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Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*

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Abstract

The objective of this work was to obtain probiotic bacterial strains with immunostimulatory qualities for shrimp. A total of 80 strains were isolated from the hepatopancreas of healthy wild shrimp (30 ± 5 g) collected in Manglaralto-Ecuador. The probiotic effect in vitro was evaluated using the agar diffusion technique. Three strains identified as *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64, showed inhibitory effects against *Vibrio harveyi* (S2). The colonization percentage in shrimp hepatopancreas was analysed using random amplified polymorphic DNA (RAPD) profiles with three primers. The strains P62, P63, and P64 achieved colonization percentages of 83%, 60% and 58%, respectively. The competitive interaction with *V. harveyi* (S2) was evaluated in shrimp using RAPDs and monoclonal antibodies. The inhibition percentage against S2 reached by strains P62, P63 and P64 was 54%, 19% and 34%, respectively. Histopathology was carried out after the colonization and interaction experiments, and confirmed that the probiotic strains had no pathogenic effects on the host. The immunostimulatory effect of P62 and P64 was evaluated in vivo using several immunity tests. *Vibrio alginolyticus* (Ili) was used as positive control. Shrimp that did not receive any probiotics served as the negative control group. The global immunity index was significantly higher ($p < 0.05$) in the shrimps stimulated with P64 and *V. alginolyticus*. For the animals stimulated with P62, the immunity index was similar to the control. Mean shrimp weights for three probiotic groups were significantly higher ($p < 0.05$) than the control. In conclusion the isolated strain *Bacillus* P64 showed both probiotic and immunostimulatory features, while *Vibrio* P62 only showed good probiotic properties.

Keywords: Probiotics; Hepatopancreas; Immunology; *Penaeus vannamei*

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1. Introduction

The occurrence of infectious diseases in shrimp culture has reduced production levels during the last decade. The increase in production in the areas that have recovered from diseases has not been able to compensate for the decrease in production in the areas that are still suffering from disease outbreaks. In Taiwan for example, shrimp production in the years 1987–1988 decreased 60% due to massive mortalities caused by pathogenic microorganisms (Wyban et al., 1992). Currently, Ecuador faces a similar situation, with shrimp production reduced by 65% in the year 2000 as a consequence of the white spot syndrome virus (WSSV) (Rosenberry, 1998). Pathogenic bacteria have also been involved in this crisis. Species such as *Vibrio harveyi*, *Vibrio anguillarum*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* have been frequently associated with mortalities both in hatcheries and grow out ponds (Baticados et al., 1990; Mohney et al., 1994). Thus, many measures have been tried to improve production levels such as the routine use of antibiotics. However, the excessive and inappropriate use of antibiotics has resulted in the presence of resistant strains of bacteria in shrimp culture.

The use of probiotic bacteria, based on the principle of competitive exclusion, and the use of immunostimulants, are two of the most promising preventive methods developed in the fight against diseases during the last few years (Fuller, 1992). One of the main challenges in developing probiotic bacteria is using appropriate selection and colonization methods. The selection criteria for probiotic bacteria should evaluate the colonization methods, competition ability against pathogens and the immunostimulatory and growth effect on shrimp (Gatesoupe, 1999; Gomez-Gil et al., 2000).

Bacteria that have been used successfully as probiotic belong to the genus *Vibrio* (Griffith, 1995; Garriques and Arevalo, 1995), *Bacillus* spp. (Moriarty, 1998; Rengpipat et al., 1998) and *Thalassobacter utilis* (Maeda and Liao, 1992). Most researchers have isolated these probiotic strains from shrimp culture water (Nogami and Maeda, 1992; Direkbusarakom et al., 1997; Tanasomwang et al., 1998), or from the intestine of different penaeid species (Rengpipat et al., 2000). Gomez-Gil et al. (1998) demonstrated the existence of a wide diversity of *Vibrio* species in the hepatopancreas of healthy *Penaeus vannamei*. However but there have been no reports of the use of any bacteria strains from the hepatopancreas as probiotics.

