

# Acuabio 1, an enhancer of the immune system in *Litopennaeus vannamei* shrimps

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## ABSTRACT

Acuabio 1 is a mixture of proteins and essential amino acids that exerts a stimulating effect in the development of aquatic organisms. It synchronizes and accelerates fish and shrimp larvae growth on early developmental stages, as well as increases the development and size of these animals. In this work, the stimulating effect of Acuabio 1 on the immune system of *Litopennaeus vannamei* shrimps was demonstrated by obtaining ( $5.4 \times 10^6$  hemocytes/mL) greater than the control group ( $4.3 \times 10^6$  hemocytes/mL). The hemolymph of treated animals showed higher lectin titers (16) than the control group (8). Animals treated with Acuabio 1 increased their phenoloxidase and peroxidase activities, indicating an enhanced oxidative defense. Furthermore, Acuabio 1 decreased the number of *Vibrio* spp. and *Pseudomonas* spp. colony forming units in the culture medium, suggesting a possible prebiotic function. All these properties make Acuabio 1 very attractive to improve the production, survival rate and quality of shrimp larvae.

Keywords: Immune system, Acuabio 1, phenoloxidase, hemocytes, shrimp

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## RESUMEN

**Acuabio 1, un estimulador del sistema inmune de camarones *Litopennaeus vannamei*.** Acuabio 1 es una mezcla de proteínas y aminoácidos esenciales que ejerce un efecto estimulador en el desarrollo de los organismos acuáticos. Sincroniza y estimula el crecimiento de larvas de peces y camarones. Con su empleo se activa y acelera el mecanismo de crecimiento desde edades muy tempranas, así como el desarrollo y la talla de los animales. En el presente trabajo se evidencia el efecto estimulador del sistema inmune del producto Acuabio 1 en camarones (*Litopennaeus vannamei*). Se determinó que al tratar los animales con este producto aumentó el número de hemocitos totales ( $5.4 \times 10^6$  hemocitos/mL) con respecto a los del grupo control ( $4.3 \times 10^6$  hemocitos/mL). Se observó que la hemolinfa de los camarones tratados tuvo mayor título de lectinas (16) comparado con el control (8). En los animales tratados con Acuabio 1 se encontró un aumento de las actividades específicas fenoloxidasas y peroxidasas comparadas con las del control, lo que indicó un aumento de la defensa oxidativa en estos animales. También se halló que Acuabio 1 disminuyó el número de unidades formadoras de colonias en el medio para *Vibrio* spp. y *Pseudomonas* spp., lo que sugiere una posible función prebiótica. Estas propiedades del producto Acuabio 1 lo hacen muy atractivo para su utilización en el mejoramiento de la producción, supervivencia y calidad de las larvas de camarones.

Palabras clave: sistema inmune, Acuabio 1, fenoloxidasas, hemocitos, shrimp

## Introduction

The total global production of shrimp culture reached more than 1.5 tonnes in 2002, accounting for 49% of the total shrimp capture. It is estimated an increase (12 to 15%) in this industry every year [1].

Although technological advances in domestication and genetic breeding of some species such as: *Penaeus monodon* [2] and *Litopennaeus vannamei* [3] have been made, there is not enough progress, due to the lack of comprehension of genetic and biochemical processes on these species, among other factors.

In order to improve the production process of one species, its genetics, reproduction, nutrition and physiology must be taken into account for the selected organism [4].

The economic relevance of shrimps has motivated its more intensive farming. However, the increased densities of growth have led to epizooties that cause production losses. The use of probiotic bacteria and immunostimulants are among the most used methods to avoid these diseases in shrimps [5].

Acuabio 1 is a precise mixture of proteins and essential amino acids which exerts a relevant nutritional effect at the initial developmental stages in aquatic organisms. Its effects synchronize and stimulate larvae growth, activating and producing the mechanisms of growth from early stages; development and size in fishes, and subsequently increasing resistance to parasites as secondary effect [6].

