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Effect of inulin and probiotic bacteria on growth, survival, immune response, and prevalence of white spot syndrome virus (WSSV) in *Litopenaeus vannamei* cultured under laboratory conditions

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The effect of inulin and probiotic bacteria on the growth, survival, immune response and viral prevalence of white spot syndrome virus (WSSV) in white shrimp was evaluated. Presumptive bacilli and lactic acid bacteria (LAB) were characterized for hemolytic and enzymatic activity, hydrophobicity and antagonism against *Vibrio*. Selected isolates (Bacilli and LAB) were included in the diet of juvenile shrimp. Two bioassays were conducted with treatments by triplicate (10 shrimp per replicate) with inulin and inulin and bacteria. Fourteen LAB and six bacilli isolates had potential as probiotics. Survival and growth was not affected by the addition of the inulin and bacteria to diet. Inulin and bacteria improved immunity in cultured shrimp. Also, these feed additives reduced the prevalence of WSSV in cultured shrimp.

Key words: Probiotics, prebiotics, white spot syndrome virus (WSSV), immune system, *Litopenaeus vannamei*.

INTRODUCTION

The farming of white shrimp (*Litopenaeus vannamei*) is an important economic activity worldwide. However, outbreak of diseases caused mainly by viruses have affected the production performance of this industry. The white spot syndrome virus (WSSV) is one of the most virulent pathogens of penaeid shrimps since it can reach accumulative mortalities of up to 100% (Chou et al., 1995; Lo et al., 2003; Leu et al., 2009).

The strategies involved in aquaculture to prevent losses caused by diseases are the basic practices of good management, chemotherapy and vaccination (Subasinghe and Barg, 1998). However, in the last few years, the use of environmentally friendly microorganisms (probiotics), prebiotics, medicinal plants and immuno-stimulants have emerged as an appropriate way to prevent infections by bacterial and viral agents and as alternative growth promoters.

In aquaculture, the term probiotic is defined as a microbial supplement consisting of simple or mixed culture of selected microorganisms that are added in order to manipulate the microbial communities present in the systems of production (Balcázar, 2002). Probiotics have a beneficial effect on the host by changes in the host-related or ambient microbial community, through an improvement in the use of feed or its nutritional value, or by enhancing the host response to disease or by improving the quality of its environment (Verschuere, 2000; Balcázar, 2002; Van Hai and Fotedar, 2010;

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Wang and Gu, 2010). As the microbiota in the gastro-intestinal tract of aquatic animals can be modified by intake of other microorganisms, microbial manipulation is a viable tool to reduce or eliminate the incidence of opportunistic pathogens (Balcázar, 2002).

Prebiotics are nondigestible food ingredients which beneficially affect the host by selectively stimulating the growth and/or activate the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host's intestinal balance (Manning and Gibson, 2004; Gibson and Roberfroid, 1995). The prebiotics include fructooligosaccharides (FOS), transgalactooligosaccharides (TOS), mannanoligosaccharides (MOS), lactose and inulin (Teitelbaum and Walker, 2002; Vulevic et al., 2004). Inulin and its derivates (oligofructose, FOS) are known as fructans and are mainly constituted by linear chains of fructose (Madrigal and Sangronis, 2007). Prebiotics have been recognized for being involved in digestion, absorption and metabolism of various nutrients in terrestrial organisms (Swanson et al., 2002; Steer et al., 2003). Dietary supplementation of FOS has been shown to enhance immune capacity and growth rate of aquatic animals such as soft-shell turtle (Ji et al., 2004), turbot larvae (Mahious et al., 2006) and white shrimp (Li et al., 2009; Yousefian and Amiri, 2009).

Probiotics and prebiotics are mostly investigated separately. The symbiotic, as a combination of probiotics and prebiotics, have not been intensively studied up to date. Their individual advantages might be additive or even synergistic, but this hypothesis needs to be qualified (Li et al., 2009).

Shrimp possess an innate immune system, consisting of cellular and humoral elements. Hemocytes play a central role in the non-specific immune response of shrimp, which rely mainly on phagocytosis, melanization, encapsulation, cytotoxicity and clotting (Sritunyalucksana et al., 1999). Humoral defence factors, such as clotting proteins, agglutinins, hydrolytic enzymes and antimicrobial peptides are released upon lysis of hemocytes, which is induced by lipopolysaccharides (LPS), peptidoglycans, and b-1,3-glucans (Johnson and Söderhäll, 1989; Söderhäll et al., 1994; Chisholm and Smith, 1995; Muta and Iwanaga, 1996; Destoumieux et al., 2000).

The aim of this study was to evaluate the effect of inulin and bacteria on growth, survival, immune response and prevalence of WSSV in *Litopenaeus vannamei* cultured under laboratory conditions.

