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Effects of Fructooligosaccharides (FOS) on the Immune Response of the Shrimp *Penaeus vannamei* and on the Reduction in *Vibrio* spp. and *Pseudomonas* spp. in Cultures of Post-Larvae

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Abstract: *Penaeus* spp. are the most cultivated type of shrimp because they have rapid growth and good adaptation to farming conditions. Due to diseases that result in high mortality and a decreased product quality, the cultivation of these shrimp globally, and in Cuba in particular, comes with the risk of significant financial losses. This study examined the effect of fructooligosaccharide (FOS, 1-kestose) on the growth and immune response of shrimp, as well as the multitude of *Vibrio* spp. and *Pseudomonas* spp. in *Penaeus vannamei* post-larvae under culture conditions. Fructooligosaccharide was applied in a completely randomized manner at a concentration of 0.4%, in both experimental groups with seventeen tanks each. In the results of this investigation, animals of greater weight (control 6.8 ± 0.2 mg; FOS 9.5 ± 0.3 mg; $p < 0.001$), length (control 1.1 ± 0.1 mm; FOS 1.3 ± 0.1 mm; $p < 0.001$), and survival (control 61.7% (95% CI of median 54.2–70.0); FOS 76.6% (95% CI of median 72.1–84.2); $p < 0.001$) were obtained when administered FOS relative to the control. An increase in the activity of proteases ($p < 0.001$), enzymes of the innate immune system such as phenoloxidase ($p < 0.001$), and lysozymes ($p < 0.001$) was observed, as well as an increase in the number of lectins ($p < 0.001$). Changes in the microbiota could be observed, with a reduction in *Vibrio* spp. and *Pseudomonas* spp. (control $2.4 \times 10^3 \pm 0.5 \times 10^3$; FOS $1.1 \times 10^2 \pm 0.3 \times 10^2$; $p < 0.001$). FOS improves the quality of the post-larvae of *P. vannamei* as reflected in the length, weight, and survival of the animals. Moreover, FOS stimulates the *P. vannamei* immune system through the enzymatic activities of phenoloxidase, lysozyme, and a number of lectins. The reduction in the population load of *Vibrio* spp. and *Pseudomonas* spp. might be a consequence of the improvement in the quality and immune system of *P. vannamei*.

Keywords: prebiotic; enzymes; microbiota; phenoloxidase; lectin

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1. Introduction

It is estimated that aquaculture represents 49% of the global production of aquatic animals. Crustaceans represent 9.7% of aquaculture production, preceded by freshwater fish and mollusks, but in terms of commercial value, they occupy the second position [1]. Due to the high demand from importing nations, shrimp aquaculture is becoming increasingly popular worldwide [2]. Shrimp in the genus *Penaeus* are the most cultivated due to the rapid growth and aquaculture adaptation of species such as *Penaeus vannamei* and *Penaeus monodon* [1].

Shrimp farming, both in Cuba and globally, faces the risk of large financial losses due to diseases that cause high mortality, and a decrease in the production quality. Moreover, crustaceans do not have specific immune responses against these infectious agents; their defensive function consists of a developed, non-specific, defense system, which is based on circulating hemocytes and various defense proteins [3]. Due to the continuous outbreaks and spread of diseases in shrimp farming systems, it is urgent to identify the effect of environmental factors on the immune response of penaeid shrimp [4]. The application of antibiotics as prophylactics was an effective strategy for controlling bacterial diseases in shrimp, but their indiscriminate use fosters the development of bacterial resistance [5]. Prebiotics are supplements that are selectively metabolized by beneficial bacteria in the digestive tract and promote the dietary modulation of the intestinal microbiota, thereby improving animal health, primarily by stimulating the growth and activity of *Bifidobacteria* and *Lactobacilli*. In addition, prebiotics do not generate dependency or negative impacts on culture organisms or the environment [6]. Immunosaccharides stimulate the immune system directly. Immunosaccharide prebiotics include fructooligosaccharide (FOS), mannan oligosaccharide, inulin, or β-glucan [7].

