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Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibrio harveyi*

[*Efeito do uso de dieta suplementada com o probiótico sobre a sobrevivência de camarões marinhos após o desafio com* Vibrio harveyi]

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ABSTRACT

The effect of a *Lactobacillus plantarum*-supplemented diet on shrimp growth, digestive tract bacterial microbiota, survival, and some hemato-immunological parameters after an experimental challenge with *Vibrio harveyi was studied*. No difference (P>0.05) was observed in shrimp survival (80±6%) and final weight (6.63±0.56g) after 60 days feeding trial. Total bacteria count and *Vibrio* spp. count in the digestive tract were not diferent (P>0.05) until day 40, but they were lower (P<0.05) in the probiotic-supplemented group on day 60. Total lactic bacteria in the shrimp digestive tract was higher after day 20 in the probiotic-supplemented group. Ten hours after *V. harveyi* challenge, survival of the probiotic-supplemented group (65.7±2.9%) was higher (P>0.05) than the control group (39.9±4.4%). Bacterial counts in hemolymph and hepatopancreas was lower (P<0.05) in the probiotic-supplemented group than in the control group after *V. harveyi* challenge. Total hemocyte count and serum agglutination activity were higher (P>0.05) in the probiotic-supplemented group after challenge with *V. harveyi.* Probiotic-supplemented diet modifies shrimp digestive tract bacterial microbiota, increasing resistance to *V. harveyi* infection.

Keywords: *Litopenaeus vannamei, Lactobacillus plantarum*, intestinal microbiota, immunostimulation

RESUMO

Avaliou-se o efeito de uma dieta suplementada com Lactobacillus plantarum *na engorda de* Litopenaeus vannamei *sobre a microbiota bacteriana do trato digestivo, a sobrevivência e alguns parâmetros hematoimunológicos após desafio com* Vibrio harveyi. *Não foi observada diferença significativa na sobrevivência (80±6%) e no peso final dos camarões (6,63±0,56g) durante a engorda (60 dias). A população de bactérias totais e* Vibrio *spp. no intestino não diferiu até o 40° dia de cultivo, mas foi inferior nos camarões alimentados com dieta suplementada com probiótico no 60° dia de cultivo. A população de bactérias lácticas foi superior no intestino de camarões alimentados com dieta suplementada com probióticos após o 20° dia de cultivo. Dez horas após o desafio, a sobrevivência dos camarões alimentados com dieta suplementada com probiótico foi superior (65,7±2,9%) a dos camarões do controle (39,9±4,4%). Camarões alimentados com dieta suplementada com probióticos e desafiados com V. harveyi apresentaram menor contagem de bactérias na hemolinfa e hepatopâncreas. A contagem total de hemócitos e a atividade aglutinante da hemolinfa foram superiores nos camarões inoculados com* V. harveyi *alimentados com dieta suplementada com probióticos. A suplementação da dieta com probióticos modifica a microbiota bacteriana intestinal dos camarões aumentando sua resistência ao desafio com* V. harveyi.

Palavras-chave: Litopenaeus vannamei, Lactobacillus plantarum, *microbiota intestinal, imunoestimulação*

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INTRODUCTION

The microbiota in the aquatic environment is usually in equilibrium and it is composed by bacteria that are either benefic or neutral to cultured animals, or by harmful obligate and opportunistic pathogenic bacteria (Schulze et al., 2006). Such equilibrium can be disturbed by inadequate management and result in the proliferation of pathogenic bacteria (Karunasagar et al., 1994).

In marine shrimp culture, the control of bacterial diseases is done mainly by the use of antibiotics (Karunasagar et al., 1994). However, their indiscriminate use has led to the appearance of antibiotic-resistant strains (Skjermo and Vadstein, 1999), further to the contamination of shrimp meat and also the environment (Holmström et al., 2003). Therefore, several countries have banned the use of antibiotics such as chloramphenicol (FAO, 2002).

Recently, an alternative that has been widely employed in the aquaculture industry is the dietary supplementation with probiotic bacteria (Gatesoupe, 1999; Vine et al., 2006). Gatesoupe (1999) defines probiotics as "live microbial cells administered to cultured organisms to colonize the digestive tract and improve their immune response".