Several mechanisms have been suggested as modes of action for probiotic bacteria. The competitive exclusion mechanism, based on the substitution of the pathogen by the beneficial population, has been considered to be important by many authors (Fuller, 1989; Moriarty, 1998; Gatesoupe, 1999). Through bacterial substitution, it is possible to reduce the adherence of pathogenic strains in the host animal and consequently reduce the risk of disease. Also, stimulation of the immune system using probiotic strains has been reported by Rengpipat et al. (2000). Immunostimulation is an alternative strategy to alert the shrimp defence system increasing the resistance against pathogenic bacteria (Rodríguez and Le Moullac, 2000). In shrimp, several microbial compounds have been reported as the main stimulants of cellular functions, such as β-glucans, lipopolysaccharides and peptidoglycans (Vargas-Albores et al., 1998). These compounds have been researched to evaluate the usefulness of their supplementation against Vibrios and WSSV (Itami et al., 1998). However, most of these studies have delivered these compounds as heat-killed *Vibrio*

(Sung et al., 1996) or cellular wall of bacteria and yeast (Sung et al., 1994; Song and Hsieht, 1994). Few researchers have studied the immunostimulatory effect of administering live probiotic bacteria in the shrimp immune system.

In this study we investigated the effect of competitive exclusion by administering live probiotic bacteria to shrimp challenged with pathogenic bacteria. In addition, the immunostimulatory effect and growth of shrimp exposed to two strains of probiotic bacteria was evaluated.

2. Materials and methods

2.1. Available bacteria

A pathogenic strain *V. harveyi* (S2 strain) involved in the "Bolitas" syndrome in Ecuador (Vandenberghe et al., 1999) was used as pathogen. *V. alginolyticus* (Ili strain), which was isolated from an Ecuadorian hatchery (Morales, personal communication) and used intensively as a probiotic for shrimp larvae (Zherdmant et al., 1997) was used as positive control.

2.2. Isolation and identification of probiotic strains

The probiotic strains were isolated from wild adult shrimp (30 ± 5 g) *P. vannamei*, collected in Manglaralto, Ecuador.

Shrimp were cut sagittally. Half of the body was used for histological analysis and the hepatopancreas (HP) of the other half was extracted and homogenised for bacterial isolation. Four serial dilutions (1/10) were performed with 2% NaCl (w/v) and plated in duplicate using the spread plate technique on Marine agar (Difco). These plates were incubated at 28 °C for 24 h. Strains were purified by streaking onto Lennox L agar (Lb agar; Sigma) supplemented with 2% NaCl (w/v).

The strains were selected for their in vitro inhibitory properties against *V. harveyi* (S2) using the agar diffusion technique described by Ruiz et al. (1996). Of a total of 80 strains, three were isolated from healthy shrimps as shown by histological examination. Two strains were identified as Vibrios and one as *Bacillus*, by phenotypic observation. This identification was confirmed using amplified fragment length polymorphism (AFLPs) and 16Sr RNA gene sequencing (Thompson et al., in press). Bacteria are maintained in the Belgium Coordinated Collections of Micro organisms, Laboratory Microbiology (BCCM™/LMG), Ghent University, Belgium, with accession numbers for the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database of AJ345063 (LMG 20362), AJ345064 (LMG 20363) and AJ413201 (LMG 20364). In this article these strains will be named as P62 (*Vibrio* sp.), P63 (*Vibrio* sp.) and P64 (*Bacillus* sp.), respectively.

2.3. Bacterial inoculums

To determine the concentration of the bacterial inoculums for the experiments, bacterial growth curves were determined for the three selected strains and *V. alginolyticus*

(Ili strain). The strains were streaked in Lb agar (2% NaCl) and incubated for 12 h. One colony was transferred into 10 ml of Lennox L broth (Lb-broth; Sigma) supplemented with 2% NaCl (w/v), incubated at 28 °C over night with continuous agitation. After this period the bacterial culture was transferred into 50 ml of Lb-broth (2% NaCl), and incubated under the same conditions for 4 h. A third transfer (T_0) (1/10) was carried out into 450 ml, under the same conditions. Then, 13 serial dilutions (1/10) were performed each hour. The dilutions 10^{-4} to 10^{-13} were both plated onto Marine agar by the spread plate technique and the optical density (OD) at 560 nm measured using a spectrophotometer (JENWAY 6400). After 8 h at 26.7 °C colonies were counted. The data were related in line graphs, obtaining the relationship CFU vs. time and vs. units of OD per strain.