The activation of enzymes such as peroxidase, superoxide dismutase, and the prophenoloxidase (proPO) system can initiate several immunological responses. In addition, other types of cytotoxic and cellular recognition mechanisms, such as coagulation proteins, lectins, peptides, antimicrobials, peroxynectins, the generation of reactive oxygen species, quinones, opsonization, phagocytosis, and nodulation, can be stimulated to eliminate the pathogen [4]. The shrimp immune system is currently described by how certain immunological parameters vary, for example, the enzymatic activities of crucial enzymes linked to the immune system such as lysozyme, phenoloxidase, peroxidase, superoxide dismutase, and lectin. The current study determined the effects of the administration of FOS under aquaculture conditions on the growth and survival, as well as on the immune response of post-larvae of *Penaeus vannamei.*

2. Materials and Methods

2.1. Location

The post-larvae (PL) were obtained from the Yagüacam hatchery center in Cumanayagüa, Cienfuegos, Cuba. The research was carried out in the Cultisur post-larval nursery area in Santa Cruz del Sur, Camagüey, Cuba. *P. vannamei* post-larvae at substage 8 days (PL 8) were stocked at 1.5 million per rectangular cement tank, having a water volume of 60 m^3.

2.2. Experimental Design

A completely randomized design was used for 34 tanks (17 with FOS treatment and 17 as control group). In the experimental group, the prebiotic fructooligosaccharide (FOS) was homogenized with sterile distilled water, then mixed with commercial shrimp feed (35% protein) using the sprayer method (40 g of FOS per kg of feed).

FOS was also added to the pond water 1 mL/m^3 every day. The control group (without FOS treatment) received the standard aquaculture treatment previously established in the center.

2.3. Culture Parameters

The temperature was maintained between 28 and 30 °C throughout the experiment, salinity at 30 ppt, pH 7.8, and dissolved oxygen at 5.00 mg/L from the third day of the PL stocking. For water exchange, 50% of each tank was replaced using a filter (1 mm^2 of mesh) attached to the outlet of the tanks to eliminate solid and organic wastes. The animals were kept for 15 days under these conditions (PL 23 substage). Commercial food (Malta Cleyton® 35% protein, Mexico) was used for feeding. Feed was offered 12 times a day in

amounts recommended by the manufacturer. The feed composition was: protein (35.03%), fat (9.04%), fiber (2.84%), water (8.84%), and ash (6.09%).

2.4. Shrimp Sample Collection and Preservation

For sample collection, 15 days after starting the treatment, three shrimp samples were taken randomly from each tank after filtering with a 1 mm nylon mesh. Shrimp samples were washed twice in phosphate-buffered saline (PBS) solution at pH 7.4. Sample preservation was carried out in vials without preservation solution at −20 °C until its evaluation in the Molecular Biology Laboratory of the Faculty of Agricultural Sciences at the University of Camagüey.

2.5. Evaluation of the Quality of Post-Larvae

The quality of the post-larvae was evaluated by determining the development parameters: length and weight. Twenty post-larvae per tank were washed with water, dried with filter paper, and weighed on an analytical balance (Sartorius, model 1205) with a precision of ±0.1 mg. The length of the post-larvae was determined by measuring them from the rostral base to the end of the telson under a microscope (±0.1mm). At the end of each bioassay, survival values per tank were recorded.

2.6. Processing of Samples

Approximately 100 PL 23 were taken from each tank per 1.5 mL vial. The post-larvae were macerated in 1.5 mL tubes with 200 µL PBS. Then, 200 µL PBS was added and homogenized in a vortex. The homogenates of the samples were centrifuged at 8000× *g* for 10 min, and the supernatant was then stored at −20 °C in new 1.5 mL vials. The concentration of total proteins was determined by the Bradford MM [8] method. The supernatant was also used to determine the presence of lectins in the larvae.

2.7. Evaluation of Immune Response Parameters

2.7.1. Phenoloxidase-Specific Enzymatic Activity

Phenoloxidase activity was determined by measuring the dopachrome produced from dihydroxyphenylalanine (L-DOPA), pH 7.6 [9]. A negative control was used to measure the spontaneous oxidation of L-DOPA. Per sample, 20 µL of supernatant was used. The assay was carried out in Nunc® 96-well flat-bottom polystyrene plates with a total volume of 400 µL. Results were expressed in U/mg protein, where U was defined as a 0.001 increase in absorbance at 490 nm per min.