Among the probiotic bacteria used in aquaculture, the lactic acid bacteria stand out for their easy multiplication, production of antimicrobial compounds (bacteriocins, hydrogen peroxide, organic and lactic acids), and the stimulation of the non-specific immune response of the host (Gatesoupe, 2008). Studies have demonstrated the beneficial effect of the addition of such bacteria in the culture of several aquatic species (Gatesoupe, 2008). Nevertheless, only a few studies have been conducted to assess the use of lactic acid bacteria strains isolated from marine shrimp.

Hemato-immunological parameters, such as the hemograms, phenoloxidase (PO) activity, and agglutinin activity of serum have been employed to monitor marine shrimp health conditions (Rengpipat et al., 2000; Gullian et al., 2004).

This study was aimed to evaluate the effect of a probiotic (*Lactobacillus plantarum*)-supplemented diet on growth, survival, digestive tract bacterial microbiota, and on some hemato-immunological parameters of a marine shrimp (*Litopenaeus vannamei*) after challenge with *Vibrio harveyi*.

MATERIAL AND METHODS

Post-larvae *L. vannamei* shrimp in the stage 20 (PL20), 0.08±0.01g (mean±SD) body weight, hatched at the Laboratório de Cultivo de Camarão Marinho - UFSC were used for the experiment. The lactic acid bacterium, *Lactobacillus plantarum*, was isolated from 30±3g *L. vannamei* broodstock shrimp reared at the same laboratory (Vieira et al., 2007). This bacterium was maintained in the CPQBA collection of microorganisms, Universidade Estadual de Campinas (UNICAMP), Brazil, under access number 007 07 DRM01. The tested pathogenic bacterium strain used was *Vibrio harveyi* ATCC 14126.

The probiotic bacterium *L. plantarum* was cultured in Man-Rogosa-Sharpe medium (MRS, Aumedia) and incubated under continuous agitation of 200rpm at 35°C for 24h to reach the concentration of 10^9CFU/mL.

The pathogenic bacterium *V. harveyi* was cultured in BHI medium (Brain Heart Infusion, Oxoid) under continuous agitation of 200rpm at 30°C for 24h and then centrifuged (1000 x g). The supernatant was discarded and the bacterial pellet was suspended in sterile saline solution (SSS; 1.5% NaCl). Bacterial concentration was estimated by counting the colonies formed on the TSA culture medium (Trypticase Soy Agar, Oxoid) after eight decimal dilutions. Inoculum concentration was adjusted to 10^7CFU/mL with 1.5% saline solution.

Two hundred milliliters of the lactic acid bacteria inoculum (10^8CFU/mL) were sprayed per kilogram of a commercial diet (35% crude protein). Probiotic incorporated diet was hermetically incubated at 35°C for 24h. The control diet was sprayed with sterile culture medium. Both diets were transferred to a forced air oven to dry at 35°C for 24h. The probiotic concentration in the final diet was $1.5\pm0.7x10^8$CFU/g.

Four hundred 20-day old shrimp post-larvae (PL 20) were stocked in eight circular tanks (1000L

capacity each). Post-larvae in four of the tanks were fed a *Lactobacillus plantarum*-supplemented diet and those in the other four tanks were fed a commercial diet without supplementation. Water temperature was kept at 28±2°C and salinity at 30%. Bottom sediment removal and 70% water exchange was done everyday (8h). Shrimp from both groups were fed *ad-libitum* four times a day (8h30min, 12h, 18h, and 22h).

The feeding trial was conducted for 60 days. Shrimp weight was determined every 15 days by sampling 30 shrimp per tank. Shrimp survival was determined at the end of the feeding trial. On days 0, 20, 40, and 60, five shrimp were randomly sampled from each tank for microbiological analysis of the digestive tract (n = 20 per treatment). Shrimp digestive tracts were removed with tweezers and scalpel, weighed, and homogenized with sterile saline solution (SSS) in a mortar and serially diluted (1/10) five times. Dilutions were spread on the following culture media: Marine agar (non-selective agar, total bacteria count, Oxoid), MRS agar (lactic bacteria selective), and TCBS agar (*Vibrio* spp. selective, Oxoid) and incubated at 30°C. On marine agar and TCBS agar, total colony forming units (CFU) were counted 24h after incubation, and 48h after incubation on MRS agar. Gram staining was performed with the colonies grown in MRS.