Aquaria were inoculated with bacteria obtained during the exponential growth phase as determined for each strain. All aquaria were inoculated at using 10^7 bacteria/ml based on the spectrophotometer data.

2.4. AP-PCR technique

Using the arbitrarily primed polymerase chain reaction (AP-PCR) technique, the profiles of RAPDs of the three isolated strains were obtained. RAPD was tested with several different random primers. The primer selection was based on three aspects with the following priority: amplification, replication and polymorphism. Therefore, three amplifications were performed per primer per strain. Three decamerous primers were selected: OPA 8, OPA 9 and OPA 10 (Operon Technologies). The DNA chromosomal extraction method of Murray and Thompson (1980) was used. The RAPD mixture reaction (25 μl) consisted of 10 mM Tris–HCL (pH 9.0), 50 mM KCl, 0.1% Triton® x-100, 2.1 mM $MgCl_2$, 0.2 μM of each deoxynucleoside triphosphate, a 0.4-μM primer concentration, 50 ng of bacterial DNA, and 1 U of Taq DNA polymerase (Promega). The amplification profile was: 1 cycle of 240 s at 94 °C, 40 cycles of 5 s at 94 °C, 45 s at 46 °C, and 90 s at 72 °C; and 1 cycle of 600 s at 72 °C. RAPD products were electrophoresed at 85 V in a 2% agarose gel, and at 105 V in 8% polyacrylamide. The amplified stock samples were used as positive controls for comparison with the bacterial profiles after the colonisation and interaction experiments.

2.5. Immunology techniques

The haemolymph was obtained from the ventral sinus. The samples were collected using a 23-gauge needle and 1 ml syringe containing 50 μl pre-cooled (4 °C) 10% sodium citrate as anticoagulant. The immunology tools used to evaluate the stimulating effect of the strains were: haemogram counts, quantification of reactive oxygen intermediates (ROIs), measurement of phenoloxidase activity (PO), antibacterial activity quantification and measurement of plasma protein concentration.

The results obtained in the five immunity tests were used to calculate an immune index according to procedures established by Echeverría (personal communication). Briefly, the values of each test were transformed using the following formula: $TV=(a-b)\times(0.2/k)$; transformed value (TV), immunology test value (a), minimum range (b), value range of

each test (k), and (0.2) which corresponds to 20%. The immune index value is the sum of the 5 TV values for each individual shrimp. The ranges considered as normal for each measured parameter used in this formula are taken from historical data (results of analysis performed during 5 years on *P. vannamei* shrimp).

The total haemocyte count (THC) and the differential haemocyte count (DHC) were performed with a haemocytometer (Neubauer chamber), using a phase-contrast microscope (40× magnification) as described by Muñoz et al. (2000). Values among 15–20×10^6 h/ml were considered acceptable. A value range (k) of 5×10^6 to 30×10^6 was established for this test.

Super oxide generation (O_2^-) was measured by reduction of Nitro blue tetrazolium (NBT) (Muñoz et al., 2000). The haemocyte concentration used was 2×10^5 h/well of a 96-well micro titre plate. Phorbol myristate acetate (PMA) (Sigma) prepared in dimethyl sulfoxide at a concentration of 30 μg/ml was used as the stimulant control. The results were expressed in rates, dividing the OD value of the sample stimulated for phagocytosis against the value of the same sample without stimulation. The results were interpreted using the following scale: rate smaller than 1, lack of activity; rate values between 1 and 1.5, low activity; and values among 1.5–2, good activity. A value range (k) of 1–2 was established for this test.

