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Use of probiotic-supplemented diet on a Pacific white shrimp farm

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ABSTRACT - The objective of this study was to evaluate the use of a diet supplemented with *Lactobacillus plantarum* on performance, immunological parameters, and bacterial microbiota of the digestive tract of white leg shrimp (*Litopenaeus vannamei*). We used six earthen ponds (1.2 ha) stocked with 14 shrimp m^{-2} (3 g mean weight). Three ponds received a diet supplemented with *L. plantarum* and three were maintained as control. The survival and feed efficiency of shrimp fed the supplemented diet were greater than that observed in shrimp fed the control diet, with survival values of 83.02±6.12% and 74.65±9.07% and feed efficiencies of 117.97±4.45% and 104.46±7.30%, respectively. However, we observed no differences in weight gain. The intestines of shrimp fed probiotics had lower counts of *Vibrio* spp. and higher counts of lactic acid bacteria, compared with those of control shrimp. Diets supplemented with *L. plantarum* alter the intestinal bacterial microbiota of shrimp, resulting in increased survival and feed efficiency.

Key Words: immune response, *Lactobacillus*, microbiology

Introduction

Recently, the interest in the use of probiotics in aquaculture to prevent diseases and increase resistance to pathogens has increased (Akhter et al., 2015). Gatesoupe (1999) defined a probiotic as "microbial cells that are administered in such a way as to be kept alive, with the aim of improving health". The use of probiotics produces benefits for marine shrimp by improving the balance of the intestinal microbiota (Vieira et al., 2008), survival (Pham et al., 2014), resistance to infection by pathogens (Aguilera-Rivera et al., 2014), immune stimulation (Ferreira et al., 2015), and diet digestibility (Buglione Neto, 2009). Unlike antibiotics, this method does not leave any residues in the shrimp meat (Kesarcodi-Watson et al., 2008).

Lactic acid bacteria are among one of the most used probiotics in aquaculture, due to their rapid reproduction, production of antimicrobial compounds (organic acids, lactic acid, bacteriocins, and hydrogen peroxide), and stimulation of non-specific immune responses in their hosts (Pandiyan et al., 2013). However, most studies on probiotics and marine crustaceans have been performed on a small laboratory scale and under controlled laboratory conditions (Vieira et al., 2007, 2008, 2010; Pham et al., 2014; Ferreira et al., 2015). Because laboratory conditions do not always match the conditions on commercial farms, there is a need for studies on the use of probiotics on commercial farms.

The objective of this study was to evaluate the use of a diet supplemented with *Lactobacillus plantarum* on performance, immunological parameters, and bacterial microbiota of the digestive tract of white leg shrimp (*Litopenaeus vannamei*) on a farm in the State of Santa Catarina, Brazil.

Material and Methods

The experiment was conducted in Balneário Barra do Sul, Santa Catarina, Brazil (26°32'S and 48°39'W), from February to April 2008.

We used the probiotic strain *L. plantarum* (accession #CPQBA 007 07 DRM01), which was isolated from an adult shrimp (*L. vannamei*) (Vieira et al., 2007).

Lactobacillus plantarum was grown in MRS liquid culture medium (Difco®; de Man, Rogosa and Sharpe, 1960) for 24 h under continuous agitation to a concentration of 10^9 cfu mL^{-1}. The probiotic was added to commercial rations (Table 1) at a ratio of 100 mL kg^{-1}, 30 min before being provided to the shrimp.

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Twenty-day-old shrimp post-larvae (PL20) were cultured in an intensive nursery (100 shrimp m^{-2}) until they reached a mean weight of 3 g. They were then transferred to six ponds of 1.20 ha at a mean stocking density of 14 shrimp m^{-2}. A randomised complete block design was used, with three blocks of two ponds stocked with shrimp from the beginning, middle, and end of the nursery tank harvest. Shrimp in one pond from each block were fed commercial diets supplemented with *L. plantarum,* and the shrimp in the other pond were fed control diets. The diets (32% protein) were provided twice daily (9.00 h and 16.00 h) in trays at approximately 3% of the biomass quantity.

The water temperature was measured daily (at 0.00 h, 5.00 h, and 17.00 h), as well as dissolved oxygen levels at the surface and bottom, using an oximeter (Alfakit® AT-150) and the concentrations of ammonia, nitrate, nitrite, and orthophosphate (according to Grasshoff et al., 1983) and pH (Alfakit® AT-300).

