura of UNESP, Campus of Jaboticabal, was distributed in 20 500 L tanks, in a water recirculating system and acclimated for 15 days. The photoperiod was 12 h light:12 h dark. Fish were fed daily (3% body weight) for 15 days, without rejecting the diet, twice a day (8:30 a.m. and 5:00 p.m.), with the following treatments: T1 (100), T2 (150), T3 (300) and T4 (500) mg kg^{-1} levamisole (LHC; 1-2-3,5,6-tetraidro-6-fenilimidazol [2,1-b]tiazol) and a control treatment (T0) without levamisole. Each treatment was performed in four repetitions with 15 fish per repetition group. The levamisole concentrations were chosen according to a previous study [36].

The chosen concentrations of LHC powder (IMEVE, http://www.imeve.com.br/azul/) were added to a commercial feed (FRI-AQUA, for omnivorous fish, 26% crude protein and 3200 kcal metabolizable energy), previously ground and mixed. After the addition of 30–40% water, the diets were pelletized in a meat grinder, dried in an oven and stored at −20 °C.

During the experimental period, water quality parameters were monitored and remained in acceptable ranges for tropical fish [37]: temperature 28 ± 0.16 °C, pH 7.46 ± 0.14, dissolved O_2 6.43 ± 0.86 mg L^{-1} and total ammonia 0.21 ± 0.03 mg L^{-1}.

2.2. Air exposure, Aeromonas hydrophila challenge and sampling

After LHC feeding, as an initial sample, blood from the caudal vessel was collected from eight fish per treatment, anesthetized using benzocaine (100 mg L^{-1}). Then, the remaining fish were captured and exposed to air for three minutes, immediately inoculated with *A. hydrophila* and sampled at 1, 3 and 24 h after stressful handling protocol to assess indicators of stress and innate immune system activity.

2.3. Stress indicators

The cortisol plasma concentrations were determined using immunoenzymatic assay kit (DRG International, Inc., USA; Cortisol ELISA, EIA 1887) and glucose plasma concentrations were assessed by an enzymatic method (Labtest kit, São Paulo, Brazil, code 84).

2.4. Immunological analysis

2.4.1. Leukocyte respiratory burst

The leukocyte respiratory burst (LRB) was determined in total blood collected with heparin, according to a previously published protocol [38] that was subsequently modified [39]. For the analysis of the production of oxygen reactive species, 0.1 mL of blood was incubated with the same volume of 0.2% nitroblue tetrazolium buffer (NBT, Sigma Aldrich, batch MKBS 6330 V) for 30 min at 25 °C. After incubation, 1 mL of N,N-dimethyl formamide (DMF, Sigma Aldrich, SHBD 7629 V) was added to 50 μL of the incubated solution and the optical density of the solution was determined at wavelength of 540 nm in a spectrophotometer (Thermo Scientific; Genesys 10S).

2.4.2. Hemolytic activity of the complement system (HAC_{50}) alternative pathway

The hemolytic activity of complement (HAC_{50}) was determined in serum [39]. A kinetic assay determined the time required for each serum sample to lyse 50% of a rabbit erythrocyte suspension. To prepare this suspension, a blood sample was mixed with an equal volume of Alsever solution to wash and separate red blood cells. The serum samples were diluted in TEA-EGTA-Mg buffer (triethanolamine ethylene glycol tetraacetic acid, 8 mM, with 2 mM of Mg^{2+} and 0.1% gelatin, pH 7.4) and mixed with the red cell suspension. A reading was taken every 20 s for 10 min at 700 nm in a spectrophotometer (Thermo Scientific Genesys 10S).

2.4.3. Serum lysozyme activity

The serum lysozyme activity (SLA) was determined by lysing a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, M3770) using lyophilized lysozyme from chicken egg (Sigma-Aldrich, L6876) as the standard. The analysis was performed by a turbidimetric assay [39]. An aliquot of 50 μL of pacu serum (in triplicate) was pipetted into a 96-well plate with 300 μL of sodium phosphate buffer (0.05 M, pH 7.4) and 300 μL of *Micrococcus* suspension. The decrease in the absorbance of each sample was compared to that obtained from the standard curve and the activity of lysozyme was expressed in ng mL^{-1}. Absorbance was measured at 450 nm using a microplate reader (Model Multiskan Ascent, Thermo Fisher Scientific Inc., Madison, WI, USA) at room temperature.