After the 60-day feeding trial, 35 shrimp (n = 140 per treatment) were challenged with *V. harveyi* (25µL SSS with 10^7CFU/mL injected in the first abdominal segment) and transferred to plastic tanks with 100L of seawater. Other 35 shrimp per tank were injected with 25µL of SSS in the first abdominal segment and equally transferred to plastic tanks with 100L of seawater, serving as negative controls. Shrimp survival was determined 10h after challenge. Five shrimp per tank (four pools of five shrimp, per treatment) were sampled for some hemato-immunological and microbiological analyses of hemolymph and hepatopancreas.

Hemolymph was collected by inserting a 21G needle (pre-cooled 4°C to avoid coagulation) coupled to a 1mL syringe, into the shrimp ventral sinus. After collection, 10µL sub-samples were fixed in 4% formalin-MAS solution (27mM sodium citrate, 336mM NaCl, 115mM glucose, 9mM EDTA, pH 7.0) to determine total hemocyte count (THC). Another 10µL sub-sample was used for microbiological analysis and the remaining hemolymph was left to clot on ice. The clot was then frozen and thawed three times, centrifuged at 10000 x g for 10min to obtain the serum, which was aliquoted and stored at -20°C.

After hemolymph collection, a sample of hepatopancreas of shrimps was removed with tweezers and scalpel, without disruption of digestive tract, to avoid contamination.

Sampled hemolymph (10µL) was spread under sterile conditions on TCBS agar (Thiosulphate Citrate Bile Salt Sucrose, Oxoid) to check for *Vibrio* sp. Shrimp hepatopancreas were weighed and homogenized with SSS and spread on TCBS agar. Total colony forming units were counted 24h after incubation at 30°C.

Total hemocyte count was directly determined using a Neubauer chamber. Serum protein concentration was determined according to the Bradford method (1976) using bovine serum albumin (BSA) as standard.

PO activity was detected spectrophotometrically (490nm) by the formation of the pigment DOPA-chrome, after oxidation of the substrate L-dihydroxyphenylalanine (L-DOPA, Sigma). Briefly, serum samples (eight pools of three shrimp) were diluted (1:9) in TBS (1mM Tris, 336mM NaCl, 5mM $CaCl_2$, 10mM $MgCl_2$, pH 7.4) and 50µL of the solution were incubated in triplicate with 50µL of enzyme inducer trypsin (Sigma, 1mg/L) for 5min in 96-microwell plates. After incubation, 50µL of L-DOPA (3mg/mL) were added in each well. Control was done with 100µL TBS added to 50µL of the 1:9 diluted serum in TBS. DOPA-chrome formation was monitored after 0, 5, and 10min. One unit of enzymatic activity is equivalent to a variation of 0.001 in the absorbance/min/milligram of protein (Söderhäll and Hall, 1984). Each test was performed in triplicates.

To determinate the agglutinating activity, samples of 50 Al of shrimp serum were added to a U-shaped 96-well microtiter plate and a twofold serial dilution was prepared using TBS (100mM Tris-HCl, pH 7.4, 150mM NaCl, 10mM $CaCl_2$) as diluent. The same volume of a 2% suspension of rat red blood cells in TBS was added to each well and incubated for 2-3h at

room temperature in a humid chamber. In controls, the shrimp serum was replaced by TBS. The titer of the natural agglutinating activity of the shrimp serum was expressed as the reciprocal of the highest dilution showing a positive pattern of agglutination. Each test was performed in duplicates.

Microbiological counts and THC were log(x+1)-transformed to homogenize variances before analysis. Agglutinating titre were $\log_2(x)$ transformed. Shrimp weigh gain and gut microbiological analysis of shrimp were analyzed by repeated measures ANOVA ($\alpha<0.05$). Experimental infection were analyzed by two-way (2x2) ANOVA ($\alpha<0.05$). Factor A levels were (P) probiotic group and (C) control group; and factor B levels were (V) challenged with *V. harveyi* and (S) negative control with sterile saline solution. When analysis of variance indicated difference between factors, Student Newmam Keuls (SNK) test was used at the significance level of 5% (Zar, 1984).

RESULTS AND DISCUSSION

After 60 days of feeding trial, shrimp survival and final weight did not differ (P>0.05) between the group fed probiotic-supplemented diet (81±5% and 6.86±0.25g for survival and weight, respectively) and the group fed the unsupplemented control diet (79±7% and 6.39±0.86g for survival and weight, respectively). This result corroborates with the results by Rengpipat et al. (2000), who added *Bacillus* S11 in *Penaeus monodon* culture water. Nevertheless, it has been reported that the dietary supplementation of *Lactobacillus acidophillus* and *L. sporogenes* for *Macrobrachium rosenbergii* increased shrimp growth rate (Venkat et al., 2004).

