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Levamisole promotes an adjuvant effect on the immunity of pacu (*Piaractus mesopotamicus*) when immunized with *Aeromonas hydrophila*, even when provided in the diet

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ABSTRACT

Dietary immunomodulators can help to defend against disease, but their dosage and time of administration are not always clear in aquaculture. In this study, we analyzed the amount of dietary levamisole required to induce an adjuvant effect. Because immunostimulants and immunization can improve the fish immune system, this study evaluated the effects of dietary levamisole administration (at 0, 125, 250 and 500 mg/kg diet) and immunization with *A. hydrophila* (1×10^9 CFU) on the acquired and innate immune systems of pacu (mean initial weight 176.03 ± 15.73 g). The results showed that levamisole can act as an adjuvant for vaccination, even when administered through the diet. Immunization and levamisole administration increased the antibody titer, serum bactericidal activity, and hematocrit as well as the numbers of red blood cells, leukocytes and thrombocytes in pacu ($P < 0.05$). However, the lysozyme activity; leukocyte respiratory activity; total protein, albumin, and globulin levels; A:G index; corpuscular hemoglobin volume; and other white blood cells showed no differences when compared with the control fish. As the immunostimulant improved, certain parameters of the acquired and innate immune systems of pacu increased, and the administration of levamisole in the diet (125 or 250 mg/kg) for seven days may be used to promote adjuvant effects during immunization with *A. hydrophila* and thus constitutes a feed protocol strategy to be used prior to immunization.

1. Introduction

The aquaculture industry in Brazil has been growing since the late 1980s, and many fish species are being farmed. However, this intensification has led to disease outbreaks. Because the use of antibiotics has been widely criticized during the past decade due to its negative impacts on the environment as well as on fish, dietary immunostimulants are being used as an alternative treatment. Immunostimulants can overcome several problems caused by the misuse of antibiotics,

Abbreviations: MHC, major histocompatibility complex; CFU, colony-forming unit; CP, crude protein; TSA, trypticase soy agar; PBS, phosphate-buffered saline; NBT, nitroblue tetrazolium; DMF, dimethylformamide; A:G, albumin:globulin; DE, digestible energy; SEM, standard error of the means; ROS, reactive oxygen species; HSI, hepatosomatic index; MCV, mean corpuscular value; SGC, special granulocyte cell.

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such as the accumulation of these drugs in the environment and the development of bacterial resistance and immune suppression (Sakai, 1999; Tort, 2011; Kiron, 2012; Baba et al., 2014). Nonetheless, there is a lack of information about the treatment period, dose and administration route for immunostimulants in several species of fish (Mulero et al., 1998; Sink and Lochmann, 2014).

The responses of the fish immune system can be modulated by certain substances, such as levamisole, a synthetic anthelmintic used in mammals that is also a strong trigger of innate and specific immunity in fish (Reverter et al., 2014; Siwicki, 1987). In fact, levamisole is considered to be an adjuvant compound upon vaccination because it improves the intensity of the immune response, although this immunostimulant is commonly administered via injection. Adjuvants are considered to enhance antigen-specific immune responses because they result in increased antibody titers. Levamisole is a T-independent antigen that prompts an acquired response based on its ability to modulate natural killer cells during cell lysis induction, MHC receptor expression and cytokine increases, including IFN-α and IL-6 (Holcombe et al., 2006; Kang et al., 2005).

Furthermore, this immunostimulant promotes improvements in innate parameters such as the cytotoxic activity of leukocytes (Cheng et al., 2008; Fierro-Castro et al., 2012), phagocytosis (Mulero et al., 1998; Findlay and Munday, 2000), and the respiratory activity of macrophages (Siwicki, 1989; Mulero et al., 1998). Levamisole also improves specific immune responses (Jeney and Anderson, 1993; Cuesta et al., 2004) and elicits increased resistance against various etiological agents, including *Vibrio anguillarum* (Kajita et al., 1990), *Aeromonas hydropila* (Baba et al., 1993), *Paramoeba* spp. (Findlay et al., 2000; Munday and Zilberg, 2003), *Edwardsiella tarda* (Sahoo and Mukherjee, 2002), *Photobacterium damselae* (Leano et al., 2003), and the nematode *Anguillicola crassus* (Geets et al., 1992).

The use of dietary immunostimulants is a viable approach to preventing the economic losses caused by disease outbreaks, such as infection by *A. hydrophila*,a gram-negative bacterium that is widely distributed in aquatic environments that has a negative economic impact on aquaculture worldwide (Austin and Austin, 1999; Janda and Abbott, 1998). However, few studies have been conducted to evaluate the immune system of Brazilian fish, and additional research into immune modulation is needed. Pacu, or *Piaractus mesopotamicus*, is a remarkable native fish with great economic importance because of its production characteristics, such as its rustic management, rapid growth rate and consumer acceptance (Takahashi et al., 2006; Castro et al., 2014). The present study was designed to explore the effects of dietary levamisole on the innate and adaptive immunity as well as the biochemical and hematological parameters of *P. mesopotamicus*.

2. Materials and methods

2.1. Animals and installations

A total of 240 pacu (176.03 ± 15.73 g; 20.29 ± 1.17 cm) were distributed among 24 100 L tanks (10 fish per tank, 6 replicate tanks for each treatment) and allowed to acclimatize for 20 days prior to experimental feeding; during this time, the fish were fed twice per day to apparent satiation with a commercial diet (28% crude protein, 3% fat, 1% fiber, levamisole-free). This study was approved by the Ethics Committee on animal use of the Universidade Estadual Paulista “Julio de Mesquita Filho” UNESP (protocol number 004206/10). A photoperiod of 12 h light:12 h dark (07.00-19.00 h) was used. The experiment was performed indoors in tanks that were supplied with a constant flux of water of 2-2.5 L per min. The culture system was also provided with continuous aeration by an air compressor. The tanks were cleaned of feces and uneaten feed every two days via aspiration. The water temperature, dissolved oxygen and pH were monitored daily using a YSI 55 Oxygen Meter (Yellow Spring Instrument, Yellow Spring, OH, USA) and a YSI 63 pH Meter (Yellow Springs Instrument, Yellow Springs, OH, USA), respectively. Total ammonia nitrogen was monitored every week (Nessler reagent). The values stayed within the appropriate ranges described for this species (Urbinati et al., 2010) as follows: temperature 27.86 ± 0.83 °C, dissolved oxygen 5.61 ± 0.66 mg/L, pH 7.57 ± 0.34 and NH_4 0.25 ± 0.15 mg/L.

2.2. Experimental diets

The diet that was used for fish acclimatization and as the base for the levamisole-containing food was a commercial diet containing 28% crude protein and an estimated digestible energy level of 12.56 MJ/kg (FRI-RIBE, Sao Paulo, Brazil). Levamisole (SIGMA, St. Louis, MO, USA) was added to the commercial diet at 0, 125, 250 or 500 mg/kg. During the conditioning and experimental periods, the fish were fed two daily meals until apparent satiety.

2.3. Feeding trials and experimental design

The experiment was divided in two trials, with the first feeding trial performed to evaluate the optimal doses and duration of levamisole administration and to determine the adjuvant effects of the drug on non-specific immune responses and the hematological and serum biochemistry parameters of pacu. In the first trial, the fish were randomly divided into eight treatments in a factorial design with two periods (seven and 15 days) and four levamisole levels (0, 125, 250 and 500 mg/kg); there were six replicates for each treatment. The experimental diets were offered to the fish in two daily meals for seven or 15 days.