2.7.2. Superoxide Dismutase-Specific Enzymatic Activity

The specific activity of SOD was determined according to the oxidation of nitroblue tetrazolium (NBT) in the presence of riboflavin [10]. The measurement sample was exposed to 8000–9000 lux, with the light-emitting source 30 cm from the reaction site. The specific enzymatic activity was obtained by dividing the enzymatic activity value by the protein concentration value in each sample. The assay was carried out in Nunc ® 96-well flat-bottom polystyrene plates with a total volume of 400 µL, using 20 µL per sample. Results were expressed in U μg^{-1} protein, where U was defined as a 0.001 increase at 630 nm per minute.

2.7.3. Peroxidase-Specific Enzymatic Activity

Peroxidase activity was quantified using a standard curve made with horseradish peroxidase (HRP) coupled to a mouse anti-polyhistidine (Sigma-Aldrich, St. Louis, MO, USA) antibody [11]. Peroxidase assessments were performed in Nunc ® 96-well polystyrene plates (Denmark) by adding 20 µL of sample and 50 µL of a solution containing 0.15% H_2O_2 (*v*/*v*) and 0.10% o-phenylenediamine (weight/volume) in phosphate buffer, pH 5, (0.05 M Na_2HPO_4, 0.02 M citric acid). The mixtures were incubated at 25 °C for 30 min.

The reactions were stopped with 50 µL of sulfuric acid (2 M) and the chromogen formation was quantified after two hours at 490 nm. The results were expressed in U/mg of protein, where U was defined as the formation of 1 µmol of 2,3-diaminophenazine by oxidation of o-phenylenediamine per minute. Per sample, 20 µL of supernatant was used. A molar extinction coefficient of 16,700 M^{-1} cm^{-1} was assumed for 2,3-diaminophenazine (Fornera and Walde, 2010).

2.7.4. Lysozyme-Specific Enzymatic Activity

The lysozyme-specific enzymatic activity was determined by the absorbance variation over time of a lyophilized solution of *Micrococcus lysodeikticus* [12]. The final solution, with a final volume of 180 µL, included the lyophilized *M. lysodeikticus* (0.4 mg/mL) dissolved in phosphate buffer (NaH_2PO_4/Na_2HPO_4) at a concentration of 100 mM, with adjustment to pH 8 with orthophosphoric acid (H_3PO_4) (85%). The solution had the desired absorbance of 0.3. Per sample, 20 µL was added, with absorbance at 490 nm measured over time. The absorbance was measured every 5 min during the incubation. One unit of enzymatic activity is equivalent to a 0.001 decrease at 490 nm per min and was reported as specific enzymatic activity by dividing the enzymatic activity value by the protein concentration value for each sample.

2.7.5. Lectin Expression

Rabbit blood was used to determine the presence of lectins and agglutinins. The blood was collected in a 15 mL centrifuge tube (Corning, Corning, NY USA) with ethylenediaminetetraacetic acid (EDTA 50 mmol L^{-1}) and stored at 4 °C until the time of the test. The blood was centrifuged at 1500× *g* for 10 min and the serum was discarded. The erythrocyte pellet was washed twice with PBS. Then, it was centrifuged at 2000× *g* for 10 min. Next, a suspension of erythrocytes (1% (*v*/*v*)) was prepared in PBS pH 7.2. Then, 25 µL of each of the samples was mixed with 25 µL of PBS pH 7.2 in 96-well concave-bottom plates (Costar®, Corning, NY, USA), and 25 µL of the erythrocyte suspension was added to each sample. The reactions were incubated for 20 min at 28 °C, and the presence of lectins was determined by observing hemagglutination. The assay was carried out in triplicate. A suspension of erythrocytes in PBS, pH 7.2, was used as a negative control [13].

2.7.6. Bacteriological Analysis

For the microbiological evaluation of bacteria, presumptively of the genus *Vibrio* spp. and *Pseudomonas* spp., the number of colony-forming units (cfu) mL^{-1} (that grew on thiosulfate–citrate–bile salts–sucrose (TCBS) agar) was determined in the samples at the end of the experiment. To determine the number of cfu mL^{-1} of bacteria in the larvae, 3 samples were taken per tank with an average of 50 individuals in each sample. The larvae were disinfested with 0.1% benzalkonium chloride [13] and rinsed with sterile $H2O_d$ to eliminate external bacteria. Whole organisms were macerated with a homogenizer (Wiggenhauser, D-130, Selangor, Malaysia) in 500 µL of sterile PBS. Serial dilutions of 1:10 were made for the macerated samples. A 10 µL subsample of each dilution was plated on the TCBS plates. All plates were incubated at 28 °C for 24 to 48 h.

