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Microbial immunostimulants reduce mortality in whiteleg shrimp (*Litopenaeus vannamei*) challenged with *Vibrio sinaloensis* strains

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ABSTRACT

The effect of microbial immunostimulants on the survival and immune response of juvenile *Litopenaeus vannamei* challenged with *Vibrio sinaloensis* strains was evaluated. Dead microorganisms were added to feed with the attractant Dry Oil® and consisted of four lactic acid bacteria (Lta2, Lta6, Lta8, and Lta10) and one yeast (Lt6). *V. sinaloensis* strains or saline solution were inoculated to shrimp by injection. The bioassay was conducted for 21 days with five treatments in triplicate: (I) shrimp fed with commercial feed + sterile saline solution at 2.5% NaCl (control group I); (II) shrimp feed with commercial feed + LD_{50} *Vibrio* (control group II); (III) shrimp fed daily with experimental diet + LD_{50} *Vibrio*; (IV) shrimp fed every 3 days with experimental diet + LD_{50} *Vibrio*; and (V) shrimp fed every 6 days with experimental diet + LD_{50} *Vibrio*. Shrimp (8.1 ± 1.4 g) were cultured in 120-L plastic tanks and fed twice a day. The activity of lysosomal enzymes in plasma and hemocytes were determined with the API ZYM kit and lysoplate assay. Survival of shrimp in treatment IV was significantly higher than those of control II. Total hemocyte count in treatment III was significantly higher than control II. The activity of nine hydrolytic enzymes was found in plasma and six in the hemocyte lysate supernatant (HLS). Shrimp fed with immunostimulants every six days were not protected against *V. sinaloensis*. The results indicate that these microbial immunostimulants administered every three days is a good feed additive against *Vibrio* spp. in shrimp culture.

1. Introduction

Aquaculture represents one of the main food providers in the world. In shrimp farming, whiteleg shrimp (*Litopenaeus vannamei*) is the primary penaeid shrimp currently being cultured in Central and South America (Burge et al., 2007; Chang-Che and Jiann-Chu, 2008). However, during the past two decades, worldwide commercial shrimp farming has suffered outbreaks of diseases caused mainly by *Vibrio* bacteria and viruses due to a deteriorated pond environment (Lo et al., 2003).

Species of *Vibrio* are well known in penaeid shrimp culture as causative agents of vibriosis, which is a serious threat to the aquaculture industry, responsible for massive mortality of cultured penaeids worldwide (Baticados et al., 1990; Lightner and Lewis, 1975). Treatment of shrimp suspected of being infected with *Vibrio* spp. is mainly based on the use of antibiotics, but the susceptibility of vibrios to antibiotics varies widely among strains of the same species. There is little information about the detailed use of antibiotics; even their use in aquaculture may develop antibiotic resistance in pathogens that infect those cultured animals and humans (Soto-Rodríguez et al., 2008).

The application of immunostimulants in shrimp aquaculture is increasingly gaining interest as an environmentally safe alternative to antibiotics and chemotherapeutics (Song et al., 1997). Shrimp possess an innate immune system, consisting of cellular and humoral elements. Hemocytes play a central role in the non-specific immune response of shrimp, which rely mainly on phagocytosis, melanization, encapsulation, cytotoxicity, and clotting (Sritunyalucksana et al., 1999). Humoral defense factors, such as clotting proteins, agglutinins, hydrolytic enzymes, and antimicrobial peptides are released upon lysis of hemocytes, which is induced by lipopolysaccharides (LPS), peptidoglycans, and β-1,3-glucans (Chisholm and Smith, 1995; Destoumieux et al., 2000; Johansson and Söderhäll, 1989; Muta and Iwanaga 1996; Söderhäll et al., 1994).

Diets containing immunostimulants are used in aquaculture in order to increase resistance to stress and diseases of cultured fishes and invertebrates by alerting the immune system (Doñate et al., 2010; Rendón and Balcázar, 2003). They can be extracted from the walls of microorganisms such as Gram-negative bacteria (lipopolysaccharides), Gram-positive bacteria (peptidoglycans), and fungi (β-1, 3-glucans). Furthermore, the whole cell can be used as immunostimulant (Sajeevan et al., 2009a; Partida-Arangure et al., unpublished data). There are

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several methods of stimulation like immersion, injection, and feeding among others (Chen and Ainsworth, 1992; Jorgensen et al., 1993; Rorstad et al., 1993). However, the most practical method of stimulation is incorporating the immunostimulating substances into the feed (Azad et al., 2005).

Currently many commercial immunostimulants are available in the shrimp aquaculture industry and are extensively used by shrimp farmers. However, scientific data in support of their function and dose/frequency of application are lacking. Information regarding the dose is essential as overdose leads to immunosuppression, rendering less protection to infection (Chang et al., 2000; Sajeevan et al., 2006, Sajeevan et al., 2009b).

This study was undertaken to examine the survival and the immune response of *L. vannamei* against *Vibrio sinaloensis* strains when shrimp were treated with whole-cell microbial immunostimulants.

2. Materials and methods

2.1. Microbial immunostimulants

Lactic acid bacteria (Lta2, Lta6, Lta8, and Lta10) and yeast (Lt6) used in this work as immunostimulants were originally isolated, characterized, and tested by Apún-Molina et al. (2009) and Peraza-Gómez et al. (2011).