To measure the phenoloxidase activity the haemocyte pellet was resuspended in a Cacodylate buffer solution at a concentration of 10×10^6 ml^{-1} (Na cacodylate 10 mM pH 7). This suspension was centrifuged (3 min, $12{,}000 \times g$) and the supernatant (prophenoloxidase source) was used as sample. A total of 50 μl of each sample were deposited by triplicate in each well of a 96-well micro titre plate. A solution of Cacodylate buffer containing 20 mM of Ca^{2+} and Laminarin (Sigma) (2 mg/ml) was used as elicitor. After 70 min of incubation, phenoloxidase activity was measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA; Sigma) as the substrate (allowing 10 min of reaction), according to Leonard et al. (1985).The results were interpreted based on the following scale; OD smaller than 0.2, low; between 0.2 and 0.35, normal; and 0.35 to 0.5, high activity. A value range (k) of 0.2–0.5 was established for this test.

Antibacterial activity was quantified using a turbid metric method (Tapia et al., in press). Bacterial growth was evaluated in presence or absence of plasma. A plasma control and a negative control in saline solution were used. The negative control corresponded to 100% of bacterial growth. Inhibition percentages: less than 20% (low); percentages 20–40% (regular), and percentages greater than 40% (good). A value range (k) of 20–40 was established for this test.

Plasma protein concentration was measured by the method of Lowry et al. (1951). Protein concentration was expressed in μg/μl. Values: less than 80 mg/ml (very low); 80–100 mg/ml (low); 100–130 mg/ml (normal) and greater than 130 mg/ml (high). A value range (k) of 50–150 was established for this test.

2.6. Shrimp experiments

For the three experiments, shrimp were acclimatized for 15 days, with continuous water exchange and constant aeration. The seawater was filtered to 0.5 μm and UV sterilized.

The temperature was maintained at 28 ± 2 °C. The shrimp were fed with commercial pellets (50% protein), which were sterilized daily by oven heating to 80 °C during 15 min.

2.6.1. Evaluation of the colonisation capacity of bacteria

The experimental design was completely randomized with four treatments and five replicates per treatment, each containing 20 shrimp (1 ± 0.3 g) randomly distributed in aquariums containing 40 l with seawater. The bacteria used were *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 and one control without bacteria. After the acclimatization only one bacterial inoculation was carried out (Section 2.3). Exposure time was 24 h without water exchange.

For the recovery isolations, 10 HP were collected, homogenized and five serial dilutions (1/10) performed. The isolate strains were plated in triplicate on Lb agar (2% NaCl) using the spread plate technique. Culture conditions were at 28 °C for 24 h. Colonization percentages were evaluated by counting the CFU/g HP based on colony morphology and identifying the strains by AP-PCR using three primers. The health condition of shrimp was determined by histology using 10 animals from each aquarium.

2.6.2. Effect of competitive interaction against V. harveyi (S2)

The experimental design was completely randomized with five treatments and five replicates per treatment. The strains *Vibrio* P62, *Vibrio* P63, *Bacillus* P64, positive (S2) and negative control were used. Stocking density, weight of shrimp, and inoculums dose (Section 2.3) were the same as in the first experiment (Section 2.6.1).

The strains were inoculated for three successive days. After 20 h of exposure a 50% water exchange was carried out. On the fourth day of the experiment *V. harveyi* (S2) was inoculated at 10^7 CFU/ml concentration and no water exchange performed during 24 h. Interaction percentages were evaluated by counting the CFU/g of HP on Lb agar (2% NaCl), differentiating strains on morphological characteristics AP-PCR and monoclonal antibodies against S2.

2.6.3. Evaluation of the strains as stimulants of the immune system

Strains *Vibrio* P62 and *Bacillus* P64 were used. A negative control and positive control (Ili strain) were also used. The experimental design was completely randomized, with five replicates per treatment. Shrimp (1.5 ± 0.2 g) were randomly distributed in aquariums of containing 50 l seawater with 10 animals each. They were fed with commercial pellets (50% crude protein) at 3% biomass, distributed over two daily feedings during the experimental period (25 days). The inoculation took place every 2 days from days 15 to 25, with 50% water exchange after 20 h of exposure. On day 25 haemolymph from each shrimp in intermoult stage was collected. The samples of each replicate were pooled and transferred to sterile micro tubes at 4 °C. Individual weight of the shrimp was evaluated in each treatment.