2.8. Statistical Analysis

The statistical analysis of the experimental data was carried out with the help of the GraphPad Prism software version 6.01 (2012, GraphPad Software, Boston, MA, USA). The data obtained for weight, height, survival, and specific enzymatic activities were verified to have a normal distribution (Kolmogórov–Smirnov). Fisher's F-test was then applied to the sample to determine the homogeneity between the variances. The difference between the means was determined using the Student's *t*-test. In the case of survival and lectin titers, the non-parametric Mann–Whitney U-test was used to detect differences between medians.

3. Results and Discussion

3.1. Length, Weight, and Survival

Prebiotics are classified as organic additives because they are derived from natural ingredients such as alginate, inulin, and various oligosaccharides [14,15]. In aquaculture, both dietary FOS and short-chain FOS (scFOS) have gained much interest due to their growth-promoting factors in several aquatic species, including *P. vannamei* [16]. In addition, they have been used as sole carbon sources to evaluate the growth, length, and weight of shrimp of similar age, relating to their intestinal bacterial composition [17]. The authors confirmed that weight, length, and survival are related to the administration of prebiotics and the change in the composition of the intestinal microbiota.

Prebiotics can be metabolized by beneficial bacteria in the genus Lactobacillus and by Bifidobacterium, producing short-chain fatty acids (scFA) and lactate [14]. However, the effect of prebiotics on shrimp growth and weight gain is controversial throughout the literature. While Zhou Z, Ding Z and Huiyuan L [16] reported an increased growth in *P. vannamei*, other reports did not find a significant increase in the growth and survival of *P. vannamei* fed with fructooligosaccharides included in the diet [18,19]. Thus, the effect of prebiotics on the growth performance of aquatic species may be ambiguous because some studies with fish have not observed positive improvements in growth performance with the dietary supplements of prebiotics [20–23].

In studies on the effect of scFOS supplementation on the beneficial microbiota of the white shrimp digestive tract [24], they did not improve weight gain, feed conversion, or shrimp survival. On the other hand, the analysis by Zhou Z, Ding Z and Huiyuan L [16] showed that scFOS supplementation in the diet at concentrations of 0.4% improved growth rate and survival, stimulated bacterial growth, and stimulated immune enzymes.

Similar results were obtained in this study, with the only difference being that FOS was also administered directly into the water over 21 days (Figure 1). Significant differences were reported in tanks treated with FOS compared to the control without FOS. The animals treated with FOS were heavier and larger than the control. However, there are no reports of the effect of FOS stimulating growth or immune response when administered directly into the water. Perhaps one of the possible effects of this prebiotic is indirectly on the intestinal microbiota due to microorganisms stimulated in the water by FOS.

When analyzing the results obtained (Figure 2), there is evidence of improved survival in the group where FOS was administered, compared to the control group. However, Ref. [25] applied this prebiotic to *P. vannamei* over 35 days of culture. No differences were observed between the experimental groups regarding survival; the same outcome occurred when these researchers [26] used this experimental design but with galactooligosaccharides (GOS). In contrast, Fuandila NN, Widanarni W and Yuhana M [27] observed an increased survival rate when using honey as a prebiotic in *P. vannamei*.

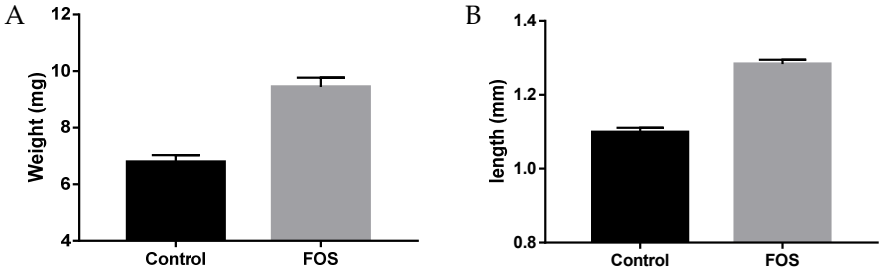

Figure 1. Quality evaluation of *Penaeus vannamei* post-larvae treated with FOS, (fructooligosaccharide, 1-kestose) (n = 340) and the control (n = 340) groups. Weight (**A**) and length (**B**) are given. Data represent mean ± SEM (standard error of the mean), and T bars indicate the SEM ($p < 0.001$, Student's *t*-test).