2.5. Acute bacterial challenge

The *A. hydrophila* strain used in this experiment, to immunostimulate the fish, was isolated from carp (*Cyprinus carpio* strain A135, LAPOA, UNESP) and identified by sequencing the 16S rDNA (similarity of 100% with GenBank access ATCC 7966). The strain was stocked in TSB (tryptic soy broth, Media) medium with 30% glycerol (sterile) at −80 °C. An aliquot of 20 μL (strain stock) was inoculated in 5 mL of autoclaved TSB medium and incubated in a bacteriological incubator at 28 °C for 24 h. Thereafter, 200 mL of autoclaved TSB medium was added and incubated again following the same procedure. The bacterial suspension was centrifuged at 8000 *g* for 10 min and the supernatant was discarded, and then PBS buffer (0.01 M) was used to wash the pellets twice followed by centrifugation. Before the inoculation, the bacteria were resuspended in PBS (0.01 M) at a concentration 7.6×10^6 CFU mL^{-1}, adjusted by UFC counting after bacterial culture, and spectrophotometry ($OD_{600} = 1.095$). This concentration was determined previously in a sub-lethal dose trial (data not shown). No mortality was recorded during the experimental period.

2.6. Statistical analysis

The experimental design was completely randomized. All data were tested for homoscedasticity and normality with the Levene test and the Cramer-Von Mises test, respectively, then analyzed using one-way ANOVA and the means were compared using a Tukey's multiple range test. When necessary, data were transformed to fit a normal distribution, using the proper transformations according to each situation. Results are expressed as mean ± SD. The statistical analyses were performed with the software R V3.0.3.

2.7. Ethical statement

All experimental procedures that involved animal use in this study were performed in accordance with ethical principles in animal experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA), Brasilia, Brazil, and approved by the Comissão de Ética no Uso de Animais (CEUA) protocol n° 002214/12, UNESP Jaboticabal Campus.

3. Results

We induced an acute stress response prior to bacterial inoculation in fish fed with LHC to evaluate its immunostimulatory activity in fish under conditions that could impair the immune system. We assessed stress (plasma cortisol and glucose levels) and innate immune activity (respiratory activity of leukocytes, complement system activity and lysozyme concentration in serum) indicators in fish after 3 min of air exposure and immediate bacterial infection (stressful handling).

3.1. Stress indicators

3.1.1. Plasma cortisol

Plasma cortisol concentrations increased (Fig. 1) at 1 h after stressful handling in fish from all treatment groups ($P = 0.0001$). However, 300 and 500 mg L^{-1} LHC attenuated the increase observed at these sampling. After 3 h, the hormone concentrations decreased in general and returned to the initial values. Only fish that received 300 mg L^{-1} LHC presented cortisol levels lower than those of control fish. At 24 h, the hormone concentrations remained similar to the initial values.

3.1.2. Plasma glucose

Plasma glucose concentrations showed the highest values (Fig. 2) at 1 and 3 h after stressful handling ($P = 0.0101$), then returned to the initial values after 24 h, without any observed effect of levamisole.

3.2. Immunological indicators

3.2.1. Leukocyte respiratory burst (LRB)

Regardless of the LHC level, the LRB increased (Fig. 3) at 1 h after stressful handling ($P = 0.0013$), and remained high at 3 and 24 h compared to the initial sampling. After 1 h, the highest LRB was observed in fish fed with 100 mg kg^{-1} LHC, whereas in those receiving 300 and 500 mg kg^{-1}, the values were intermediate compared to control fish.

3.2.2. Hemolytic activity of the complement system (HAC_{50}) alternative pathway

The HAC_{50} decreased in fish from all treatment groups (Fig. 4) at 3 h after stressful handling ($P = 0.0102$), as observed by the increase in the time required to promote the lysis of rabbit erythrocytes, and returned to initial values 24 h after stressful handling. This reduction was attenuated in fish that received 100 mg kg^{-1} LHC compared to control fish. Fish that received the other LHC concentrations presented intermediate values. At 24 h, the HAC_{50} was higher in all fish that received LHC compared to the control group.

3.2.3. Serum lysozyme activity (SLA)

The SLA was not influenced by LHC (Fig. 5), but only by stressful handling ($P = 0.0113$). There was a gradual reduction at 1 and 3 h after the stressor in all treatments, followed by an increase at 24 h compared to the values observed at 1 h.