2.7. Statistical analysis

The data obtained during the experiments was analysed using the Duncan multiple range test at 95% confidence level with Statistica 4.3 program.

3. Results

Of the 80 strains isolated from wild shrimp HP, 2 fulfilled the probiotic criteria of originating from healthy shrimps, reaching high colonization percentages (>50%), inhibiting the in vivo growth of *V. harveyi*, and not causing histological damages in inoculated shrimps at 10^7 CFU/ml bacterial concentration.

3.1. Colonisation capacity of bacteria

The colonization experiment demonstrated the capacity of the strains to enter the shrimp HP and their competitive exclusion power. For *Vibrio* P62 and *Vibrio* P63, the total quantity of CFU/g HP was not significantly different from the control ($p>0.05$), indicating the high capacity of both Vibrios to inhibit autochthonous bacteria or to carry out competitive substitution. The mean bacterial count reached at these treatments was $4.2 \times 10^4 \pm 8.5 \times 10^3$ CFU/g HP but, the colonization percentage reached by *Vibrio* P62 was 83%, demonstrating a stronger antibacterial effect than *Vibrio* P63. In the case of the shrimps inoculated with *Bacillus* P64, the total bacterial number was significantly higher ($5.3 \times 10^4 \pm 7.6 \times 10^3$ CFU/g HP), 68.4% higher than the control. Although the colonization percentage reached by this strain (58%) was similar to the one reached by *Vibrio* P63 (60%), P63 was more efficient performing the competitive substitution of the indigenous flora (Fig. 1). No histological damage was registered in the inoculated shrimp after 12 h.

3.2. Competitive interaction against V. harveyi (S2)

The total bacterial count of HP was significantly higher ($p<0.05$) in the shrimps inoculated with P62, P63, P64 and S2 (control) compared to the control treatment without bacteria. The mean bacterial concentration reached in the treatments with inoculated bacteria, was $5.2 \times 10^4 \pm 8.2 \times 10^3$ CFU/g HP. The control reached $3.1 \times 10^4 \pm 5.6 \times 10^3$

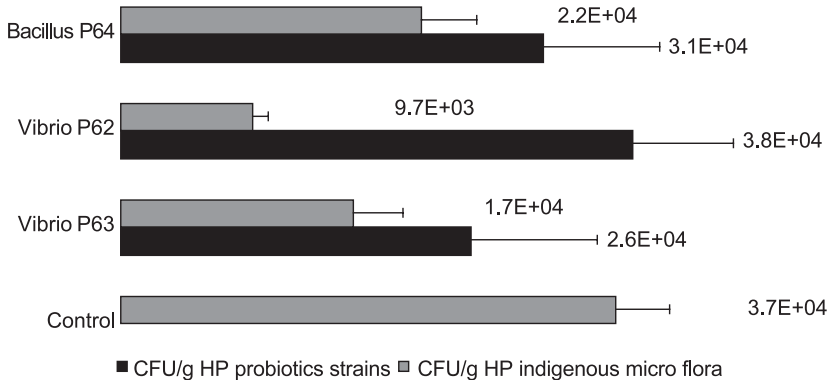

Fig. 1. Percentages of recovered bacteria (inoculated and indigenous) from *P. vannamei* shrimp hepatopancreas after the colonisation experiment using *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64.

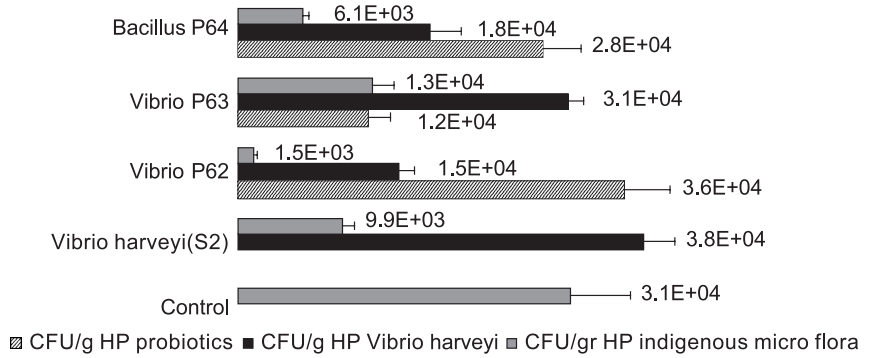

Fig. 2. Bacterial concentration reached by *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 in the interaction experiment against *Vibrio harveyi* (S2), in *Penaeus vannamei* shrimp hepatopancreas.