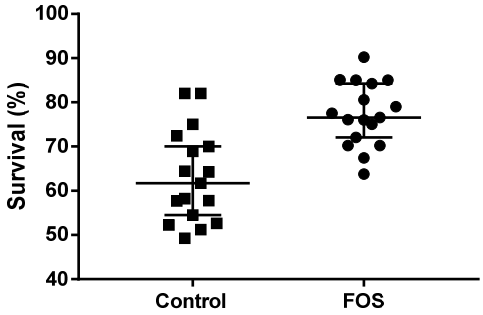

Figure 2. Survival of *P. vannamei* post-larvae. The bars represent the median in the control (n = 17) and the FOS-treated groups (n = 17) ($p < 0.001$, Mann–Whitney U-test). The T bars indicate the range of reported survival values.

3.2. Innate Immune System

Phenoloxidase is an essential enzyme for the immune response of invertebrates. Its activity is not only limited to the production of compounds with bactericidal action but also to a wide range of immune functions, which makes it a central component of the innate immune response [28]. Phenoloxidase is the main enzyme in shrimp that enables wound repair and cuticle sclerotization due to its microbicidal actions [29]. Immunostimulants, as molecules and/or whole cells of bacteria and yeasts [30], can be used to increase the stimulation of the immune response and resistance to stress and diseases, through direct interaction with the cells of the immune system and their activation [31].

High phenoloxidase activity improved the ability of Pacific white shrimp to recognize foreign entities entering their bodies [32]. A better immune response would protect shrimp against pathogen infections [33]. Dong C and Wang J [34] reported that FOS increased the activity of phenoloxidase or superoxide dismutase, and can positively influence the survival rate of *P. vannamei*.

In the experiment, the animals treated with FOS showed phenoloxidase activity values higher than the untreated control group (Figure 3). Similarly, Safari O, Shahsavani D, Paolucci M and Atash MMS [35] reported that the administration of FOS can stimulate the rate of hemocyte proliferation, because an increase in the number of hemocytes explains the high activity of phenoloxidase, as they are the main producers of this enzyme.

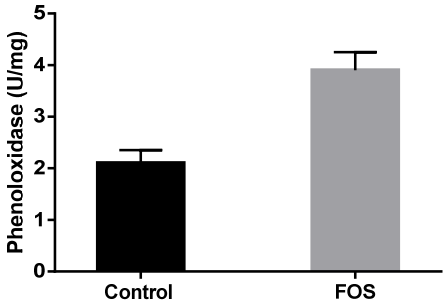

Figure 3. Specific activity of phenoloxidase (U mg^{-1}) in *Penaeus vannamei* post-larvae treated with FOS (fructooligosaccharide, 1-kestose) (n = 17) and the control (n = 17) groups. Data represent mean ± standard deviation (SD). T bars indicates the SD ($p < 0.001$, Student's *t*-test).

Peroxidase activity is closely related to the generation and removal of reactive oxygen species (ROS) generated by oxidative metabolism during phagocytosis of foreign particles. It may have cytotoxic activity against foreign cell types that invade the organism, which would provide it with an additional immune function [36]. However, it is unclear

whether there are distinct mechanisms for shrimp glutathione peroxidase to participate in immunity other than their antioxidant activity [37].

Peroxidase activity is related to environmental stressors such as exposure to viral and bacterial pathogens [38]. A decrease in total peroxidase activity has been reported under stress conditions due to high cadmium levels and pH stress in *P. vannamei* [39].

The total peroxidase activity after two hours did not show any significant differences between the tanks treated with FOS and the control tanks (control group $1.84 \times 10^4 \pm 0.08 \times 10^4$ U/mg and FOS group $1.75 \times 10^4 \pm 0.06 \times 10^4$ U/mg). The lack of any difference between the two groups may be due to similarities in the oxidative status of shrimp larvae in the tanks. The result suggests that the effect of FOS on the immune response does not follow a stimulation product of oxidative stress in shrimp larvae since the stimulation of immune parameters is evident without altering their oxidative status.