MATERIALS AND METHODS

Isolation and culture of presumptive lactic acid bacteria (LAB)

Guts of 10 juvenile shrimp were removed (*L. vannamei*, n = 5; *Farfantepenaeus californiensis*, n = 5). Each gut was dissected aseptically and homogenized in an Eppenforf tube with 400 µl of sterile saline solution with 2% NaCl. The homogenate (100 µl) was inoculated on Rogosa agar plates (RA, Difco, USA) with 2% NaCl and incubated at 30°C for 120 h. de Man, Rogosa and Sharpe (MRS) agar plates (BD Difco, USA) with aniline blue (200 mg/l) and NaCl (2%) were used for LAB isolation. Each growing colony on Rogosa plates was spread on MRS plates by the cross-streak method and incubated at 30°C for 24 h. Each blue isolate was stored at -80°C in MRS broth (BD Difco, USA) with 15% (v/v) glycerol.

Isolation and count of presumptive *Bacillus*

Guts and hepatopancreas of six juvenile shrimp (*L. vannamei*) were removed. Each sample was dissected aseptically and homogenized in an Eppendorf tube with 400 µl of sterile saline solution with 2% NaCl. The samples were incubated at 80°C for 10 min in order to eliminate vegetative bacterial cells and to favour the spore-forming bacteria such as *Bacillus*. The samples were spread on trypticase soy agar (TSA, BD Bioxon, USA) plates with NaCl (2%) by the cross-streak method. The plates were incubated for 24 h at 37°C, and growing colonies were purified and stored at -80°C in trypticase soy broth (TSB) with 15% (v/v) glycerol.

Characterization of the isolates

The isolates were characterized using Gram stain and cellular morphology. In addition, hemolysis, growth, hydrophobicity, inhibitory activity against presumptive vibrios and enzyme production were studied as selection criteria for potential probionts.

Hemolysis on human blood

The supernatant fraction of the 24 h-culture of each isolate in MRS (LAB) and TSB (bacilli) was obtained by centrifugation at 10,000 *g* for 10 min and tested for its hemolytic activity on blood agar (BA, BD Bioxon, USA) plates prepared with 1 ml of heparinized human blood. Supernatant pH was adjusted to 6.5 with NaOH (1 M) to avoid false positives (Balcázar et al., 2008). Wells of 6 mm diameter were made on BA plates. Each well was filled with 50 µl of supernatant or MRS and TS broth (negative controls) and incubated for 48 h at 37°C. Three types of hemolytic activity were expected: α-hemolysis (incomplete hemolysis) when the agar around the well is dark and greenish; β-hemolysis (complete hemolysis) when the agar around the well appears lightened and transparent and; γ-hemolysis (lack of hemolysis) when the agar around the well show no changes. The strains showing α and β-hemolysis were discarded as potential probionts and were not used for further analysis.

Bacterial growth kinetics

Growth curves were determined for each isolate to identify the log phase. The isolate stock (20 µl) was inoculated into 50 ml of MRS (LAB) and TS (bacilli) broth with 2% NaCl. Cultures were incubated at 30 (LAB) or 37°C (bacilli) to determine growth by reading the absorbance of cultures in a Thermo Spectronic Genesys 2 Spectrophotometer (Thermo Scientific, USA) at 580 nm for 96 h. MRS or TS broth with 2% NaCl was used as a blank.

Colony forming unit counts (CFU)

Each isolate was grown as in the kinetics assay, and 1 ml of the culture was centrifuged at 10,000 *g* for 20 min. The cellular pellet was resuspended in 1 ml of sterile saline solution at 2% NaCl. The bacterial suspension was adjusted to an optical density of 1.0 in a

Thermo Spectronic Genesys 2 Spectrophotometer at 580 nm. The serial dilution method was used to determine the CFU/ml of bacterial suspension.

Hydrophobicity test

To determine the hydrophobicity of the bacteria, Petri plates were prepared with MRS or TSA with 2% NaCl and 0.03% Congo red (Sigma, USA). Congo red was added after sterilization of mediums. Each isolate was spread on plates by the cross-streak method and incubated at 30 or 37°C for 24 to 48 h. Red colonies were considered positive and white or colorless colonies were considered negative (Sharma et al., 2006).

Extracellular enzymatic activity

Extracellular protease and lipase activities were determined (León et al., 2000). Isolates were grown as in kinetic assay, the supernatant pH of LAB was adjusted as in human blood hemolysis assay. Proteolytic activity was tested in basal medium (1.5% agar and 0.5% yeast extract) added with 2% skimmed milk (Fluka, Switzerland) or 1% gelatin (Knox).

Lipolytic activity was tested in basal medium plates supplemented with Tween 80 (1%) (Sigma, Germany). Wells (6.0-mm diameter) were sunk on every plate. The wells were filled with 50 µL of bacterial supernatant (LAB or bacilli) and incubated at 30 to 37°C for 24 h. MRS or TS broth with 2% NaCl was used as a negative control and a β-hemolytic isolate was used as a positive control. A clear zone surrounding the well indicated proteolytic activity and opacity of the medium surrounding the well indicated lipolytic activity.

Inhibitory activity

The bacterial supernatant of 24-h cultures was obtained as described above and tested for its inhibitory activity against *Vibrio sinaloensis* VHPC18 and *V. sinaloensis* VHPC23 (Flores-Miranda et al., 2012) by the well-diffusion assay (Bauer et al., 1966). TSA plates with 3% NaCl were overlaid with approximately 1×10^6 CFU. Wells (6 mm diameter) were sunk on every Petri plate and filled with 50 µl of bacterial supernatant. The plates were incubated at 37°C for 48 h. MRS or TSB with 2% NaCl was used as control. The inhibition zone around the wells was recorded.