The properties described make Acuabio 1 very attractive for increasing production, survival and quality of aquatic organisms' larvae, the very essential step when culturing them.

Variations have been previously reported in invertebrates in response to several external and artificial stimuli such as: temperature and salinity changes, contaminants, natural or artificially induced infections, among others [7, 8].

In the present work, the effect of Acuabio 1 in parameters of the immune system in *Litopennaeus vannamei* shrimps is described, including total number of

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hemocytes, phenoloxidase and peroxidase specific activities and lectin titers, and counts of *Vibrio* spp. and *Pseudomonas* spp. in the hepatopancreas.

## Materials and methods

### Animal culture and treatment

Juvenile,  $5.0 \pm 0.2$  g average weight shrimps were randomly distributed in two groups, with two replicates each. Replicates were seeded in 50 L tanks at 10 animals per aquarium. The control group was treated with 100 µg/L of bovine serum albumin, and the second group was incubated with Acuabio 1 at 100 µg/L. Animals were kept under constant illumination and aeration, at 34 g/L of salinity and 50% of the water was changed daily. Twenty five days later, hemolymph was collected from the ventral sinus at the cephalothorax of all the animals by using 1 mL syringes filled with 387 mM sodium citrate anticoagulant. Samples were transferred to eppendorf tubes and kept in ice (4 °C) for cell preservation.

### Total hemocyte counts

The hemocyte numbers were determined in plasma by cellular counting in a Neubauer hemocytometer by using a phase contrast microscope (Olympus; 40x). The following formula was used for calculation:

$$NH = \frac{\sum_{i=1}^8 CTH}{8} \times \text{dilución} \times 10^4$$

Where:

NH: Number of hemocytes ( $10^6$  cells/mL)

CTH = hemocytes count in 8 cuadrants

### Lectin titers

The used rabbit blood was collected in a solution containing 110 mM D-glucose, 37 mM sodium citrate, 72 mM NaCl and 2.9 mM citric acid, at 1:1 v/v ratio and stored at 4 °C. The collected erythrocytes was washed twice in PBS 1X (136 mM NaCl, 2.6 mM KCl, 8.0 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ ), pH 7.2. Washes were centrifuging at 1500 rpm for 5 min. A 2% v/v erythrocyte suspension was prepared in PBS 1X, pH 7.2. Serial dilutions were prepared of the samples in PBS 1X, pH 7.2, by using a 96-well U bottom plate (Costar®, USA), to a total volume of 100 µL per well. One hundred microliters of the 2% v/v rabbit erythrocyte suspension were added per well, and the plate was incubated for 1 h at 28 °C. A negative control of 100 µL of PBS 1X, pH 7.2, was included. The hemagglutination was determined for each sample, and the lectin titer was the highest dilution of the sample where hemagglutination occurred [9].

### Antiprotease activity

Twenty microliters of hemolymph were collected per animal and later incubated with a 5 mg/mL trypsin solution for 10 min at 22 °C. Two hundred microliters of 100 mM phosphate buffer (pH 7.0) and 250 µL of azocasein at 2% w/v were subsequently added, then incubated for 1 h at 22 °C. After that time, 500 µL of 10% w/v trichloroacetic acid were added and incubated for 30 min at 22 °C. The mixture was centrifuged

at 6000 rpm for 5 min and 100 µL of the supernatant was transferred to a 96-well plate filled with 100 µL of 1 M NaOH per well. The reaction was read at 450 nm. Phosphate buffer was used as 100% control instead of hemolymph, and phosphate buffer instead of hemolymph and trypsin. The inhibition percent of trypsin activity was calculated for each sample by comparing the control value. All the samples were analyzed in duplicates [10].