From the current results, the SOD activity between the experimental groups also did not show significant differences (control group 0.88 ± 0.06 U/mg and FOS group 0.95 ± 0.08 U/mg). It may be because, in both groups, the physiological states were not exposed to stress. This result coincides with the one obtained by Varela-Granados Y, Frías-Gómez SA, Hernández-Hernández LH, Powell MS and Vega-Villasante F [40], with no differentiation of SOD activity between treated (fructo- and mannan oligosaccharides) and untreated groups. However, it does not coincide with that obtained by Hu X, Yang HL, Yan YY, Zhang CX, Ye Jd, Lu KL, Hu LH, Zhang JJ, Ruan L and Sun YZ [41], showing that the form of administration of FOS within the diet can have effects dependent on the concentration of FOS and other environmental factors. It has been discussed that SOD activities improved by prebiotics seem to be dose-dependent [40]. However, mannan and fructooligosaccharides were able to stimulate SOD in crayfish, with higher SOD activities observed for the combination of both prebiotics [35]. Therefore, more research is needed to resolve the effect of FOS on the antioxidant response of cultured species.

Two members of the superoxide dismutase family exist in invertebrates Cu/Zn (SOD 1) and Mn (SOD 2). In addition, it is known that environmental factors such as hypoxia and high temperatures can stimulate the expression of SOD 2 in shrimp. However, SOD 1 expression is continuous, because its expression is constitutive [42]. Even though the SOD 2 expression increases faster against pathogens, both enzymes (SOD 1 and SOD 2) increase their expression upon challenge against pathogens [43].

Superoxide dismutase possesses the ability to decrease superoxide anions, which occur in water and hydrogen peroxide. Extracellular SOD co-operates in the destruction of ingested or encapsulated parasites during the respiratory burst generated with phagocytosis [44]. The antioxidant system is frequently used as a potential indicator of oxidative stress in marine organisms [45]. Discoveries related to the cellular activity of SOD show that it plays a crucial role as a modulator of the immune response [46]. This distribution is closely related to the antioxidant function and is found at higher levels in the places where the respiratory burst occurs, confirming its role in preventing and eliminating oxidative damage [47].

Lysozymes are proteins that are specifically synthesized by hemocytes, mainly granular hemocytes [48]. They are well-characterized in penaeid shrimp, where they have lytic activity against Gram-negative and Gram-positive bacterial species, including pathogenic *Vibrio* spp. [12]. There are three types of lysozymes, c-lysozyme, g-lysozyme, and i-lysozyme, of which c-lysozyme and i-lysozyme have been reported in *P. vannamei*, both with potent antimicrobial activity [49].

FOS administration in the freshwater prawn *Macrobrachium* a*canthurus* does not affect lysozyme activity, compared to the control [40]. In contrast, the results of the current study showed a stimulation of lysozyme activity in the group treated with FOS as compared to the untreated control group (Figure 4). On the other hand, research by Elshopakey GE, Risha EF, Abdalla OA, Okamura Y, Hanh VD, Ibuki M, Sudhakaran R and Itami T [50] showed similar results to this study with the prebiotic β-1,4-mannobiose increasing lysozyme expression. Within their research, the increase in the number of

circulating hemocytes in the hemolymph, specifically granular hemocytes, was also verified. This increase in the number of granular hemocytes, the main producers of lysozyme in shrimp, could explain the increase in lysozyme activity in the current experiment. Previous studies have shown that supplementing mannan oligosaccharide and FOS in a juvenile narrow-clawed crayfish leads to a higher activity of lysozymes [35]. These authors observed a higher lysozyme activity when both oligosaccharides were combined, compared to the individual supplementations.

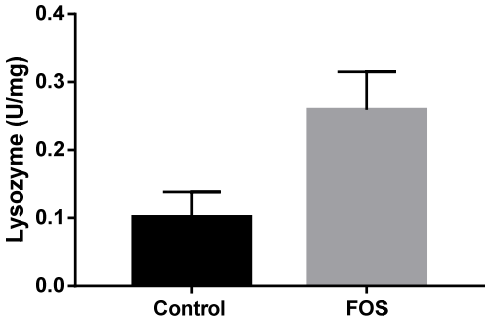

Figure 4. Specific activity of lysozyme in *Penaeus vannamei* post-larvae treated with FOS (fructooligosaccharide, 1-kestose) (n = 17) and the control (n = 17) groups. Data represent mean ± standard deviation (SD). T bars indicate the SD ($p < 0.001$, Student's *t*-test).