CFU/g HP. The total bacterial concentration increased by 64% with S2 inoculation and up to 80% with the inclusion of probiotics. The strain *Vibrio* P62 achieved the greatest inhibitory effect. P62 reduced by 60% the S2 colonization, besides displacing the indigenous micro flora of the HP. The inhibitory effect of *Vibrio* P63 was greater on the natural flora than on the pathogen S2, achieving only 19% of inhibition. *Bacillus* P64 inhibited the natural flora and competed with the pathogen, reducing the S2 colonization by 34% (Fig. 2).

3.3. Immune system stimulation

The total haemocyte count did not show significant differences among the treatments. The total haemocyte average was $19.9 \pm 5.6 \times 10^6$ haemocyte/ml. The hyaline cell quantity was significantly lower ($p < 0.05$) in the animals inoculated with *Vibrio* P62 ($2.6 \pm 1.2 \times 10^6$ cell/ml) than *V. alginolyticus* (Ili) ($5.9 \pm 2.4 \times 10^6$ cell/ml) and *Bacillus* P64 ($4.8 \pm 1.7 \times 10^6$ cell/ml). Although the quantity of granulose (GR) and semigranulose (SG) cells was not significantly different in any treatment, the animals inoculated with

Table 1
Mean immunogical values of control and probiotic treatments

Immunitary values	*V. alginolyticus*	*Vibrio* P62	*Bacillus* P64	Control
Total haemocytes (cells/ml)	$22 \pm 9.9 \times 10^6$	$17 \pm 4.9 \times 10^6$	$21 \pm 3.1 \times 10^6$	$20 \pm 3.9 \times 10^6$
Granular cells (cells/ml)	$5.9 \pm \times 3.710^6$	$4.4 \pm 1.3 \times 10^6$	$7.5 \pm 1.7 \times 10^6$	$6 \pm 3 \times 10^6$
Semigranular cells (cells/ml)	$7.4 \pm 4 \times 10^6$	$7.4 \pm 1.8 \times 10^6$	$6.9 \pm 1.6 \times 10^6$	$9.6 \pm 0.7 \times 10^6$
Hyaline cells (cells/ml)	$5.9 \pm 2.4 \times 10^{6\ a}$	$2.6 \pm 1.2 \times 10^{6\ b}$	$4.8 \pm 1.7 \times 10^{6\ a}$	$2.3 \pm 1 \times 10^{6\ b}$
Atypical cells (cells/ml)	$2.2 \pm 1.3 \times 10^6$	$2 \pm 2.4 \times 10^6$	$1.5 \pm 0.9 \times 10^6$	$1.9 \pm 1.4 \times 10^6$
O_2^- rate	1.18 ± 0.08	1.15 ± 0.11	1.20 ± 0.09	1.11 ± 0.05
Antibacterial activity (%)	8.5 ± 5.6	25.4 ± 13.7	20.6 ± 8.7	30.0 ± 9.0
Plasmatic protein (mg/ml)	112.1 ± 8.1	97.7 ± 6.0	102.6 ± 3.9	104.3 ± 8.6
Phenoloxidase activity (O.D.)	670 ± 0.02^a	661 ± 0.07^a	738 ± 0.08^a	449 ± 0.05^b

Superscripts with different letters (a,b) indicate significant differences among treatments ($p < 0.05$). Atypical cells: haemocytes with altered morphology.