### Specific peroxidase activity

It was measured by using pyrogallol (Sigma, USA) as enzyme substrate. Briefly, 64 µL of 100 mM phosphate buffer, pH 6.0, 32 µL of 147 mM  $\text{H}_2\text{O}_2$ , 64 µL of the 180 mM pyrogallol solution and 420 µL of distilled water were mixed in a 1 cm path length cuvette. 20 µL of the sample was added to the mixture; then homogenized; later, read its absorbance at 420 nm after 20 s. Distilled water was used as baseline. One peroxidase unit was defined as the amount of enzyme required to catalyze the production of 1 mg of purpurogallin from pyrogallol after 0.20 s at 20 °C [11]. The specific enzyme activity was calculated as the ratio of the enzyme activity over protein concentration. The total protein concentration was previously determined using in 20 µL of hemolymph by the Bradford's method [12].

### Phenoloxidase specific activity

It was measured by detecting spectrophotometrically the formation of a chromogen from L-dihydroxyphenylalanine (L-DOPA). Fifteen microliters of hemolymph plasma was placed in a 96-well plate and 50 µL of 7.5 mM L-DOPA were added. The absorbance was measured at 490 after 10 min at 25 °C. One unit of phenoloxidase was considered as the variation of 0.001 absorbance units per minute [13]. The specific enzyme activity was calculated as the ratio of enzyme activity over protein concentration.

### *Vibrio* spp. and *Pseudomonas* spp. colony number in the hepatopancreas

One hundred milligrams of hepatopancreas were collected and suspended in 1 mL of sterile PBS 1X. Serial dilutions were carried out in saline (150 mM NaCl) until  $10^{-10}$ , and 100 µL of each dilution were seeded on three plates with agar-thiosulphate-citratebile-sucrose (TCBS; 5 g/L yeast extract, 5 g/L peptone, 10 g/L sodium citrate, 10 g/L  $\text{Na}_2\text{S}_2\text{O}_8$ , 8 g/L ox bile, 20 g/L sucrose, 10 g/L NaCl, 1 g/L ferric citrate, 40 mg/L bromothymol blue, 40 mg/L thymol blue, 15 g/L agar; pH  $8.6 \pm 0.2$ ). This medium was designed to detect *Vibrio* spp. and *Pseudomonas* spp. [14]. Once the suspension was absorbed, the plate was incubated at 28 °C. When growth was observed, the colony forming units (c.f.u.) were estimated by counting the colonies in the dilution showing from 30 to 300 of them.

### Statistical analyses

The median of values corresponding to the total hemocyte counts, hemagglutination activity and the number of *Vibrio* spp and *Pseudomonas* spp. colonies, were compared by using the Mann-Whitney test. Normality was evaluated for the specific peroxidase and phenoloxidase activities by the Kolmogorov-Smirnov test,

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followed by a variance analysis by using the F test, also differences between groups were detected. In the case of antiprotease activity, percentage values were transformed by a scale  $\arcsen(X/100)^{1/2}$ , and the specific enzyme activities were similarly analyzed. Experimental data were statistically evaluated with the aid of the programs Statistical version 6.0 (StatSoft, USA) and GraphPad Prism version 4.00 (GraphPad Software, USA).

## Results

To evaluate the effect of Acuabio 1 on the immune system of *L. vannamei*, several parameters were measured in the hemolymph to characterize the response against pathogens in penaeid shrimps. Animals treated with Acuabio 1 showed a 25% increase in hemocyte counts per milliliter ( $P < 0.05$ ) compared to untreated animals (Figure 1). This indicates that Acuabio 1 stimulates the proliferation of hemocytes in *L. vannamei*.

Lectins are among the innate immunity parameters that could be functionally involved in host defense. The agglutinating capacity of hemolymph in shrimps treated with Acuabio 1 was analyzed in rabbit erythrocytes in the presence of lectins. It was observed that the hemolymph lectin titers were twice greater than the titers detected in untreated animals ( $P < 0.05$ ; Figure 2).

Many bacteria produce proteolytic toxins to digest host tissue proteins as amino acid source. Therefore, the antiprotease activity is another relevant factor of the innate immunity. When *L. vannamei* shrimps were treated with Acuabio 1, the antiprotease activity in the hemolymph was 50% ( $P < 0.05$ ) higher than in the control animals (Figure 3).