4. Discussion

The primary result of the current study is that levamisole (LHC) added to feed, and provided to fish for 15 days, prevented the increase in circulating cortisol and improved the profile of the innate immune response in pacu after fish were exposed to air and inoculated with *A. hydrophila*. We used the strategy of stimulating the

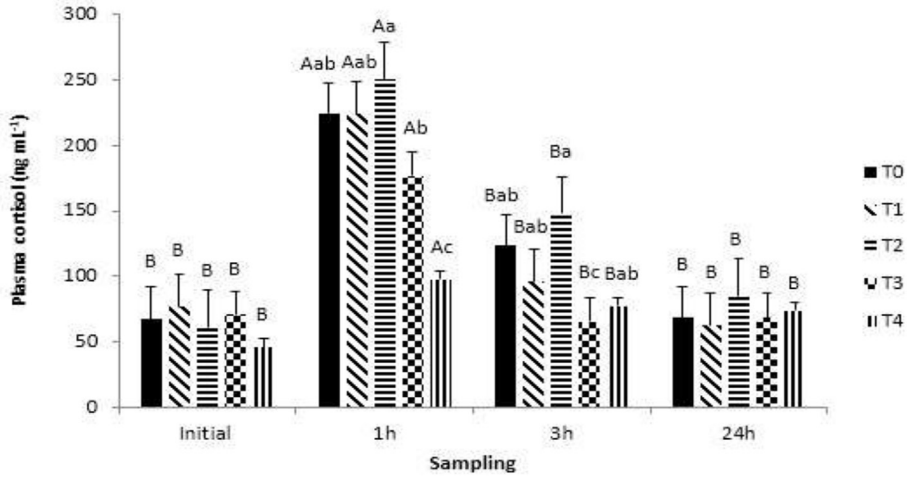

Fig. 1. Plasma cortisol concentrations of pacu (n = 8) fed for 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}) and T4 (500 mg kg^{-1}), submitted to 3 min of air exposure, inoculated with *Aeromonas hydrophila* and sampled 1, 3 and 24 h later. Different capital letters indicate differences between sampling and different lower case letters indicate differences between treatments.

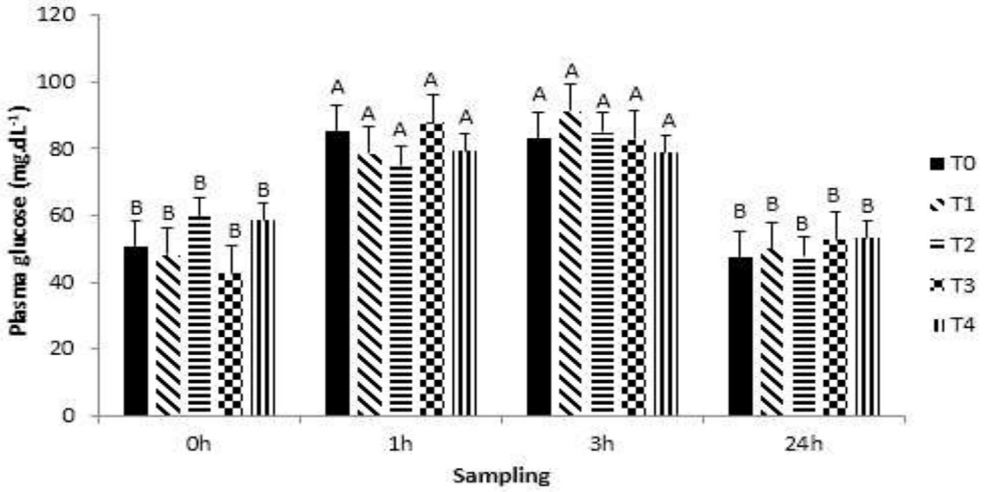

Fig. 2. Plasma glucose concentrations of pacu (n = 8) fed for 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}) and T4 (500 mg kg^{-1}), submitted to 3 min of air exposure, inoculated with *Aeromonas hydrophila* and sampled 1, 3 and 24 h later. Different capital letters indicate differences between sampling and different lower case letters indicate differences between treatments.