To evaluate weight gain, 30 shrimp from three points in the pond were collected every 15 days. The survival rates and feed efficiencies at the end of the culture period were evaluated. Thirty and sixty days after stocking, 20 shrimp (two pools of 10 animals) were collected per tank to evaluate immunological parameters (total haemocyte count, phenoloxidase enzyme activity, and the antimicrobial activity of the serum), the presence of bacteria in the haemolymph and hepatopancreas, as well as the bacterial microbiota of the digestive tract. Furthermore, the pleopods from 150 shrimp per pond were collected to detect white spot syndrome virus (WSSV) and the water sample from each pond was evaluated for its environmental bacterial microbiota.

The water samples were 1:10 serially diluted five times and plated on marine agar (Difco®), Thiosulfate Citrate Bile Sucrose Agar (TCBS, Difco®), and MRS agar for counts of total bacteria, vibrionaceae, and lactic acid bacteria, respectively. Colony-forming units (cfu) were counted after a 24-h incubation period for marine agar and TCBS agar plates and after 48 h for MRS agar plates.

Table 1 - Composition of the commercial feed

Ingredient	Final percentage (%)
Soybean meal	19.40
Fishmeal	21.00
43% meat	8.80
Wheat bran	10.00
33% sugarcane dry yeast	10.00
Rice bran (solvent extracted)	5.00
Liquid molasses	4.00
Broken rice	8.30
Hydrolysed feather meal	4.00
Fish oil	1.50
Tuna flavouring	4.00
Mono-dicalcium phosphate	1.35
Refined salt	0.50
Mineral-vitamin premix	2.15
Total	100.00

From the ventral sinus of each shrimp, 300 µL haemolymph were collected using sterile 1-mL syringes with 21-G needles, previously cooled at 4 °C to prevent coagulation. One 10-µL sample of the collected haemolymph was fixed in modified Alsever's solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) supplemented with 4% of formalin for the total haemocyte count (THC). The other 10-µL aliquots of haemolymph were used for microbiological evaluations, and the rest of the sample was left at 4 °C to coagulate. The clot was congealed (−20 °C) and defrosted (3×) to allow for cellular rupture and the release of the granular content, and was then repeatedly centrifuged at 4 °C (2,000 × *g* for 10 min). The supernatant or serum was aliquoted and stored at −20 °C for subsequent analyses.

After haemolymph collection, a hepatopancreas sample was carefully removed to avoid digestive tract rupture and, consequently, contaminating the sample (n = 60 per treatment). Finally, the intestines of the shrimp were excised and weighed in pools of five animals.

A sample of the collected haemolymph (10 µL) was plated on Petri dishes with TCBS agar under sterile conditions. The hepatopancreas samples were macerated in SSE and plated on TCBS agar Petri dishes under sterile conditions. The plates were incubated at 30 °C for 24 h to detect the presence of *Vibrio* spp.

The digestive tract samples were macerated in sterile saline solution at a 1:1 ratio (w:v). The samples were then 1:10 serially diluted five times in saline and plated onto Petri dishes with marine agar, TCBS agar, and MRS agar (Difco®). The cfu were counted after a 24-h incubation period for the marine agar and TCBS agar and after 48 h for the MRS agar. The colonies that grew on MRS agar were Gram-stained to confirm that their morphology was similar to that of the original probiotic.

The THC was estimated using a Neubauer chamber, and the serum total protein concentration was estimated via a Bradford (1976) assay, using bovine serum albumen (22%) as a standard.

Phenoloxidase enzymatic activity was determined by a colorimetric method by the formation of the pigment DOPA-chrome, after the enzymatic oxidation of the substrate L-dihydroxyphenylalanine (L-DOPA, Sigma), according to Söderhäll and Häll (1984).

For antimicrobial analysis, the bacterial strains *Vibrio harveyi* ATCC 14126 (pathogenic to the marine shrimp),

Escherichia coli D363 (a Gram-negative standard), and *Staphylococcus aureus* A270 (a Gram-positive standard) were used. V*ibrio alginolyticus* was cultured in saline peptone water (SPW) (1.5% peptone, 1.5% NaCl, pH 7.2), whereas the other bacteria were cultured in Luria Bertani (1% peptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) for 24 h at 30 °C. The assays were performed by serially diluting 50 µL serum in SPW for *V. harveyi* or poor broth (PB) (1% peptone, 0.5% NaCl, pH 7.4) for the other bacteria in 96 well, flat bottomed microtiter plates. Each well received 10 µL of a 10^3 cfu mL^{-1} bacterial suspension in the log phase. For the controls, the serum was substituted by saline. Bacterial growth was determined in a microplate reader (OD_{630}). The antimicrobial activity was estimated to be the reciprocal of the last well that inhibited bacterial growth.