Total bacteria counts in shrimp digestive tract was not different (P>0.05) between groups on days 0, 20, and 40. However, on day 60, total bacteria counts in the group fed probiotic-supplemented diet was lower (P<0.05) than in the group fed the control diet (Figure 1a). *Vibrio* spp. count in shrimp digestive tract followed the same trend of total bacteria count, being lower (P<0.05) in the group fed the probiotic-supplemented diet only on day 60 day of feeding trial (Figure 1b).

Lactic bacteria count in the digestive tract was higher (P<0.05) in shrimp fed the probiotic-supplemented diet after 20 days of feeding trial (Figure 1c). In the probiotic group, Gram staining revealed Gram-positive bacilli-cocci colonies grouped in pairs, morphologically similar to those bacteria used as probiotic, whereas the control group presented different types of Gram-positive bacteria, mostly cocci.

In aquatic organisms, gut bacterial microbiota is mainly composed of Gram-negative bacteria, being the genera *Vibrio*, *Pseudomonas*, and *Aeromonas* predominant (Vine et al., 2006). Nevertheless, several studies have demonstrated that such bacterial microbiota can be modified by the addition of Gram-positive bacteria in the diet (Ziaei-Nejad et al., 2006; Vieira et al, 2008) or in the culture water (Rengpipat et al. 1998; 2000). Accordingly, in the present study, shrimp fed *L. plantarum* supplemented diet showed less viable total and vibrio bacteria, and high amounts of lactic bacteria. This would be related to the capacity of lactic bacteria to produce antimicrobial compounds such as hydrogen peroxide, bacteriocins (like reuterins), lactic acid, and other organic acids. In addition, lactic bacteria present in the digestive tract can also produce immunostimulating substances (Gatesoupe, 2008).

Modification in the gut microbiota can be an important tool to prevent diseases as the ecological niche occupied by benefic bacteria can avoid the fixation of possibly pathogenic ones (Gómez-Gil et al., 2000).

Survival of the group fed probiotic-supplemented diet was higher (P>0.05) than the group fed the control diet after 10h of challenge with *V. harveyi*. No mortality was observed in shrimp of the negative control group injected with SSS only (Table 1). Also, for *P. monodon*, a similar result was observed, as survival to *V. harveyi* infection was higher when *Bacillus* S11 was added to the water culture (Rengpipat et al., 1998). Higher survival of shrimp fed probiotic-supplemented diet might be related to an immune reactive effect of probiotics on the host. Lactic acid bacteria are known to produce extracellular compounds that can stimulate the non-specific immune response in vertebrates (Marteau et al., 2002; Gill, 2003).