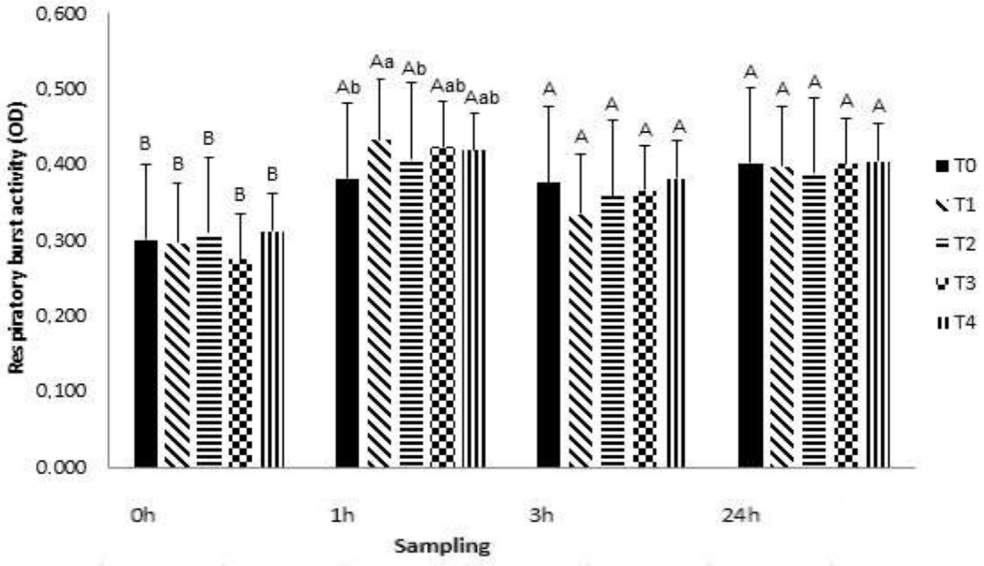

Fig. 3. Leukocyte respiratory burst (LRB) of pacu (n = 8) fed for 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}) and T4 (500 mg kg^{-1}), submitted to 3 min of air exposure, inoculated with *Aeromonas hydrophila* and sampled 1, 3 and 24 h later. Different capital letters indicate differences between sampling and different lower case letters indicate differences between treatments.

immune system with bacteria under stress conditions in fish previously fed with LHC.

The stress condition in our study was characterized by increased levels of circulating cortisol and glucose. Cortisol secreted during stress mediates a biological adjustment to adapt fish to the homeostatic alteration caused by the stressor. However, depending on severity and chronicity, the mortality risk increases due to the loss of the adaptive capacity of fish [3,40]. Stress caused by the practices of fish farming is a potential factor that causes increased susceptibility of fish to pathogens [3], thus increasing the probability of parasitic infections as a tertiary response [9] and even contributing to mortality [41]. High levels of cortisol are associated with immunosuppression in the winter months [42] and crowding [43] in gilthead seabream (*Sparus aurata*), as well as to a reduction in leukocyte populations after the onset of single or multiple cold shocks [44]. Elevated blood cortisol also suppresses, *in vitro*, the expression of interleukin-1β, tumor necrosis factor-α, serum amyloid A and inducible nitric oxide synthase induced by infection with *Trypanoplasma borreli* [44,45] and depresses the respiratory burst activity of head-kidney leukocytes and the phagocytosis of yeast cells [46]. Additionally, plasma cortisol elevated by oral administration decreases the percentage of circulating B lymphocytes in carp [47].

In our study, LHC reduced the stress-induced elevation of plasma cortisol at 1 h after air exposure and bacterial challenge in fish fed with 500 mg kg^{-1} and after 3 h in fish fed with 300 mg kg^{-1}. The reduction may have attenuated the immunosuppressive effects of cortisol, observed with high concentrations of this hormone [42–47]. Cortisol returned to initial values within 3 h in fish fed with LHC. To the best of our knowledge, no similar results were described in fish. These data are innovative in aquaculture, suggesting the dietary supplementation of pacu with levamisole as a