Preparation of experimental diet with bacteria with potential as probiotic and inulin

A bacterial mixture with potential as probiotic [LAB (BAL3 and BAL7), bacilli (BC1 and CIB1)] and inulin from blue agave (*Agave tequilana*, IIDEAL, S.A. de C.V., Guadalajara, Jalisco, Mexico) were sprayed on commercial feed (Purina®, 35% protein). Bacteria were counted as in colony forming unit counts. Bacteria were added at 1×10^5 CFU/g feed (5×10^4 CFU/isolate) and inulin at 0.4 or 0.8 g kg/feed. The concentrations of bacteria and prebiotic were based on the works of Apún-Molina et al. (2009) and Zhou et al. (2007, 2009). Dry Oil (DO, Innovaciones Acuícolas, Mexico) was used in all treatments (including a control group) as an adhesive and feed attractant following manufacturer's instructions. Feed was dried at room temperature for 4 h and then stored at 4°C. The viability of bacteria at 4°C was evaluated (data not shown). In agreement with the analysis of viability, the feed with bacteria was prepared every five days.

Animals

Two batches of 220 and 200 apparently healthy shrimp where collected from a commercial farm (Acuícola Cuate Machado, Guasave, Sinaloa, Mexico) and immediately transported to the laboratory facilities of CIIDIR Sinaloa in a plastic container (250 l) provided with sea water and aeration. The collected shrimp had no signs of WSSV and/or bacterial infections.

Shrimp acclimation to culture conditions

Shrimp was acclimated to culture conditions for five days in 120-L indoor plastic tanks containing 80 L of filtered (20 µm) sea water (34 to 35 g/l) and constant aeration in groups of 10 organisms per tank. Shrimp was fed twice daily at 09:00 and 17:00 h with commercial feed (Purina, Mexico, 35% protein). Feeding ration was 7% of mean body weight. Half of the water was exchanged at day three. Uneaten food and waste matter were removed daily before feeding.

Experimental design

Two bioassays were conducted to evaluate the effect of feed supplemented with bacteria and/or inulin. Prebiotic and probiotic bacteria were evaluated in terms of growth performance, survival, immune response and prevalence of WSSV in experimental shrimp. Before the bioassays, prevalence of WSSV in experimental shrimp was done. Shrimp was WSSV positive (data not shown). The specific growth rate (SGR) was determined using the following equation (Ziaei-Nejad et al., 2006):

$$SGR = (LN\ W_t - LN\ W_0) \times 100/t$$

Where t, is the culture period in days, LN W0 is the natural logarithm of the shrimp weight at the beginning of the bioassay and LN Wt is the natural logarithm of the shrimp weight at day t (W_0 and W_t are in grams).

Animals were maintained in an indoor culture system in 120-L plastic tanks with 80 L filtered (20 µm) sea water and constant aeration. Each treatment had three replicates with 10 shrimps per tank. Shrimp was fed with commercial feed (Purina, 35% protein) twice daily at 09:00 and 16:00 h. Initially, animals were fed 6% of the mean body weight and adjusted thereafter according to the feeding response of shrimp in each tank. Uneaten food and waste matter were removed every three days before feeding, and 50% of the water was exchanged. Values of pH (HI 98127 pHep, Hanna Instruments, USA), salinity (Refractometer W/ATC 300011, Sper Scientific, USA), dissolved oxygen and temperature (YSI model 55 oxygen meter, Spring Instruments, USA) were monitored every three days. Water samples were analysed for nitrites, nitrates and ammonia at the beginning, at the middle and at the end of each experiment, following the Strickland and Parsons (1968) method.

The first bioassay was conducted for 60 days with shrimp weighing 1.4 ± 0.31 g. During the first five days of experimental condition, animals were fed with commercial feed only. At days six to 60, animals were fed with commercial feed supplemented with inulin and inulin and bacteria (LAB and bacilli mixture). The experiment was conducted as a completely randomized design with five treatments: I) shrimp fed with commercial feed (control group); II) shrimp fed with commercial feed + inulin (0.4 g/kg feed); III) shrimp fed with commercial feed + inulin (8.0 g/kg feed); IV) shrimp fed with commercial feed + inulin (0.4 g/kg feed) and bacteria (1×10^5 CFU/g feed); V) shrimp fed with commercial feed + inulin (8.0 g/kg feed) and bacteria (1×10^5 CFU/g feed).

At the end of the bioassay, survival was determined as a percentage and shrimp were weighed. In addition, hemolymph was

collected for the analysis of the immune system.