The presence of WSSV was determined by nested PCR, using the primers and conditions described by Lo et al. (1996).

The total haemocyte counts and bacterial counts were transformed by log(x+1) for standardization and homogeneity of variances. The antimicrobial activity values were transformed by $\log_2(x)$. We used ANOVA with a randomized complete block design ($\alpha = 0.05$) for the animal performance indices, and we used ANOVA with a randomized complete block design and repeated measures ($\alpha = 0.05$) for the remaining analyses (Zar, 1984).

Results and Discussion

The bottom temperature (23.2-30.2 °C), surface temperature (24.5-31.3 °C), bottom dissolved oxygen (2.5-6.7 mg L^{-1}), surface oxygen (3.1-8.9 mg L^{-1}), pH (7.8-8.6), ammonia (0.018-0.023 mg L^{-1}), nitrite (0-0.01 mg L^{-1}), nitrate (0.66-0.89 mg L^{-1}), and orthophosphate (0.007-0.019 mg L^{-1}) remained within the recommended limits for culturing *L. vannamei*.

The total bacterial counts ($1.51 \pm 0.74 \times 10^4$ cfu mL^{-1}) and *Vibrio* spp. counts ($1.22 \pm 0.52 \times 10^2$ cfu mL^{-1}) in the culture water did not vary between treatments or collection periods ($P \geq 0.05$) and were similar to the values observed by Sung et al. (2001) on a *P. monodom* farm. As expected, we did not observe the presence of lactic acid bacteria in the culture water. Lactic acid bacteria are normally associated with the microbiota of the digestive tract of animals (Kongnun and Hongpattarakere, 2012); thus, they should not interfere with bacterial populations in the water column.

Lactic acid bacteria counts in the digestive tract of shrimp fed diets supplemented with *L. plantarum* were higher ($P < 0.05$) than those in the control shrimp, for both collection periods. There were also fewer *Vibrio* spp. ($P < 0.05$) in the digestive tract of shrimp fed the supplemented diets compared with the controls. There was no difference ($P \geq 0.05$) in total bacterial counts from the digestive tract of the treated and control shrimp, for both collection periods (Table 2).

As observed by Vieira et al. (2008) in laboratory conditions, the present study demonstrated that the diets supplemented with *L. plantarum* also altered the microbiota of shrimp on farm. The presence of lactic acid bacteria in the intestinal tract can enhance the animal immune system, resulting in shrimp more resistant to disease in the cultivation environment.

The alteration of the bacterial microbiota in the intestinal tract of marine shrimp by dietary probiotics is an important preventive tool against disease (Kongnun and Hongpattarakere, 2012). A significant reduction in the abundance of *Vibrio* spp. was found in the intestinal tract of shrimp fed the probiotic-supplemented diet, followed by an increase in the abundance of lactic acid bacteria (Table 2). This result is related to the ability of lactic acid bacteria such as *L. plantarum* to inhibit the adhesion of pathogenic bacteria to the intestinal mucosa (Balcázar et al., 2008) and to produce antimicrobial substances such as plantaricin (Hernández et al., 2005), hydrogen peroxide, and organic acids (Kongnun and Hongpattarakere, 2012). Therefore, the use of *L. plantarum* in the diet can help in the prevention of *Vibrio* disease, such as the acute hepatopancreatic necrosis disease (AHPND), which is

Table 2 - Bacterial microbiota in the digestive tract of shrimp (*Litopenaeus vannamei*) fed a control diet or a diet supplemented with *L. plantarum*, after 30 and 60 days of cultivation

Collection	Treatment	Microbiological evaluation of the digestive tract (cfu g^{-1})		
		Total bacteria	Total *Vibrio* spp.	Total lactic acid
30 days	Probiotic	$1.56 \pm 0.21 \times 10^7$	$4.98 \pm 0.08 \times 10^4$*	$5.64 \pm 1.50 \times 10^4$*
	Control	$2.36 \pm 0.28 \times 10^7$	$1.10 \pm 0.48 \times 10^5$	$1.67 \pm 0.24 \times 10^1$
60 days	Probiotic	$2.75 \pm 1.51 \times 10^5$	$1.33 \pm 0.81 \times 10^3$*	$2.15 \pm 1.02 \times 10^4$*
	Control	$8.83 \pm 1.21 \times 10^6$	$1.17 \times 0.03 \times 10^4$	0

*Significantly different in the analysis of variance (P<0.05).