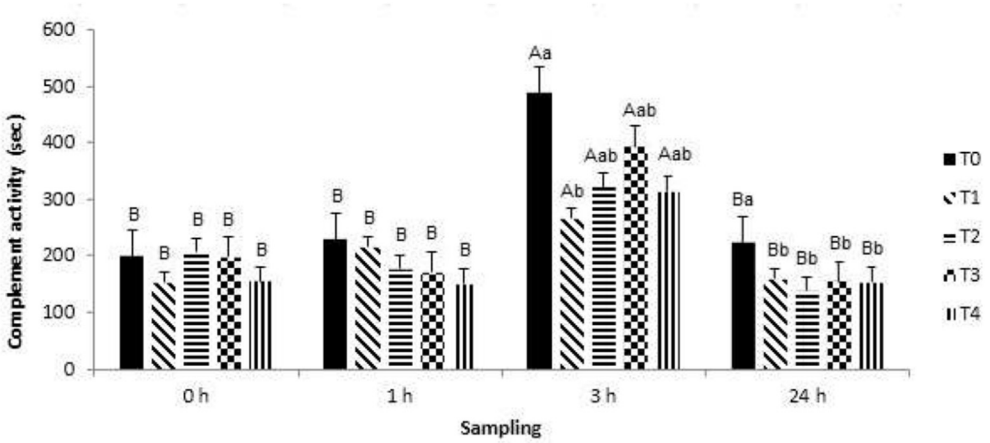

Fig. 4. Hemolytic activity of complement system (HAC_{50}) of pacu (n = 8) fed for 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}) and T4 (500 mg kg^{-1}), submitted to 3 min of air exposure, inoculated with *Aeromonas hydrophila* and sampled 1, 3 and 24 h later. Different capital letters indicate differences between sampling and different lower case letters indicate differences between treatments.

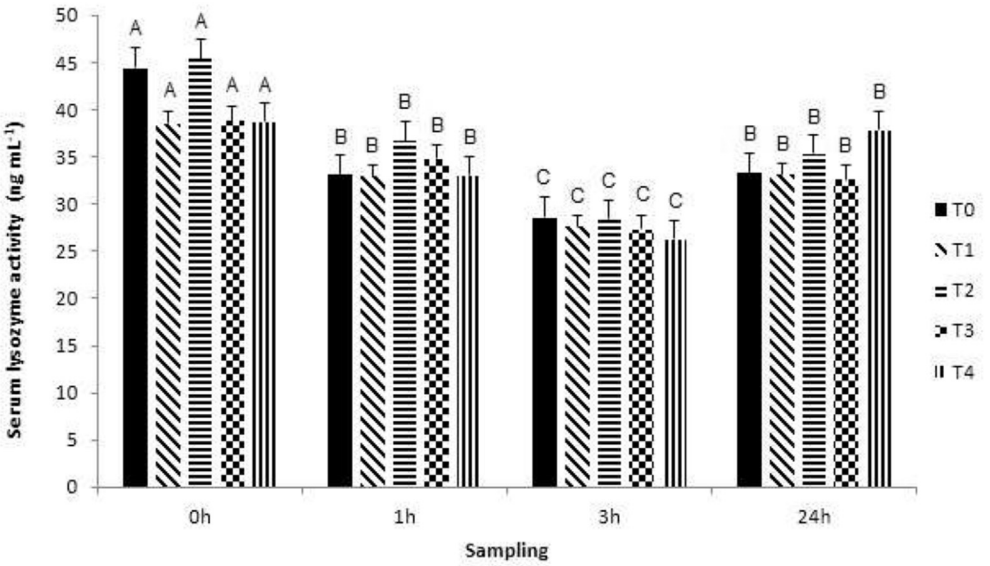

Fig. 5. Serum lysozyme activity (SLA) of pacu (n = 8) fed for 15 days with levamisole hydrochloride: T0 (control), T1 (100 mg kg^{-1}), T2 (150 mg kg^{-1}), T3 (300 mg kg^{-1}) and T4 (500 mg kg^{-1}), submitted to 3 min of air exposure, inoculated with *Aeromonas hydrophila* and sampled 1, 3 and 24 h later. Different capital letters indicate differences between sampling and different lower case letters indicate differences between treatments.

viable alternative, to avoid the deleterious effects of stress management of transport and biometrics, in intensive production systems. In this line, a previous study showed a reduction in plasma corticosterone in rats following antigen challenge with ovalbumin, when fed a diet containing levamisole (18 mg kg^{-1}) for 14 days, although the animals were not stressed [27]. The authors suggested that immune stimulation caused by levamisole was mediated by a reduction in blood corticosterone, either due to the inhibition of hormone synthesis or faster metabolic clearance.

Despite the effect of LHC on cortisol levels, it had no influence on elevated plasma glucose concentrations 1 h after stress, which with cortisol confirmed the stress caused by handling of the fish. Elevated concentrations of cortisol and glucose are reliable stress indicators [1], suggesting that air exposure and the presence of bacteria in the body were stressful to pacu. Fish submitted to a stressor factor, through the neuroendocrine system, release catecholamines, and secondarily cortisol, in an attempt to fight for their survival [48]. According to [1], increased plasma glucose concentration in the body can help to relieve the effects caused by stress by increasing the concentration of energy and oxygen available to the tissues and brain.