The second bioassay was conducted for 21 days with shrimp weighing 12.8 ± 1.8 g. During the first seven days of experimental condition, animals were fed with commercial feed (treatments I and II) and commercial feed plus inulin and bacteria (treatments III and IV). At day eight, animals from all treatments were fed with only 1 g per tank of muscle shrimp paste positive for WSSV (one-step PCR). From day nine to 21 animals were fed as the first seven days. The experiment was conducted as a completely randomized design with four treatments: I) shrimp fed with commercial feed (control group); II) shrimp fed with commercial feed + WSSV; III) shrimp fed with commercial feed + WSSV + bacteria (1 x 10^5 CFU/g feed); IV) shrimp fed with commercial feed + WSSV + inulin (8.0 g/kg feed) and bacteria (1 x 10^5 CFU/g feed).

At the end of the bioassay, survival was determined as a percentage and shrimp were weighed. Also, the screening of live and dead shrimp for WSSV (single and nested PCR) was done for all treatments.

During the first bioassay, the water temperature was maintained between 25.07 ± 0.06 and 26.32 ± 0.06°C; oxygen between 5.00 ± 0.17 and 5.82 ± 0.19 mg/l; pH between 8.15 ± 0.42 and 8.37 ± 0.10; and salinity between 35.10 ± 0.1 and 35.82 ± 0.1‰. The total ammonium concentration was 0.77 ± 0.01 and 0.97 ± 0.05 mg/l; nitrites 0.06 ± 0.00 and 0.12 ± 0.05 mg/l and nitrates between 0.68 ± 0.05 and 0.80 ± 0.10 mg/l.

During the second bioassay, the water temperature was maintained between 28.91 ± 0.01 and 29.60 ± 0.02°C; oxygen between 5.02 ± 0.10 and 5.80 ± 0.11 mg/l; pH between 8.10 ± 0.53 and 8.42 ± 0.10; and salinity between 35.10 ± 0.1 and 35.52 ± 0.1‰. The total ammonium concentration was 0.76 ± 0.01 and 0.99 ± 0.05 mg/l; nitrites 0.18 ± 0.01 and 0.36 ± 0.00 mg/l, and nitrates between 0.62 ± 0.07 and 0.80 ± 0.10 mg/l.

Water parameters measured during both bioassays remained within the optimum ranges for shrimp (Boyd and Tucker, 1998).

Prevalence of WSSV

WSSV detection was performed by nested PCR, using the primers WSSVout-1/WSSVout-2 and WSSVin-1/WSSVin-2 (Kimura et al., 1996), which amplified genome fragments of 982 and 570 bp. Negative samples were tested with an internal control that amplified a 298 bp segment of shrimp GAPDH DNA using the primers GAPDH298F and GAPDH298R by one-step PCR (Tang and Lightner, 2001).

Hemolymph collection

Hemolymph from 12 intermoult shrimp per treatment was analyzed. Hemolymph (200 to 300 µl) of individual shrimp was withdrawn from the pleopod base of the first abdominal segment using a sterile 1 ml syringe with a 25 gauge needle. Before hemolymph extraction, the syringe was loaded with a precooled (4°C) solution (SIC-EDTA) (450 mM NaCl, 10 mM KCl, Hepes 10 Mm, EDTA-Na_2 10 mM, pH 7.3) used as anticoagulant (Vargas-Albores et al., 1993). Fifty microliters of the anticoagulant-hemolymph mixture were diluted in 150 µl of formaldehyde (4%) and then 20 µl were placed on a hemocytometer (Neubauer chamber) to determine the total hemocytes count (THC) using a compound microscope.

Separation of plasma and hemocytes

Samples of hemolymph were centrifuged at 800 *g* for 5 min at 4°C and the supernatant was considered plasma. The hemocyte pellet was resuspended and washed twice in precooled cacodylate buffer [$Na(CH3)_2$ AsO_2 • $3H_2O$] (10 mM, pH 7) from Sigma by centrifugation at 800 *g* for 5 min at 4°C. Finally, the hemocytes were resuspended in 600 µl cacodylate buffer.

Preparation of hemocyte lysate supernatant (HLS)

Samples were frozen at -70°C to break the hemocytes and then thawed twice. Individual samples were centrifuged at 15,000 *g* for 5 min at 4°C and the HLS was used immediately to run the immunological analysis or stored at -70°C.

API ZYM system

The API ZYM commercial kit for enzymatic activity detection (BioMerieux, Durham, NC, USA) is a semiquantitative colorimetric micromethod to assess 19 hydrolytic enzymes. This was used according to the instruction manual of the manufacturer. Samples (one pool, each one of six animals) of HLS or plasma were added to the reactions strips, 65 µl/well, and incubated at 37°C for 4 h. Five to ten minutes after addition of reagents from the API ZYM kit at room temperature, the resulting colours were estimated under natural light and recorded as 0 to 5, according to a colour scale provided by the manufacturer, and transformed to the amount (nM) of hydrolyzed substrate. The activity was expressed as units, where one unit represents the substrate hydrolyzed in nM/mg protein. A one-time measurement was performed for each pool (one strip).