Table 3 - Survival, feed efficiency, days of cultivation, final weight, and productivity in shrimp (*Litopenaues vannamei*) fed diets supplemented (probiotic) or not supplemented (control) with *L. plantarum*

Treatment	Survival (%)	Feed efficiency (%)	Days of cultivation	Final weight (g)	Productivity (kg ha^{-1})
Probiotic	83.02±6.12*	117.97±4.45*	77.00±6.42	11.2±0.62	1325.61±74.44
Control	74.65±9.07	104.46±7.30	72.33±9.7	11.96±0.35	1291.13±192.92

*Significant difference for the analysis of variance (P<0.05).

Table 4 - Phenoloxidase enzyme activity (PO), total haemocyte count (THC), and antimicrobial activity against *Vibrio alginolyticus*, *Staphylococcus aureus*, and *Escherichia coli* of the haemolymph from shrimp (*Litopenaeus vannamei*) fed a diet supplemented with *L. plantarum* or control diet after 30 and 60 days of cultivation

Collection	Treatment	Immunological parameter		Minimum inhibitory concentration (mg of protein mL^{-1})		
		PO (U min^{-1} mg^{-1})	THC (× 10^6 mL^{-1})	*V. alginolyticus*	*S. aureus*	*E. coli*
30 days	Probiotic	71.37±9.93	36.26±8.58	8.44±1.81	9.05±0.18	10.84±1.22
	Control	80.95±3.92	35.60±11.77	15.38±7.55	8.02±1.69	13.31±0.98
60 days	Probiotic	82.46±4.67	41.67±28.66	2.66±2.66	8.71±1.30	13.89±5.66
	Control	78.16±13.30	40.93±13.53	4.66±2.40	11.02±3.94	10.66±2.59

dramatically affecting the culture of shrimp in Asia and Mexico (Nunan et al., 2013).

The survival of shrimp supplemented with *L. plantarum* was higher (P<0.05) (Table 3). Bacteria of the genus *Vibrio* are known pathogens of marine shrimp (Vanmaele et al., 2015). Thus, the increased survival of shrimp supplemented with *L. plantarum* might be related to the decreased abundance of *Vibrio* spp. in the digestive tract. In *L. stylirostris*, diets supplemented with *Pediococcus acidilactici* also resulted in increased survival and reduced the populations of *Vibrio* spp. in the digestive tract, under similar field conditions (Castex et al., 2008). Wang et al. (2007) reported an increase in the survival of *L. vannamei* in commercial culture tanks using a commercial probiotic *Bacillus* sp.

Feed efficiency was higher (P<0.05) in shrimp fed supplemented diets (Table 3). Probiotic bacteria can produce or stimulate the production of digestive enzymes in the host (Kongnun and Hongpattarakere, 2012). Buglione Neto (2009) demonstrated an enhanced apparent digestibility of crude protein in *L. vannamei* supplemented with *L. plantarum* in commercial diets, which might explain the improved feed efficiency we observed here. Similar improvements in feed efficiency have been reported by Wang (2007) in the commercial culture of *L. vannamei* in China using commercial probiotics. However, there was no significant difference among the treatments in the final weight of shrimp, productivity or the number of days of culture (P≥0.05) (Table 3).

Lactic acid bacteria in the intestinal tract can also produce immunostimulatory substances (Gill, 2003). In *L. vannamei*, the use of a diet supplemented with *L. plantarum* stimulated an immunological response as reported by Vieira et al. (2008, 2010). However, we observed no differences in immunological parameters between treatments (P≥0.05) (Table 4).

In studies by Vieira et al. (2008), the immunological parameters of shrimp supplemented with *L. plantarum* changed in relation to control shrimp only after experimental infection with *V. harveyi*. In the present study, neither *Vibrio* spp. (haemolymph and hepatopancreas) nor WSSV was detected in the shrimp. Thus, the shrimp we sampled were healthy, which might explain why no changes were observed in the immunological parameters evaluated.

Conclusions

The use of a diet supplemented with *L. plantarum* increases the survival and feed efficiency of shrimp raised on farm.

Diets supplemented with *L. plantarum* modify the intestinal bacterial microbiota of shrimp raised on a commercial farm, decreasing the abundance of *Vibrio* spp. and increasing the abundance of lactic acid bacteria.

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