The fish immune system is divided into the innate (non-specific) and the acquired (specific) immune system and is of primary importance in combating infections in fish. The components of the innate immune system are commonly divided into physical parameters, cellular and humoral factors. Among the cellular and humoral factors, different phagocytes, lysozyme and the lytic pathway of the complement system act together in the identification and destruction of invading pathogens [49,50]. Thus, the quantification of the physiological parameter as respiratory activity of leukocytes, complement system, and lysozyme assists the understanding of the functioning of the innate immune system, when the fish ingest diet supplemented with immunostimulant in aquaculture systems [11].

In our study, LHC (100 mg kg^{-1}) enhanced the leukocyte respiratory burst (LRB) 1 h after stress and bacterial inoculation in fish. During phagocytosis, phagocytic cells such as neutrophils, monocytes and macrophages increase their consumption of molecular oxygen and respiratory burst activity to produce large quantities of antimicrobial reactive oxygen species (ROS) [51].

Our results are consistent with previous studies that showed activation of the LRB by levamisole. Leukocyte functions, including phagocytosis and respiratory burst activity in phagocytes, were enhanced by the dietary intake of levamisole in gilthead seabream (*Sparus aurata*) [22]. Other studies also showed stimulating effect of levamisole, however through routes of administration different from that we used. Rainbow trout (*Oncorhynchus mykiss*) were exposed by immersion to different concentrations of levamisole solution and the activity of phagocytic cells against *Yersinia ruckeri* in fish exposed to the drug solutions was found higher than that in controls [23]. In another study, trout injected intraperitonially with levamisole presented *in vitro* increase in the phagocytic activity of leukocytes [52]. However, levamisole failed to increase the phagocyte function *in vitro* in seabream (*Sparus aurata*) [21].

Levamisole also influenced the hemolytic activity of complement system (alternative pathway - HAC_{50}) in our study. At 3 h after stress and bacterial challenge the HAC_{50} was reduced in fish from all groups, though the reduction has been attenuated by levamisole, especially in 100 mg kg^{-1} treatment. At 24 h, HAC_{50} returned to initial values, before the bacterial challenge, and was higher in all fish fed with LHC, to control the bacterial infection, confirming the immune stimulant effect of the drug in pacu. Previous studies showed influence of levamisole in the activation of complement system in fish, though using different experimental protocols. In gilthead seabream (*Sparus aurata*), serum complement activity was found to be enhanced by 10 days of dietary treatment with levamisole, up to 10 weeks after feeding [22]; in contrast, in our study, we provided the drug for 15 days and sampled fish immediately after feeding. Recently, striped catfish (*Pangasianodon hypophthalmus*) intraperitonially injected with levamisole and challenged with *Edwardsiella ictaluri* showed activation of the complement system [19].

Lysozyme is an important defense molecule of the innate immune system, which is important in mediating protection against microbial invasion. It is well-documented that fish lysozyme possesses lytic activity against both Gram-positive bacteria and Gram-negative bacteria [53]. In our study, however, lysozyme serum activity was reduced in stressed and bacteria-challenged fish without an effect of levamisole. On the other hand, carps fed with 250 mg L^{-1} levamisole, for a prolonged period (90 days), and challenge with *A. hydrophila* showed higher lysozyme activity, but with a low survival rate of 66.7% [27]. Similarly, in another study, juvenile striped catfish that received 5 mg kg^{-1} levamisole intraperitoneal injection, and challenge with *Edwardsiella ictaluri*, after 21 days presented higher lysozyme activity, compared to control fish, evidencing the protective lytic activity of this enzyme against pathogens [19].

Considering all our results together, dietary levamisole provided for 15 days modulated the circulating levels of cortisol during the stress response and enhanced the innate immune response (leukocyte respiratory burst and complement system) of pacu against *A. hydrophila.* The air exposure followed by bacterial challenge was a stressful condition and impaired lysozyme activity without an effect of levamisole. These results contribute positively to pacu aquaculture, especially in intensive production systems, in which the stressful management normally results in immunosuppression. In addition, levamisole is an inexpensive immunostimulant and easily accepted in the diet, making viable its offer to the fish.

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