Lysozyme-like activity assay

To detect lysozyme-like activity, the inoculated substrate in glass Petri dishes (90 mm in diameter; 15 mm high) was used as a standard assay. One millilitre was taken from a 4 mg/ml suspension of dried *Micrococcus lysodeikticus* (Sigma) and diluted in 14 ml of 50 mM Tris-HCl buffer-1% agarose at pH 5.2, and then spread on a Petri dish. Once the agarose had solidified, 6.0-mm diameter wells were sunk in the substrate. Human saliva, diluted 1:9 in a 0.1% NaCl solution, was used as a positive control. Tris-HCl buffer was used as a negative control. Wells were filled with 30 µl of sample (HLS) and controls. After 24-h incubation at 37°C, the diameter of the clear zone surrounding the wells was measured. The diameter of each clearance zone was obtained by measuring the total diameter minus the diameter of the well. Results were expressed in units (0.1 mm = 1 U) per mg protein (U/mg protein). A one-time measurement was done for each pool, each one with three replicates (Canicatti, 1990).

Protein determination

The protein concentration was determined according to the method described by Bradford (1976), with bovine serum albumin (BSA) from Sigma as standard. In HLS, protein ranged from 0.08 ± 0.01 to 0.32 ± 0.01 mg/ml. In plasma, protein ranged from 34.99 ± 1.47 to 52.05 ± 0.04 mg/ml.

Statistical analysis

A one-way analysis of variance (ANOVA) using the *F* test was applied to examine the differences in growth and survival (%) among treatments. Survival data were arcsine transformed according to Daniel (1997). Where significant ANOVA differences were found, Tukey's HSD test was used to identify the nature of these differences at $P < 0.05$.

Table 1. Weight at harvesting, specific growth rate and survival of shrimp fed diets supplemented with inulin and probiotic bacteria in the first bioassay (mean ± SD).

Mean value	Treatment				
	I	II	III	IV	V
Weight at harvesting (g ± SD)	8.9 ± 2.2	8.8 ± 1.1	8.7 ± 1.5	8.8 ± 1.2	8.8 ± 1.2
Specific growth rate (%/day ± SD)	3.2 ± 0.1	3.1 ± 0.1	3.1 ± 0.3	3.1 ± 0.1	3.1 ± 0.1
Survival (%)	97	97	97	90	97

Treatments: I, Shrimp fed with commercial feed (control group); II, shrimp fed with commercial feed + inulin (0.4 g/kg feed); III, shrimp fed with commercial feed + inulin (8.0 g/kg feed); IV, shrimp fed with commercial feed + inulin (0.4 g/kg feed) and bacteria (1 x 10^5 CFU/g feed); V, shrimp fed with commercial feed + inulin (8.0 g/kg feed) and bacteria (1 x 10^5 CFU/g feed).

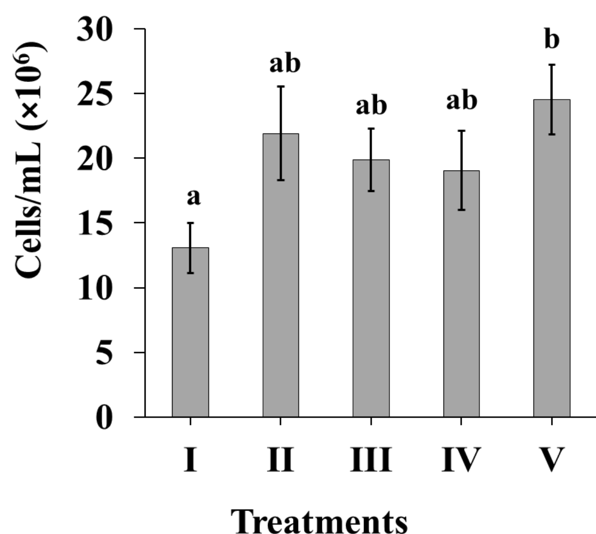

Figure 1. Total hemocyte counts of *L. vannamei* in the first bioassay. Treatments: I, Shrimp fed with commercial feed (control group); II, shrimp fed with commercial feed + inulin (0.4 g/kg feed); III, shrimp fed with commercial feed + inulin (8.0 g/kg feed); IV, shrimp fed with commercial feed + inulin (0.4 g/kg feed) and bacteria (1 x 10^5 CFU/g feed); V, shrimp fed with commercial feed + inulin (8.0 g/kg feed) and bacteria (1 x 10^5 CFU/g feed). Different superscripts indicate significant differences ($P < 0.05$). Error bars = mean ± standard error.

RESULTS

Bioassay I

Forty-five (45) presumptive bacilli (rod shaped) were isolated from *L. vannamei,* 27 from the gut and 18 from hepatopancreas. In human blood, 28 isolates depicted β-hemolysis, 11 isolates depicted α-hemolysis and six isolates depicted γ-hemolysis. From selected isolates with gamma hemolysis, only the supernatant of the isolate CIB1 showed inhibitory activity against *V. sinaloensis* VHPC18 and *V. sinaloensis* VHPC23 (Flores-Miranda et al., 2012) with a halo of 3 mm in diameter. Twenty (20) presumptive LAB (cocci) were isolated from brown shrimp gut (*F. californiensis*), five strains depicted β-hemolysis, one depicted α-hemolysis, and 14 depicted γ-hemolysis. Isolates with gamma hemolysis did not show hemolysis in shrimp hemolymph or inhibitory activity against vibrios. Only the gamma hemolysis isolates (six bacilli and fourteen LAB) were selected for successive characterization.