2.2. Preparation of experimental diet with microorganisms

A mixture of four selected lactic acid bacteria (LAB) and one heat-killed yeast (74 °C) was sprayed on commercial feed (Purina®, Ciudad Obregón, Mexico, 40% protein) at 8.4 mg kg feed^{-1} (1.68 mg per microbial isolate). The amount of immunostimulants was based on the work of Partida-Arangure et al. (unpublished data) whose work with the same microorganisms at a concentration of 2×10^6 CFU g feed^{-1} (4×10^5 CFU strain^{-1}). Microorganisms were grown, washed, and count as in Apún-Molina et al. (2009). Cells (2×10^6) from each isolate were centrifuged at 12,000 *g*, dried in an oven (Felisa, Jalisco, Mexico) at 74 °C for 4 h, and weighed. The dried cell pellet was ground in a mortar. Dry Oil® (Innovaciones Acuícolas, S.A. de C.V., Culiacán, Mexico) was used as adhesive and as feed attractant. Feed was dried at room temperature and stored at 4 °C for 6 days (Peraza-Gómez et al., 2011; Partida-Arangure et al., unpublished data).

2.3. V. sinaloensis strains

A mixture of *V. sinaloensis* strains (VHPC18, VHPC23, VHPC24, and VIC30), isolated from *L. vannamei*, was used to challenge white shrimp with a lethal doses 50 value (LD_{50}) of 1.178×10^5 CFU g^{-1} of body weight. Overnight cultures (TS broth) of the bacterial strains to be tested were washed by centrifugation (10,000 *g* for 10 min) and suspended in sterile saline solution (2.5% NaCl). The bacterial suspensions were adjusted to an optical density of one. The experimental inoculation of bacteria was performed with a mixture containing isolates VHPC18, VHPC23, VHPC24, and VIC30 at the same proportion. Shrimp were injected into the first abdominal segment with 40 μL of either bacterial mixture or saline solution (2.5% NaCl) using a sterile 1-mL syringe with a 25-gauge needle (Flores-Miranda, unpublished data).

2.4. Shrimp acclimation to laboratory conditions

The healthy shrimp selection was done for visible features. Shrimp were acclimated to ambient laboratory conditions for 5 days in 120-L indoor plastic tanks containing 80 L of filtered (20 mm) sea water (34–35‰) and constant aeration in groups of 10 organisms per tank. Shrimp were fed twice daily at 09:00 and 17:00 h with commercial feed (Purina®, 40% protein). Ration was 6% of the body weight. Half of the water was changed at day 3 and uneaten food and waste matter were removed daily before feeding.

2.5. Disease resistance trial

Shrimp weighing 8.1 ± 1.4 g were fed with experimental diet (Purina®, 40% protein) with immunostimulants at different frequencies. The bioassay was conducted for 21 days as a completely randomized design with five treatments in triplicate: (I) shrimp fed with commercial feed + sterile saline solution at 2.5% NaCl (control group I); (II) shrimp feed with commercial feed + LD_{50} *Vibrio* (control group II); (III) shrimp fed daily with experimental diet + LD_{50} *Vibrio*; (IV) shrimp fed every 3 days with experimental diet + LD_{50} *Vibrio*; and (V) shrimp fed every 6 days with experimental diet + LD_{50} *Vibrio*. During the first 6 days, animals in all treatments were fed with their respective diet, on the 7th day the shrimp were injected intramuscularly with 40 μL of either vibrio mixture (treatments II–VI) or saline solution (treatment I). During the bioassay, the water temperature was maintained at 23.3 ± 0.1 °C, oxygen at 6.2 ± 0.2 mg mL^{-1}, and salinity 35‰. Water parameters measured during the trial period remained within acceptable ranges (Brock and Main, 1994).

At the end of the bioassay, the survival percentage was determined and hemolymph samples were collected to determine immunological parameters.

2.6. Hemolymph collection and total hemocytes count

Hemolymph was sampled from 12 intermolt shrimp per treatment and total hemocytes count was determined. Hemolymph (100 μL) of individual shrimp was withdrawn from the pleopod base of the first abdominal segment with a sterile 1-mL syringe (25 G × 13 mm needle). Before hemolymph extraction, the syringe was loaded with a precooled (4 °C) solution (SIC-EDTA, Na_2) (450 mM NaCl, 10 mM KCl, 10 mM hepes and 10 mM EDTA, Na_2 at pH 7.3) used as an anticoagulant (Vargas-Albores et al., 1993). Fifty microliters of the anticoagulant–hemolymph mixture were diluted in 150 μL of formaldehyde (4%) and then 20 μL were placed on a hemocytometer (Neubauer) to determine the total hemocytes count (THC) using a compound microscope. The remainder of the hemolymph was stored individually in Eppendorf tubes and kept on ice for separation of plasma and hemocytes.

2.7. Separation of plasma and hemocytes

Samples of hemolymph were immediately centrifuged at 800 *g* for 10 min at 4 °C and the plasma was frozen at −80 °C. The hemocyte pellet was re-suspended and washed once in precooled anticoagulant solution by centrifugation at 800 *g* for 10 min at 4 °C. Finally, the hemocytes were re-suspended in 200–600 μL cacodylate bufer (10 mM, pH 7).

2.8. Preparation of hemocyte lysate supernatant (HLS)

Samples were frozen at −80 °C to break the hemocytes and then thawed; this procedure was carried out twice. Individual samples were centrifuged at 15,000 *g* for 10 min at 4 °C and the HLS was used immediately to run the immunological analysis or stored at −80 °C.

2.9. Enzymatic activities in plasma and HLS (The API ZYM system)

The API ZYM® commercial kit for enzymatic activity detection (BioMerieux, Durham, NC, USA) is a semiquantitative colorimetric micromethod to assess 19 hydrolytic enzymes (proteases: leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, a-chymotrypsin; lipases: lipase esterase (C8), lipase (C14); glycosidases: a-galactosidase, b-galactosidase, b-glucuronidase, a-glucosidase,