Peroxidase and phenoloxidase activities characterize the oxidative defense in the hemolymph of penaeid shrimps. An enhanced specific peroxidase activity (67%;  $P < 0.05$ ) was detected in the hemolymph of *L. vannamei* shrimps treated with Acuabio 1, compared to the hemolymph of negative control-treated animals (Figure 4A). The same animals receiving the product increased the specific phenoloxidase activity in 40% ( $P < 0.05$ ).

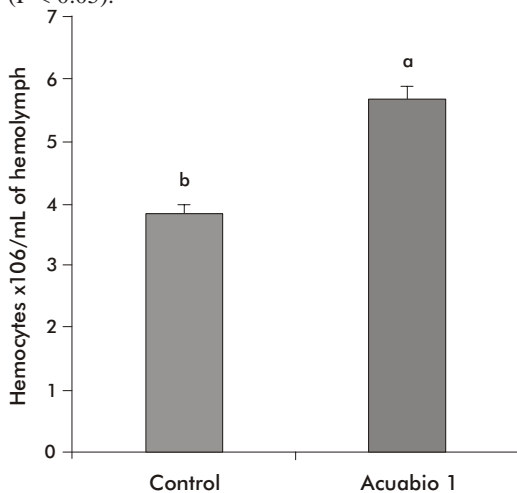


Figure 1. Number of hemocytes per milliliter of hemolymph in the control and Acuabio 1-treated groups. Data are expressed as the mean  $\pm$  standard error. Different letters represent significant statistical differences as analyzed by the Mann-Whitney test ( $p < 0.05$ ;  $n = 10$ ).

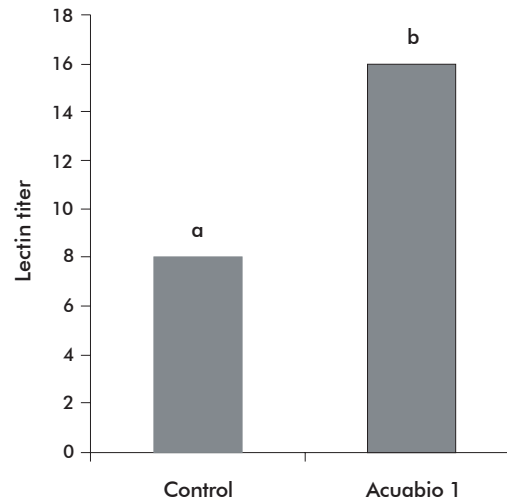


Figure 2. Lectin titers in the hemolymph of negative control and Acuabio-treated groups. Data are expressed as the median. Different letters represent significant statistical differences as analyzed by the Mann-Whitney test ( $p < 0.05$ ;  $n = 10$ ).

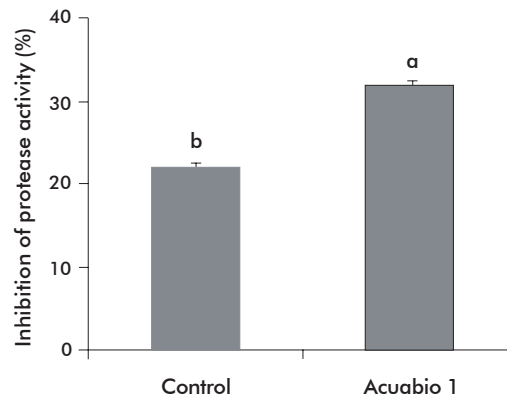


Figure 3. Antiprotease activity in the negative control and Acuabio 1-treated groups. A scale transformation of  $\arcsen(X/100)^{1/2}$  was used. Data are expressed as the median  $\pm$  standard error, and statistically significant differences were analyzed by T test ( $p < 0.05$ ;  $n = 10$ ).