All the isolates were Gram (+). The log phase was found between 6 to 48 h. When the bacterial suspension was adjusted to an optical density of 1, the CFU of the isolates ranged from 157.5 x 10^6 to 610 x 10^6/ml. The bacilli isolate CIB1 and CHB10 were congo red-negative. The isolates CIB3, BC1, BA13 and BA4 were congo red-positive. The fourteen LAB isolates were congo red-positive. Positive isolates have hydrophobic structures in their cellular wall. Proteolytic activity was found in all the bacilli isolates; however, proteolytic activity in LAB was negative. None of the isolates showed lipolytic activity.

Table 1 summarizes the results of weight at harvesting, specific growth rate and survival obtained in the first bioassay. No significant differences among treatments were found ($P > 0.05$).

In treatment V, total hemocyte count was significantly higher than the control group (P = 0.04). THC in treatments II, III and IV were similar to treatment I and V. The combination of inulin (8.0 g/kg feed) and bacteria (1 x 10^5 CFU/g feed) increased the immune capacity of shrimp since hemocytes are the first line of defense (Figure 1).

Twelve (12) hydrolytic enzymes included in the API ZYM kit were detected in samples of *L. vannamei* (Table 2). Twelve (12) enzymes were found in plasma, and six were found in HLS. The highest levels of enzymatic activity were found in the HLS. In plasma, only the activity of β-galactosidase showed a clear pattern among treatments. The activity of β-galactosidase in the control group (I) was 8.8 U, in treatment II (inulin 0.4 g/kg feed) 6.5 U, and zero in treatments III, IV, and V. In HLS, only the activity of naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glu-cosaminidase showed a clear pattern. The activity of naphthol-AS-BI-phosphohydrolase in the con-

Table 2. Enzymatic activity (one unit represents the substrate hydrolyzed in nM/mg protein) using the API ZYM kit in the plasma and HLS of *L. vannamei* in the first bioassay.

Enzyme	Plasma					HLS				
	I	II	III	IV	V	I	II	III	IV	V
Alkaline phosphatase	13.2	9.8	8.9	9.3	10.4	0	0	0	256.4	240.4
Esterase (C4)	2.2	1.6	1.5	1.5	1.7	961.5	427.4	295.9	1025.6	961.5
Esterase lipase (C8)	2.2	3.3	1.5	3.1	1.7	961.5	854.7	591.7	1025.6	961.5
Leucine arylamidase	2.2	1.6	1.5	1.5	1.7	0	0	0	0	0
Cystine arylamidase	2.2	1.6	1.5	1.5	1.7	0	0	0	0	0
Acid phosphatase	8.8	6.5	8.9	9.3	10.4	961.5	427.4	591.7	1025.6	480.8
Naphthol-AS-BI-phosphohydrolase	15.5	1.6	1.5	15.6	1.7	961.5	427.4	295.9	256.4	240.4
β-Galactosidase	8.8	6.5	0	0	0	0	0	0	0	0
β-Glucuronidase	2.2	1.6	1.5	1.5	1.7	0	0	0	0	0
α-Glucosidase	2.2	3.3	1.5	0	1.7	0	0	0	0	0
N-acetyl-β-glucosaminidase	2.2	1.6	1.5	1.5	3.5	961.5	427.4	295.9	1538.5	1923.1
α-Fucosidase	4.4	6.5	3.0	3.1	3.5	0	0	0	0	0
Lysozyme	0	0	0	0	0	0	0	0	0	0

Treatments: I, Shrimp fed with commercial feed (control group); II, shrimp fed with commercial feed + inulin (0.4 g/kg feed); III, shrimp fed with commercial feed + inulin (8.0 g/kg feed); IV, shrimp fed with commercial feed + inulin (0.4 g/kg feed) and bacteria (1 x 10^5 CFU/g feed); V, shrimp fed with commercial feed + inulin (8.0 g/kg feed) and bacteria (1 x 10^5 CFU/g feed).

trol group (I) was two and threefold higher than treatments with inulin (II, III) and inulin and bacteria (IV, V). The activity of N-acetyl-β-glucosaminidase in the control group was two and threefold higher than treatments with inulin; however it was twofold lower than treatments with inulin and bacteria. In this work, some enzymes (leucine arylamidase, cystine arylamidase, β-galactosidase, β-glucuronidase, α-glucosidase and α-fucosidase) were found in plasma but not in HLS. In lysoplate assay, lysozyme activity was not found in both plasma and HLS (Table 2).