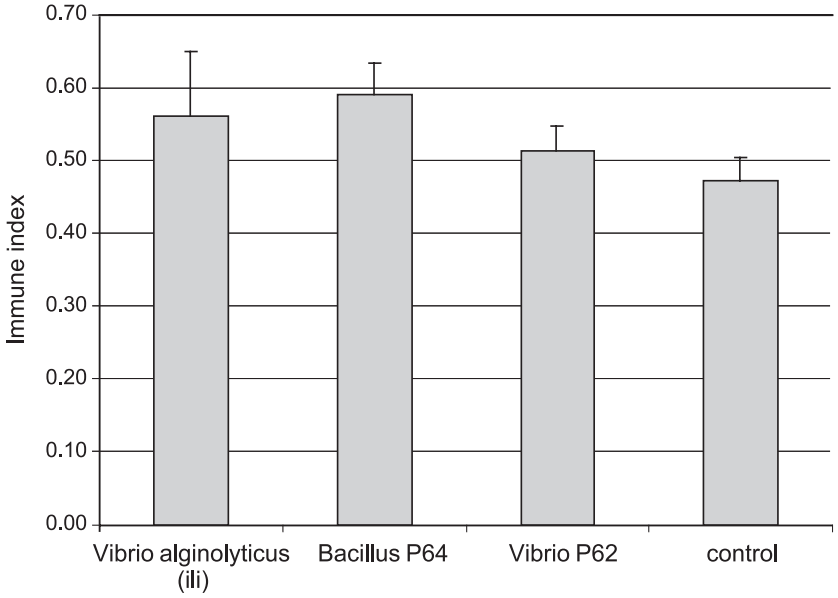

Fig. 3. Immunity index of shrimps treated with *Vibrio alginolyticus* (Ili strain), *Bacillus* P64 and *Vibrio* P62 after 25 days.

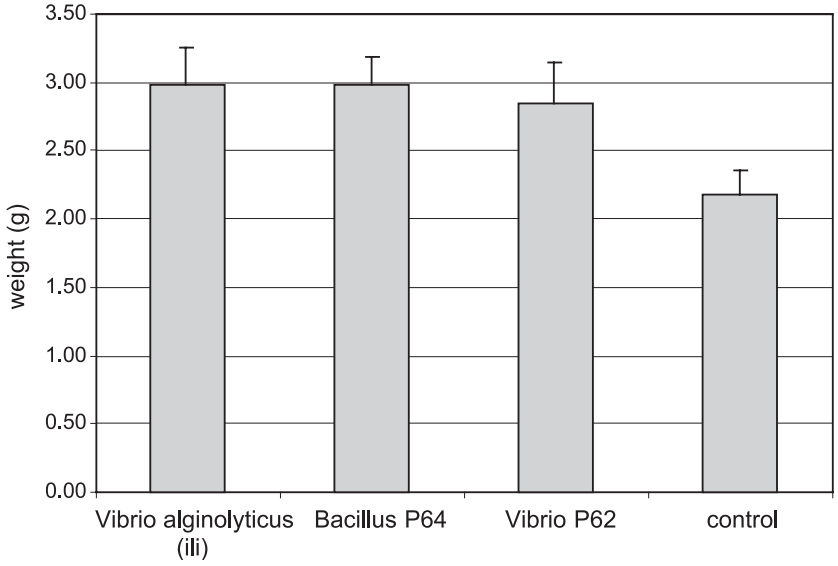

Fig. 4. Mean weight of shrimp treated with *Vibrio alginolyticus* (Ili strain), *Bacillus* P64 and *Vibrio* P62 after 25 days.

Bacillus P64 showed a decrease in the quantity of SG cells concurrent with an increase of the GR cells compared to the control (Table 1). The phagocyte stimulation rate was low for all treatments, not registering significant differences ($p>0.05$) of the reactive oxygen intermediate rate (Table 1). The phenoloxidase activity was significantly higher ($p<0.05$) in the shrimps stimulated with *Bacillus* P64, *Vibrio* P62 and *V. alginolyticus* (Ili). The antibacterial inhibition percentage of the plasma was lower than the control for all treatments. The quantity of plasmatic proteins stayed within the normal range for healthy shrimp (Table 1). The immune index was significantly greater ($p<0.05$) in the shrimps stimulated with *Bacillus* P64 and *V. alginolyticus* compared to Vibrio P62 and control, while no significant differences were found between P62 and control (Fig. 3). Mean shrimp weights for all probiotic groups were significantly higher ($p<0.05$) than the control (Fig. 4).