Due to the properties of Acuabio 1, we decided to evaluate its influence on the bacterial flora of the hepatopancreas, specifically *Vibrio* spp. and *Pseudomonas* spp. which are potential pathogens in shrimps. Total counts were expressed as c.f.u. per milligram of hepatopancreas, after bacterial growth in TCBS media. It was found that shrimps treated with Acuabio 1 showed a decrease of 40% in the load of *Vibrio* spp. and *Pseudomonas* spp. ( $P < 0.05$ ), compared to animals treated with the negative control (Figure 5).

## Discussion

Among invertebrates, crustaceans are a very high economical group due to their easy adaptation to aquaculture. Several crustacean species are affected generally by opportunistic pathogens, being lowered their production. In this work, we showed, for the first time, the influence of Acuabio 1 on the immune system of *L. vannamei* shrimps. Invertebrates lack not only a humoral immune response based on antibodies but al-

so cellular immune response based on specialized cells as lymphocytes. Therefore, they do not have immune specificity and memory [15]. Immunity in crustaceans is mediated by humoral and cellular effectors. Hemocytes remove strange particles in the hemolymph by encapsulation and forming nodular aggregates [16]. These cells are additionally involved in wound healing by encapsulation and cellular agglutination, thus initiating the clotting process by releasing the plasma factors required [17]. The results obtained evidenced that animals treated with Acuabio 1 significantly increased their total hemocytes in the hemolymph, compared to the control group. Similar results were previously reported for probiotics stimulating the immune system in shrimps [18], the Bio-Mos® and  $\beta$ -1,3-D-glucan prebiotics [19]. A higher total number of hemocytes also increased immune capacity in periods of high metabolic activity [20]. Circulating hemocytes counts in the hemolymph has been an indicator of shrimps' health because infectious diseases would be modified the composition of hemolymph [21].

Another parameter of crustacean immunity studied was lectin titers. Lectins in decapod crustaceans determine structural properties and specificity as serum lectins in humans. There have being almost completely identified the lectin types of cultured species of shrimp. Lectins in invertebrates are part of the recognition process of strange agents [15, 22], inducing agglutination on pathogenic cells, triggering as opsonins, several cellular events such as: phagocytosis [23]. Results showed that shrimps treated with Acuabio 1 developed the highest lectin titers, indicating of an increased agglutinating capacity against pathogens. It was recently identified that the levels of mannose-binding lectins are increased in humans by the administration of immunostimulants [24]. Multiple sequence alignments showed that the first carbohydrate recognition domains in the lectin (L v LT) of *L. vannamei* completely comprise the 23 amino acids of mannose-binding lectins and other types of lectins in fish. The binding of the four residues relevant to form the three carbohydrate recognition domains are completely conserved [25]. On the other hand, it has been demonstrated that *L. setiferus* lectins are involved in the activation of the immune response of granular hemocytes induced by NADPH and other oxidative pathways through a specific receptor [26].

In this study, the antiprotease activity in the hemolymph of animals treated with Acuabio 1 was higher than in the negative control group. There have been described that shrimp plasma contains several protease inhibitors, mainly  $\alpha$ 1-antiproteinase and  $\alpha$ 2-microglobulin [27]. There were also established that differences in the activity  $\alpha$ 1-microglobulin in two trout species are related to resistance to *Aeromonas salmonicida* infections [28]. Therefore, an increase in this parameter is beneficial to shrimps' health treated with Acuabio 1.

The oxidative defense, represented by the peroxidase and phenoloxydase activities, could be used to measure the health state of the animals [29]. Herein, the animals treated with Acuabio 1 showed the highest activities for both oxidative enzymes. The cascade of phenoloxydase activity could derive into the formation of melanin, stimulation of phagocytosis, nodule forma-