Bioassay II

No significant differences among treatments were found ($P > 0.05$) in growth (data not shown). Figure 2 summarizes the results obtained in the second bioassay. The survival of shrimp in treatment I was 89.0%, and the prevalence of WSSV-infected shrimp determined by nested PCR was 44.4%, out of which 11.1% were dead, and 33.33% were alive by the end of the experiment. It is possible that about 44.4% of farmed shrimp in the earthen pond were WSSV positive. The survival of shrimp in treatment II was 92.6%, and the prevalence of WSSV-infected shrimp determined by nested PCR was 51.85%, out of which 7.4% were dead, and 44.4% were alive by the end of the experiment. The survival of shrimp in treatment III was 88.9%, and the prevalence of WSSV-infected shrimp determined by nested PCR was 51.8%, out of which 11.1% were dead and 40.7% were alive by the end of the experiment. The survival of shrimp in treatment IV was 100%, and the prevalence of WSSV-infected shrimp determined by nested PCR was 22.2%, out of which 0% was dead, and 22.2% were alive by the end of the experiment. Results show that inulin (8.0 g/kg feed) and bacteria (1 x 10^5 CFU/g feed) reduced prevalence of WSSV in experimental infected shrimp from 51.8 to 22.2%.

DISCUSSION

In shrimp farming, probiotics and prebiotics act as an alternative treatment to antibiotics and chemicals, and play the role of alarm molecules to activate the immune system (López et al., 2003). The benefits of the use of prebiotics and probiotics to the host have been mentioned by several authors (Douglas and Sanders, 2008; Reid, 2008; Wang et al., 2008). In this work, we found 45 presumptive bacilli and 20 presumptive LAB, and they were evaluated for their probiotic

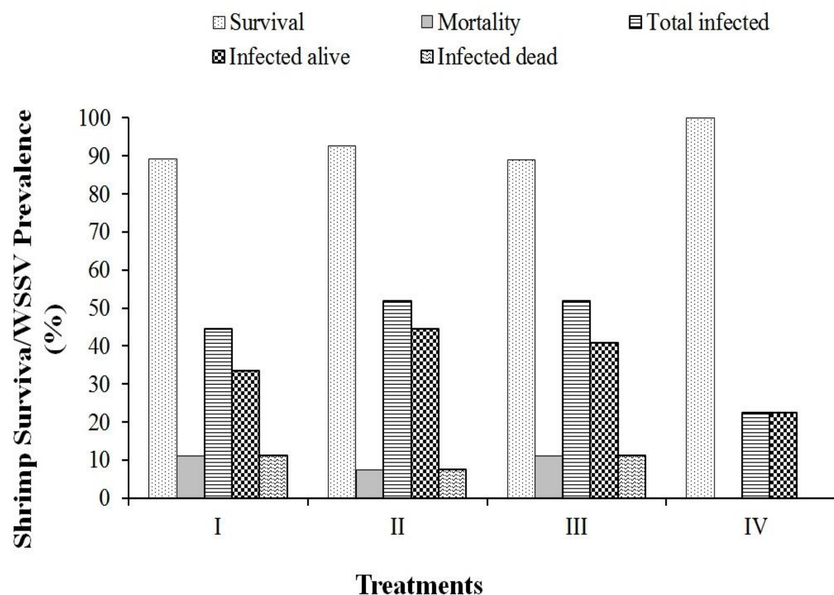

Figure 2. Percentage of shrimp survival, mortality and WSSV prevalence in the second bioassay. Treatments: I, Shrimp fed with commercial feed (control group); II, shrimp fed with commercial feed + WSSV; III, shrimp fed with commercial feed + WSSV + bacteria (1 x 10^5 CFU/g feed); IV, shrimp fed with commercial feed + WWSV + inulin (8.0 g/kg feed) and bacteria (1 x 10^5 CFU/g feed). Data in percentage are expressed as means.

potential in white shrimp. A criterion to discard potential harmful bacteria was their ability to produce toxins that induce lysis of erythrocytes and hemocytes (Apún-Molina et al., 2009). According to this, the isolates displaying β or α-hemolysis were discarded because of their possible negative effect on humans and shrimp, and bacteria with gamma hemolysis (14 LAB and six bacilli isolates) were selected as potential probiotics.

On the other hand, other criterions to select potential probiotic bacteria include the adherence capability (hydrophobicity), antagonism against pathogens and production of extracellular enzymes. In this work, from bacteria with gamma hemolysis, 14 LAB isolates and four bacilli were hydrophobic. According to An and Friedman (2000) and Rinkinen (2004), adherence capability is necessary for a probiotic agent to exert its positive impact on the host since hydrophobic bacteria have the ability to bind nonspecifically to the epithelium of the shrimp intestine by hydrophobic interactions. Only the bacilli isolate CIB1 showed inhibitory activity against *V. sinaloensis* VHPC18 and *V. sinaloensis* VHPC23. Although this feature is desirable for a potential probiotic bacteria, the lack of antagonism against pathogenic bacteria can be reinforced with pH changes due to the production of organic acids, competition for nutrients, production of hydrogen peroxide or adhesion inhibitors (Farzanfar, 2006; Mota et al., 2006). In this sense, extracellular bacterial enzymes such as proteases and lipases can help in the nutrition of the host (Moriarty, 1996; Balcázar et al., 2006; Farzanfar, 2006). In this work, neither bacilli nor LAB isolates showed extracellular lipolytic activity. On the other hand, extracellular proteolytic activity was found in all the bacilli isolates but not in LAB. Moriarty (1996, 1998) mentions that Gram-positive bacteria as members of the genus *Bacillus*, do secrete a wide range of exoenzymes. Also, the presence of the probiont may in some way stimulate endogenous enzymes produced by the shrimp (Saeed et al., 2006).

