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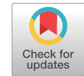
Antioxidant enzymes, hematology and histology of spleen in Nile tilapia fed supplemented diet with natural extracts challenged with *Aeromonas hydrophila*



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ABSTRACT

This study investigated the effects of dietary supplementation with the extrats of propolis and *Aloe barbadensis* (aloe) on the antioxydant enzime activity, hematology and histology of the spleen of Nile tilapia challenged with *Aeromonas hydrophila*. Seventy two juvenile Nile tilapia were divided in four treatments and three replicates and fed extract mixture for 15 days: fish fed supplemented diet with 1% of the mixture of extracts of propolis and aloe (1:1) injected with phosphate-buffered saline (PBS); fish fed suplemented diet with 1% of the mixture of extracts of propolis and aloe (1:1) injected with the *A. hydrophila*, fish fed supplemented diet with the mixture of propolis extracts and aloe, injected with PBS and injected with *A. hydrophila*. The influence of the supplementation of propolis and Aloe extracts on the immunomodulation in tilapias was observed by the evaluation of the survival of the animals after challenge with *A. hydrophila*. Non-supplemented fish had a 44.5% survival rate and those supplemented with 1% of the mixture of extracts showed 55.6% survival 7 days after challenge. The supplemented animals also showed a significant increase in the number of lymphocytes in the evaluation of the blood parameters and, consequently, in the histopathological evaluation, presented greater presence of centers of melanomacrophages. In addition, the activity of the antioxidant enzymes glutathione reductase (GR) in the spleen presented a significant difference in fish supplemented with 1% of the extracts mixture, being superior in the animals injected with PBS when compared to those challenged with *A. hydrophila*.

1. Introduction

Bacterial diseases are among the most important causes of economic losses in fish farming [1], *Aeromonas* spp., *Pseudomonas fluorescens*, *Vibrio anguillarum*, *Flavobacterium columnare*, *Edwardsiella tarda*, *Streptococcus* spp. and *Enterococcus* sp. are the main cause of mortality [2]. Excessive use of antibacterial drugs in fish farming to combat pathogens is responsible for changes such as immunosuppression, nephrotoxicity, low growth, development of resistant bacterial strains, environmental problems such as effluents from fish farming and bioaccumulation in fish [3].

According to Yonar et al. [4], these products may interact with lymphoid tissues and may alter immune system functions and balance, leading to undesirable effects such as immunosuppression, uncontrolled cell proliferation, and other changes that may unbalance the body's defense mechanisms against pathogens. Some substances have been used to stimulate the immune system, acting through different mechanisms seeking for the improvement of the defense response.

Substances of plant origin have been studied for the purpose of immunostimulating fish against possible bacterial infections [5–7]. The extracts of propolis and *Aloe barbadensis* Miller have shown good results as immunostimulants [7–9]. The administration of immunostimulatory substances prior to infection can improve the fish defense system and protect against infections [10]. Biochemical, metabolic and hematological characteristics can be used to evaluate the influence of these products on the immune system. Studies with experimental bacterial infection in fish are usually carried out [11,12], but few demonstrate the hematological and enzymatic changes that the infection can cause, especially in fish supplemented with natural extracts of propolis and aloe.

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Nile tilapia (*Oreochromis niloticus*) fed with diet containing 1% propolis-ethanolic-extract, presented increase in the specific growth rate, food efficiency, hematocrit percentage, monocyte number, lysozyme activity, bactericidal activity of the serum and protection against *A. hydrophila*, when compared to those fed unsuppelemented diet or with diet containing crude propolis [13]. Similarly, studies have shown that Nile tilapia (GIFT variety) fed diet containing different concentrations of *Aloe vera* (0.5% 1%, 2%, and 4%) presented enhanced growth, food conversion and hemato-biochemical parameters, but *Aloe* had no significant effect on the survival of fish when compared to control [14].

The present study evaluated the effects of supplemented diet with propolis and aloe on the activity of antioxidant enzymes, hemato-immunological parameters and histology of the spleen in Nile tilapia after challenge with *A. hydrophila*.

2. Material and methods

2.1. Experimental conditions

Nile tilapia juveniles (n = 72; 57.3 ± 11.2 g weight and 17.6 ± 4.2 cm of total length) from the same spawning, were stocked in polyethylene water tanks with 100 L capacity, equipped with biological filter, heater and constant aeration, maintained in the following conditions: average temperature of 24.0 ± 2.8 °C, pH 6.51 ± 0.43 (Alfakit®, AT-350), total ammonia 0.90 ± 0.33 mg L^{-1} (Alfakit®, colorimetric method) and dissolved oxygen 6.0 ± 0.7 mg L^{-1} (Hanna®, HI 9146). Seven days after acclimatization, the experiment was started with the supplemented diets for a period of 15 days. For this, the animals were randomly distributed in tanks, with four treatments in triplicate: fish fed supplemented diet with 1% of the mixture of extracts of propolis and aloe (1:1) injected with phosphate-buffered saline (PBS); fish fed suplemented diet with 1% of the mixture of extracts of propolis and aloe (1:1) injected with the *A. hydrophila*, fish fed supplemented diet with the mixture of propolis extracts and aloe, injected with PBS and injected with *A. hydrophila*.