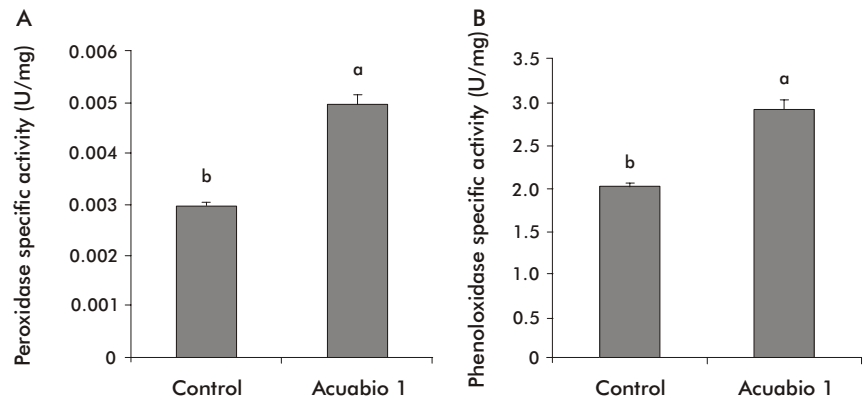


Figure 4. Oxidative defense in *L. vannamei* shrimps treated with Acuabio 1. A) Specific peroxidase (A) and phenoloxydase (B) activities in control and Acuabio 1-treated groups. Data are expressed as the median  $\pm$  standard error. Different letters indicate significant statistical differences as analyzed by the T test ( $p < 0.05$ ;  $n = 10$ ).

tion encapsulation and agglutination [30], as well as mediating the synthesis of proteins as antibacterial peptides [31]. Plasma peroxidase activity is related to the generation and elimination of reactive oxygen species during phagocytosis [32]. An increased oxidative defense in shrimps have been reported after using probiotics [33] and immunostimulants as  $\beta$ -1,3-D-glucan [34, 35].

These results with factors associated to the innate response in shrimps indicated the usefulness of Acuabio 1 as immunostimulant, suggesting that shrimps treated with this product have higher resistance to pathogens under culture conditions. Evidences have been published on the immunostimulating effect of several substances administered to shrimps at different regimes. Nevertheless, data presented were not enough to support their conclusions. In much of these studies, the experimental designs were deficient or statistical limits were absent, being insufficient to validate conclusions [36].

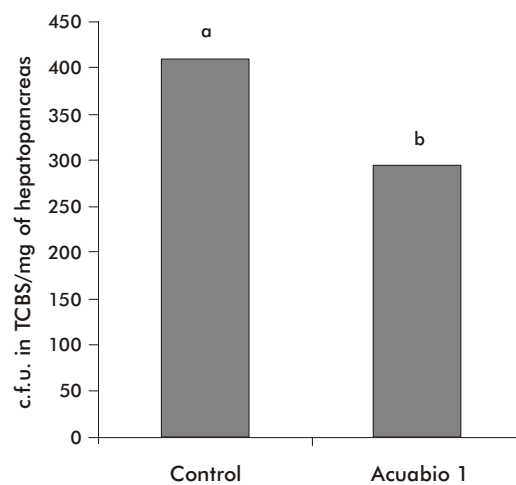


Figure 5. Number of c.f.u. per milligram of hepatopancreas in the negative control and Acuabio 1-treated groups. Data are expressed as the median. Different letters indicate significant statistical differences as analyzed by the Mann-Whitney test ( $p < 0.05$ ;  $n = 10$ ).

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A decreased total number of c.f. u. per milligram of hepatopancreas in TCBS indicated a reduced *Vibrio* spp. and *Pseudomonas* spp. flora. Similar results were reported by using the Timsen Ôimmunostimulant (40% alkyldimethylbenzylammonium chloride) in *P. monodon* shrimps [37]. The use of an endogenous immunostimulant, the antilipopolisaccharide factor 3,

avoided vibriosis in *P. monodon* shrimps [38]. Our results suggest a prebiotic function of Acuabio1, due to a decrease population of pathogenic bacteria in the hepato-pancreas of shrimps after its administration. Both the prebiotic and the immunostimulating functions make Acuabio 1 in shrimps a highly attractive product for shrimp aquaculture.

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