Substances or probiotics applied to shrimp culture systems or feed should not affect their productive performance (Courvalin, 2006). In this study, inulin and bacteria did not affect shrimp survival. However, prebiotics and probiotics may improve survival as occurred in the work of Van Hai and Fotedar (2009) when they fed the prawn *Penaeus latisulcatus* with Bio-Mos®, 1,3-D-glucan and combination of *Pseudomonas synxantha* and *Pseudomonas aeruginosa.*

In aquaculture, growth (in weight or size) rate is a key factor because it reflects the production yield. Growth rate is influenced by the conditions under which organisms are cultured (temperature, pH, ammonia, dissolved oxygen and stock density), by genetic factors and the quantity and quality of the food given (Oduleye, 1982). In this study, physicochemical parameters were within the optimum range for shrimp (Boyd and Tucker, 1998), but growth was not affected in a positive way by the administration of the inulin and bacteria. Conversely, Van Hai and Fotedar (2009) found a positive effect of

prebiotic and probiotic on growth performance of *P. latisulcatus*.

Prebiotics and probiotics can promote immune boosting effects (Reid, 2008). In the first bioassay, a significant increase in circulating hemocytes (total hemocyte count) was found in shrimp fed with bacterial mixture (1×10^5 CFU/g feed) and inulin (8.0 g/kg feed) as compared to the control group. Conversely, Van Hai and Fotedar (2009) did not find a marked increase in the total hemocyte count in *P. latisulcatus* fed with prebiotics and probiotics. However, they found a significant increase in the semi-granulocytes (SGC) and granulocytes (GC) in prawns fed with probiotics. The effect of bacteria and inulin on hemocytes found in this work may provide increased immunity to shrimp during periods of higher activity or increased pathogen loads since hemocytes are the first line of defense in invertebrates (Johansson and Söderhäll, 1989) and individuals with a high amount of hemocytes in circulation resist the presence of a pathogen (Le Moullac et al., 1997).

This is the first report on the effect of inulin and probiotic bacteria on the activity of lysosomal enzymes from hemocytes of *L. vannamei.* In this sense, it is known that lysosomal hydrolytic enzymes have been scarcely studied in crustaceans, but they have a key role in mollusks and shrimp's defense system since they are involved in the death and degradation of microorganisms and particles inside the hemocytes and some cases are released to other tissues and/or plasma where they change the molecular conformation of the cellular surface pathogens, encouraging the recognition and phagocytosis (Carajaraville et al., 1995; López et al., 1997). In this work, 12 hydrolytic enzymes included in the API ZYM kit were detected in samples of *L. vannamei*, 12 enzymes were found in plasma and six in HLS. The highest levels of enzymatic activity were found in the HLS. In plasma, the activity of β-galactosidase decreased in shrimp treated with inulin and inulin and the bacteria. In HLS, the activity of naphthol-AS-BI-phosphohydrolase decreased in shrimp treated with inulin and inulin and bacteria. On the other hand, the activity of N-acetyl-β-glucosaminidase in the control group was two and threefold higher than that in the treatments with inulin; however, it was twofold lower than that in the treatments with inulin and bacteria. It is clear that the enzymatic activity increases due to LAB and bacilli but not by inulin. Similarly, Peraza-Gómez (2011) reported that the enzymatic activity of N-acetyl-β- glucosaminidase in *L. vannamei* fed with probiotics (LAB and yeast) was higher (two to fourfold higher) than in shrimp fed commercial feed alone. Shiva-Ramayoni (2007) reported that N-acetyl-β-glucosaminidase has significant antibacterial activity against Gram-positive bacteria. Lysozyme activity (lysoplate assay) was not found in both plasma and HLS. The lysozymes of penaeid have lytic activity against several species of Gram positive and negative bacteria, including pathogenic species of *Vibrio* (Hikima et al., 2003; de-la-Re-Vega et al., 2006).

In the second bioassay, shrimp survival of 100% and a decrease in the prevalence of WSSV (22.2%) in shrimp fed inulin (8.0 g/kg feed) and bacteria (1×10^5 CFU/g feed) was observed when compared with control shrimp (44.4 and 51.8%). There are no reports on the effect of prebiotics and probiotics on WSSV prevalence in *L. vannamei* or other penaeid shrimp. However, further research is needed on the effect of prebiotics against viral infections in cultured shrimp since human milk and commercially available oligosaccharides may act as competitive receptors on the host cell surface, thereby preventing adhesion of a number of bacterial and viral pathogens (Shoaf et al., 2006; Vitoria-Miñana, 2007).

Inulin and bacteria improved immunity in cultured shrimp. Also, these feed additives reduce the prevalence of WSSV in cultured shrimp. However, further research is needed on the effect of prebiotics on viral infections in cultured shrimp. Also, further research is needed on molecular identification of bacteria.

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