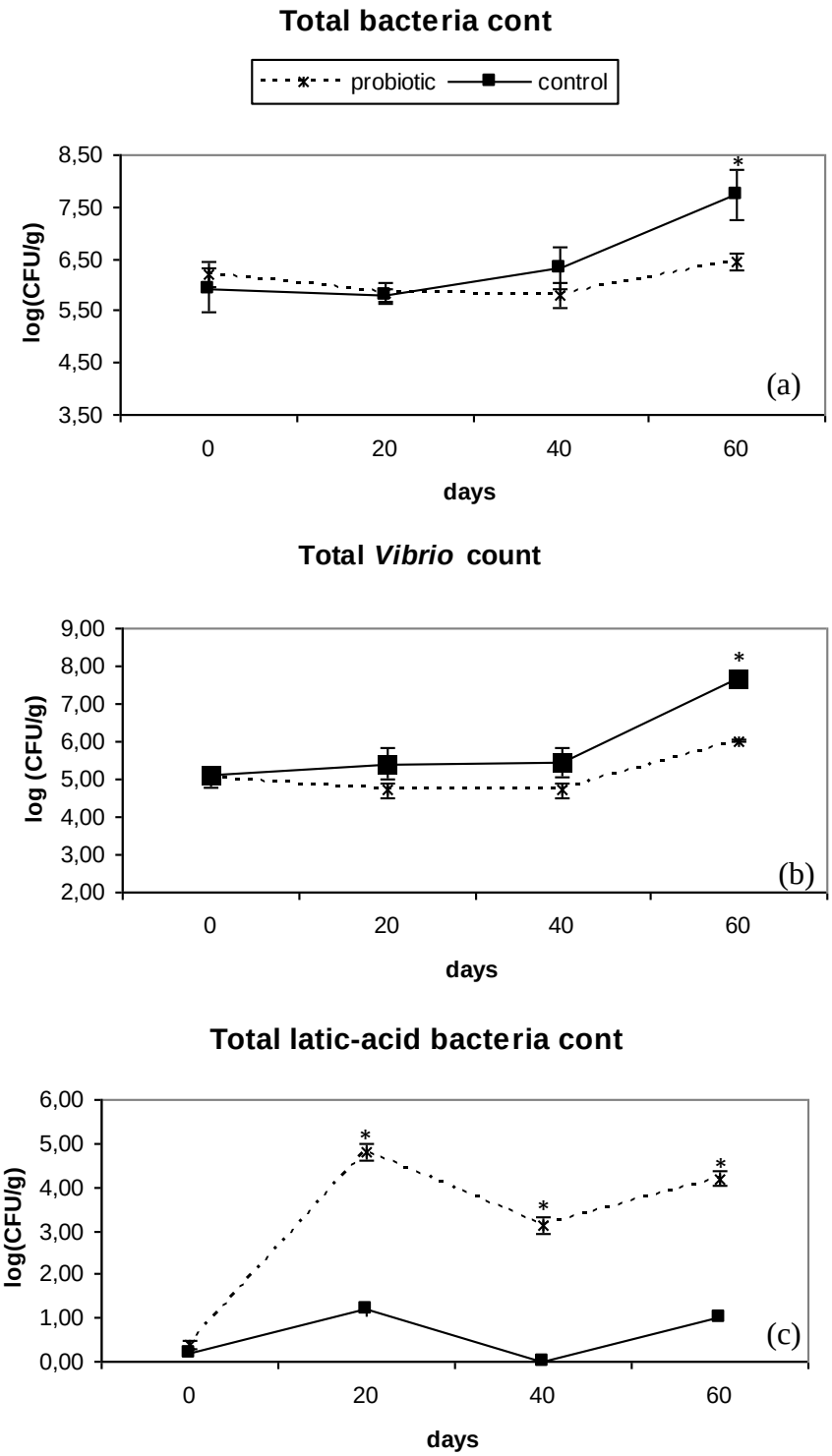

Figure 1. Bacterial microbiota of the digestive tract of shrimp fed probiotic-supplemented diet or control diet - a: total bacteria count; b: *Vibrio* spp. count; c: lactic-acid bacteria count.
*Significant difference after SNK mean comparison test (P<0.05).

Table 1: Survival, *Vibrio* spp. count in hemolymph and hepatopancreas, total hemocyte count (THC), phenoloxidase (PO) activity, and serum agglutination activity in shrimp fed probiotic-supplemented diet or control diet and challenged with *V. harveyi* or negative control (sterile saline solution injection - SSS)

Treatment	Survival (%)	*Vibrio* spp. (log CFU/mL)		THC ($x10^6.mL^{-1}$)	PO activity ($U/min/mg^{-1}$)	Agglutinating activity
		Hemolymph	Hepatopancreas			
Diet + *L. plantarum* and SSS injection	100±0a	0±0a*	0.28±0.1a	46.9±6.0a	22.1±6.6a	24,576.0±8192a
Control diet and SSS injection	100±0a	0±0a	2.5±0.1a	25.9±8.0a	37.3±10.2a	32,768.0±0a
Diet + *L. plantarum* and *V. harveyi* injection	65.7±2.9b	2.0±1.0b	2.9±0.2a	9.9±9.7c	57.4±14.5a	109,226.7±21,845.3b
Control diet and *V. harveyi* injection	39.9±4.4c	3.5±0.3c	3.90±0.02b	3.2±2.5b	29.7±9.3a	43,690.7±10,922.7a

*Means followed by distinct letters are different according to SNK test ($P<0.05$).

Vibrio spp. count in hemolymph and hepatopancreas of shrimps fed probiotic-supplemented diet was lower ($P<0.05$) than that from shrimp from the control group after the *V. harveyi* challenge. Shrimp injected with SSS (negative control) did not present *Vibrio* spp. in hemolymph and only a few in the hepatopancreas. *Vibrio* spp. count in the hemolymph was lower than in the hepatopancreas in all shrimp examined (Table 1).