2.2. Preparation of experimental diet

Commercial diet Nicoluzzi® 4 mm 36% crude protein was used throughout the experimental period. The extracts of propolis and aloe were prepared at the Laboratory of Morphogenesis and Plant Biochemistry, Department of Plant Science, UFSC; being the propolis used in this experiment was from apiaries of the state of Santa Catarina, from *Eucalyptus grandis* shoots and from *Araucaria angustifolia* resin. For each experimental unit used, the biomass of fish was calculated to determine the amount of feed at equivalent to 3% of live weight. A dilution of the extracts in 50% alcohol was prepared to form the 1:1 mixture in concentration 1%, included by spraying the feed pellets.

2.3. Challenge with Aeromonas hydrophila

After 15 days of feeding, the fish were challenged with *A. hydrophila*. The strain prepared in the Marine Shrimp Laboratory, Department of Aquaculture, UFSC, was incubated in test tubes with brain culture infusion (BHI) liquid culture medium at 30 °C for 24 h, after which the contents were centrifuged for 15 min at 10000 g and the precipitate was resuspended in 10 mL of sterile physiological solution. For the bacterial count present in the inoculum, five serial 1:10 dilutions were performed, seeded on plates containing tryptone soya agar (TSA) culture medium. After checking the concentration of the bacteria in the inoculum, the dilution was performed in order to reach the ideal concentration for the challenge. The experimental infection was performed by injecting 100 μL of *A. hydrofila* per fish, at a concentration of 5×10^6 CFU mL^{-1} [15].

2.4. Hematological analysis

After the feeding period, nine animals from each treatment were anesthetized with eugenol Vetec® (100 mg L^{-1}) for collection of blood by puncture of the caudal vessel using a syringe containing 10% EDTA solution. The collected blood was separated into aliquots for different analyzes: percentage of phagocytosis, erythrocyte counts in Neubauer's chamber and confection of duplicate blood extensions later stained with May-Grunwald/Giemsa by the Rosenfeld method [16], later used for differential counting of leukocytes and total count of the number of thrombocytes and leukocytes [17]. The hematocrit rate was performed according to [18] and, after reading, the capillary was broken slightly above the white blood cell range and the plasma was transferred to the total protein refractometer for total plasma protein (PPT) according to [19].

2.5. Collection and preparation of spleen samples

After blood collection, the animals were euthanized by deepening the anesthetic state (Ethics Committee on the Use of Animals: no 23080.009240/CEUA/UFSC) for the collection of spleen samples individually packed in cryogenic tubes rapidly frozen in nitrogen liquid, stored in a freezer −80 °C. For biochemical assays, the samples were homogenized in 20 mM HEPES buffer, pH 7.4 and centrifuged at 20,000 g for 30 min (4 °C). The supernatant was used for the determination of the enzymatic activities [22].

For the histological analysis, fragments of the spleen were fixed in 10% buffered formalin solution and submitted to routine histological procedures of dehydration, diaphanization and paraffin inclusion. Duplicate cuts, 4 μm thick were stained with Harris hematoxylin and 1% aqueous eosin [20]. Based on the quantification of the melanomacrophage centers (CMM), the histopathological analysis was done by stereology using the Weibel graticule coupled to the optical microscope and the fraction of the organ volume calculated according to formula [21].

2.6. Biochemical analyzes

The quantification of total protein levels was determined by the Bradford method [22] using bovine serum albumin as standard, in order to normalize the enzymatic activity.

Samples homogenized in buffer solution were used to determine the biochemical parameters. The enzymatic assays were performed in a final volume of 500 μL, with the exception of catalase activity, whose final volume was 1 mL. It was also used between 5 and 100 μL of the sample, depending on the parameter to be analyzed.

2.6.1. Glutathione reductase (GR)

The activity of glutathione reductase was based on the method described by Carlberg and Mannervik [23]. By reducing the glutathione disulfide substrate (GSSG), GR oxidizes NADPH, which can be monitored by decreasing absorbance at the wavelength of 340nm. Thus, the rate of NADPH consumption expresses the activity of this enzyme.

2.6.2. Glutathione-S-transferase (GST)

The conjugation of glutationa (GSH) to the chlorodinitrobenzene (CDNB) substrate catalyzed by GST yields a compound that can be detected at 340nm ($\varepsilon = 9600\,M^{-1}\,cm^{-1}$). The enzymatic activity is proportional to the rate of production of the conjugated compound [24]. From this activity is taken the basal reaction obtained by reading the reaction between the GSH of the assay and the CDNB, without the presence of the sample. The 5 min enzyme assay was performed in 100 mM potassium phosphate buffer (KPi), 1 mM EDTA, pH 7.0, containing 1 mM GSH. As the starting substrate 1 mM of CDNB was used. The basal absorbance was discounted from reading the test reaction in the absence of the sample.

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