However, it is worth highlighting the differences in the methodology used here, which constitutes the first determination of the effect of FOS administered in water on lysozyme activity. The induction of lysozyme expression is linked to several biotic and abiotic factors. It is known that exposure to *Vibrio anguillarum* or *Micrococcus lysodeikticus* induces a significant upregulation of lysozyme gene expression, with no change in total hemocyte counts. Furthermore, even when temperature stress does not change the lysozyme expression profile [51], it is affected by culture parameters such as diet composition and pH [52]. Therefore, thorough research is needed to study the number of hemocytes under these culture conditions, as well as their expression of lysozyme.

Lectins are proteins that play a role in the pattern recognition receptor (PRR) reaction in invertebrates and recognize all or parts of sugar molecules [53]. According to Song SK, Beck BR, Kim D, Park J, Kim J, Kim HD and Ringø E [54], prebiotics such as FOS, mannan oligosaccharides, inulin, and β- glucan are also classified as immunosaccharides, which could directly enhance non-specific immune responses through interactions with the pattern recognition receptors. Furthermore, beneficial bacteria in the intestines may interact with gut-associated lymphoid tissue (GALT), which could stimulate the immune system and improve the host's ability to prevent infection [55].

The hemagglutination capacity of the group treated with FOS was higher ($p < 0.001$) compared to the control group (Figure 5). Thus, the results of the study show that FOS addition directly in the water improves the immune response parameters in the bacterial recognition of *P. vannamei*. This might be because the carbohydrate components of the bacterial cell wall and membrane attach to lectin receptors in shrimp, which could directly enhance shrimp immune system parameters such as phenoloxidase and lysozyme. These enzymes were found to undergo a significant increase in their activity in the tanks treated with FOS, compared to the untreated ones. Both are synthesized in hemocytes upon pathogen recognition patterns through an interaction with lectins on the surface of the hemocytes (Liu et al., 2020). In addition, a greater stimulation of the defense cells, due to an increase in the growth of beneficial bacteria in the culture media, could induce overexpression of lectins in the tanks treated with FOS [56].

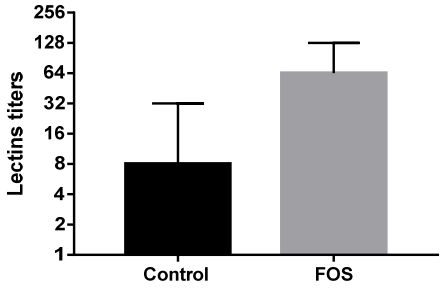

Figure 5. Rabbit erythrocyte hemagglutination titers measuring lectins from *P. vannamei* post-larvae (Log_2, scale). The bars represent the median in the control (n = 17) and the FOS-treated (fructooligosaccharide, 1-kestose) groups (n = 17) ($p < 0.001$, Mann–Whitney U-test). The T bars indicate the range of reported hemagglutination titer values.

3.3. Protease and Amylase-Specific Enzymatic Activity

When comparing the behavior of the specific enzymatic activity of proteases in this study (Figure 6), it is evident that the response obtained in the group treated with FOS was superior, with statistically significant differences ($p < 0.001$). Fuandila NN, Widanarni W and Yuhana M [27] found that using bee honey stimulates digestive enzymes, such as proteases, due to the concentration of prebiotic oligosaccharides in honey. Although some studies [27,57] support the ability of some oligosaccharides to stimulate amylases, in this study, there was no stimulation of amylase activity using the prebiotic FOS (control group 0.74 ± 0.09 U/mg and FOS group 0.65 ± 0.07 U/mg). The supplementation with mannan oligosaccharides and FOS in diets of the juvenile narrow-clawed crayfish results in a higher activity of amylases [35]. These authors demonstrated that the combination of both oligosaccharides results in an even higher amylase activity. Recently, amylase activity was stimulated in *P. vannamei* using a diet containing 0.5% FOS. In that study, however, FOS appeared to lead to some hepatopancreatic abnormalities, such as hypertrophied B-cells [58].