Crustaceans immune system is formed by strong physical barriers (thick cuticle, for example) further to cellular and humoral defenses mediated by the hemolymph (Jiravanichpaisal et al., 2006). When bacteria cross the barrier of the cuticle, the immune system acts by quickly reducing the concentration of circulating microrganisms that fix in the shrimp organs (Van de Braak et al., 2002). It was observed in this study thnat bacterial concentration in the hepatopancreas was higher than in the hemolymph 10h after challenge. The presence of bacteria in the negative control group might be explained by the entrance of microorganisms from the water through the wound caused by the injection.

Total hemocyte count (THC) of shrimp fed probiotic-supplemented diet was three times higher than shrimp from the control group after the *V. harveyi* challenge. On the other hand, THC of shrimp injected with SSS was not different between the groups, but higher than the *V. harveyi* challenged group (Table 1). This result seems to be associated with an inflammatory response of hemocytes that leave the circulation and migrate to the site of injection (Van de Braak et al., 2002). Additionally, hemocytes can aggregate into haemocytic nodules in which cell adhesion molecules, such as peroxinectin, apprehend microorganisms between them (Jiravanichpaisal et al., 2006) and then are mechanically eliminated from the circulation through the gills (Martin et al., 2000).

The fact that shrimp fed probiotic-supplemented diet and challenged with *V. harveyi* presented higher number of circulating hemocytes than the control shrimp equally challenged can be related to the faster reposition of such cells by the hematopoietic tissue, which is possibly a sign of stimulation of the immune response.

Serum agglutination activity in the control group challenged with *V. harveyi* tended to increase, although not different ($P>0.05$) from the negative control group injected with SSS. Serum agglutination activity was higher ($P<0.05$) in the group fed *L. plantarum* supplemented diet and challenged with *V. harveyi* than in the other groups (Table 1). This may be due to a higher production of lectins in the shrimp fed the probiotic-supplemented diet in response to the infection, which indicates a possible immune reactive effect of *L. plantarum*. *P. monodon* shrimp presented elevation of the agglutination activity in the hemolymph when infected with *V. vulnificus* (Ratanapo and Chulavatnatol, 1992).

Lectins would work as specific molecular pattern recognition proteins (PRP) linking to specific carbohydrates present in the pathogen surface and initiating the effective immune responses, as agglutination of the invading microorganisms and/or their facilitated fagocitosis (Marques and Barracco, 2000). Lectins isolated from *L. vannamei* showed agglutinating activity against

Vibrio bacteria (Sun et al., 2007). The most effective elimination of bacteria from the hemolymph and the hepatopancreas by the shrimp fed the probiotic-supplemented diet could be related to the elevated agglutinating activity observed in these shrimp.

Phenoloxidase (PO) activity varied from 22 to 57U.min^{-1}.mg protein^{-1}, but no differences ($P>0.05$) were observed between groups, probably because of high variations observed in this parameter. However, after the experimental challenge with *V. harveyi*, PO activity tended to be twice as high in the group previously fed the probiotic-supplemented diet than in the group fed the control diet (Table 1).

Some studies have reported alterations in the PO enzyme activity in shrimp treated with probiotics, such as in *L. vannamei* (Gulian et al., 2004) and *P. monodon* (Rengpipat et al., 2000). Phenoloxidase is an enzyme that catalizes the oxidation of phenolic compounds as tyrosine and DOPA, which originate a complex molecular cascade that culminates with the formation of a dark pigment, melanine. During the cascade, several toxic intermediate compounds are formed as quinones and reactive oxygen species that are highly microbicidal (Sritunyalucksana and Söderhäll, 2000). In the present study, although no significant alteration in the PO activity was observed in shrimp treated with the probiotic *L. plantarum* and challenged with *V. harveyi*, there was a clear trend of increased enzyme activity (about twice as high), when compared to the PO activity in shrimp fed the commercial diet. This elevated trend may have contributed to the more efficient elimination of the bacteria from the hemolymph in the shrimp fed the *L. plantarum* supplemented diet and challenged with *V. harveyi* in comparsion with the control group.

This study confirmed the immunostimulatory effect of the probiotic tested, which allows to recommend its addition in commercial diets, specially in areas affected by diseases to strengthen shrimp defense conditions and minimize the effects of pathogenic agents.

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