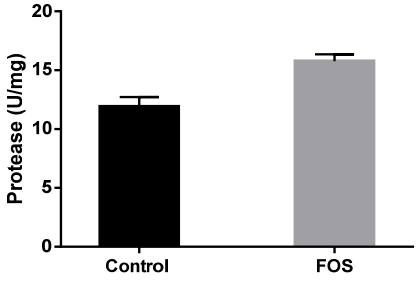

Figure 6. Specific activity of protease in *Penaeus vannamei* post-larvae treated with FOS (fructooligosaccharide, 1-kestose) (n = 17) and the control (n = 17) groups. Data represent mean ± standard deviation (SD). T bars indicate the SD ($p < 0.001$, Student's *t*-test).

3.4. Bacteriological Analysis

Figure 7 shows the values obtained in cfu/ mL on TCBS agar for potential opportunistic pathogens. Changes in the microbiota were observed when applying the FOS prebiotic, with a reduction in *Vibrio* spp. and *Pseudomonas* spp., compared to the control group (control $2.4 \times 10^3 \pm 0.5\ 10^3$ cfu/mL; FOS $1.1 \times 10^2 \pm 0.3 \times 10^2$ cfu/mL). The results obtained by Mustafa A, Buentello A, Gatlin D, Lightner D, Hume M and Lawrence A [26] support the ability of fructooligosaccharides to modify microbial communities and that they can suppress or improve the growth of specific bacteria to favor a healthy physiology of *P. vannamei* shrimp post-larvae.

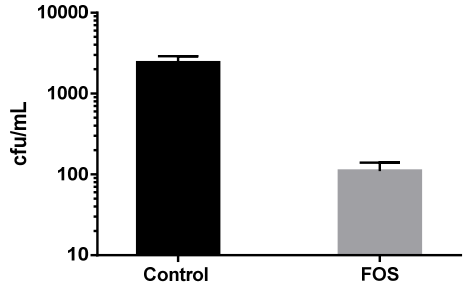

Figure 7. Bacterial count (*Vibrio* spp. and *Pseudomonas* spp.) in *P. vannamei* post-larvae (colony-forming units (cfu/mL); Log_{10}, scale). The bars represent the median in the control (n = 17) and the FOS-treated (fructooligosaccharide, 1-kestose) groups (n = 17). Data represent the mean and T bars indicate the SD ($p < 0.001$, Student's *t*-test).

The results of the current study suggest that FOS can not only change the populations of the intestinal microbiota, in particular, a significant reduction in *Vibrio* spp. and *Pseudomonas* spp., but can also create a positive association with the gut microbiota and digestive tract cells. Therefore, it can improve the physiological performance and stimulate the innate immune system of *P. vannamei* shrimp post-larvae.

Previous studies have shown that oligosaccharide supplementation in the culture water of the animals confers health benefits [59,60]. Similarly, other authors have shown that small molecules (GHRP-6) added to the culture water of shrimp and tilapia can improve their immune systems and growth [61,62]. The authors demonstrated that, in one hour, the molecules reach the highest levels in the target animal. According to those results, one can hypothesize that FOS can follow the same route after their supplementation in the water. However, further studies are necessary to corroborate their mechanism of entrance into the host and their effect on the whole microbiota of the host and the pond water.

4. Conclusions

The administration of the prebiotic FOS during the culture of post-larvae of *P. vannamei* augments their length, weight, and survival, which indicates an overall improvement in the quality of the post-larvae. Moreover, FOS enhanced the innate immune system in *P. vannamei* post-larvae by stimulating the activity of the phenoloxidase and lysozyme enzymes, as well as the ability to recognize pathogens by increasing the lectin titers, which demonstrates a better health condition. Referring to *P. vannamei* metabolism, FOS increases the activity of proteases in the post-larvae of *P. vannamei*, which consequently improves their digestive capacity to process shrimp feed. Also, FOS reduces the populations of *Vibrio* spp. and *Pseudomonas* spp. in *P. vannamei* shrimp post-larvae.

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Informed Consent Statement: Following Cuban Legislation, No. 180/07 (Gaceta Oficial 084, 19 December 2007), this field study on commercial farms did not require ethical approval. Consent to use farm production data was given by the National Shrimp Organization company.

Data Availability Statement: The data presented in this study are openly available in Zenodo at [doi: 10.5281/zenodo.8122374], reference number [8122374].

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