4. Discussion

In this study we demonstrated that the isolated beneficial bacteria of the natural micro flora are potential competitors of pathogenic bacteria. The results of the interaction with *V. harveyi* (S2) confirmed that it is possible to decrease the colonisation of this strain in the shrimp hepatopancreas (HP). Therefore, we propose that the probiotic nature of *Vibrio* P62 and *Bacillus* P64 is based on the competitive exclusion of the pathogen establishment inside the host.

The use of *Vibrio* species as probiotics is controversial because within this genus there are species that have been associated to shrimp pathologies. In Ecuador *V. alginolyticus* was associated with both healthy and unhealthy larvae and juvenile shrimp (Vandenberghe et al., 1999). Nevertheless, *V. alginolyticus* (Ili strain) has been used in the CENAIM hatchery to prevent infectious diseases related to *V. harveyi* (Zherdmant et al., 1997). The risk of using different *V. alginolyticus* strains, other than the Ili strain as a probiotic is real. Thus, in accordance to Vandenberghe et al. (1999) the genotypic identification of all the strains to be used, as probiotics must be a requirement is an indispensable step.

The *Bacillus* genus has not been associated with aquatic organism pathologies. For this reason its use has been promoted (Moriarty, 1998) and more widely accepted within the industry. Although the *Bacillus* genus does not belong to the common genera in the marine environment, it has been isolated from crustacean intestine (Rengpipat et al., 2000), marine fish (Sugita et al., 1998) and bivalves (Sugita et al., 1981). Rengpipat et al. (1998) reported that *Bacillus* S11 strain had an inhibitory effect in vitro against *V. parahaemolyticus* and *V. harveyi*. Similar results were reported by Sugita et al. (1998) for *Bacillus* sp. against *V. vulnificus*.

There are several ways by which probiotic bacteria can induce bacterial antagonism, by producing antimicrobial agents, such as antibiotics (ref), antimicrobial peptides (ref) or siderophore substances (Sugita et al., 1998), for example. Most of the discovered antibiotics have been isolated from fungi or terrestrial bacteria and only few marine organisms have been reported as antibiotic producers, most of them being *Alteromonas* (Dopazo et al., 1988; Tanasomwang et al., 1998). Thus, the use of marine probiotic bacteria may be less controversial. *Bacillus* have been linked to polymyxin, bacitracin and gramicidin antibiotic production (Rhodehamel and Harmon, 1998; Chitta et al., 2002).

However *Bacillus* bacterial antagonism also can be provoked by competition to obtain nutrients with other fast growing bacteria (Moriarty, 1998).

Skjermo and Vadstein (1999) pointed out that bacterial colonization depends on several factors such as adhesion properties, bacterial attachment site, stress factors, diet and environmental factors. Gatesoupe (1999) considered it was improbable that *Bacillus* sp. could multiply in the digestive tract of marine organisms. In our study, the presence of *Bacillus* P64 in the HP indicated that it was been able to resist physiologic and anatomical processes. However, may be probable that the antagonistic needs to be maintained by repeated inoculations. More studies under different experimental conditions are necessary to establish the restrictions of bacterial colonization.

A significant growth increase was observed in the shrimps inoculated with *Bacillus* P64, *Vibrio* P62 and *V. alginolyticus* (Ili) compared with the control. Rengpipat et al. (1998) reported similar results in pl 30 of *P. monodon* using *Bacillus* S11 as probiotic in the feed. After feeding for 100 days they found significant growth differences ($p < 0.05$) among the probiotic treatments and the control. Recently, after feeding *P. monodon* shrimps for 90 days with *Bacillus* S11, the same authors found that the probiotic treatments increased the survival (Rengpipat et al., 2000). However, they did not find significant growth differences, attributing these results to different culture conditions than in the previous experiment (Rengpipat et al., 1998). Garriques and Arevalo (1995), using *V. alginolyticus* in commercial *P. vannamei* hatcheries in Ecuador, reported less shrimp growth in the control group (7.1 mg) than in the probiotic group (7.8 mg), although statistical data was not reported.

Probiotics may improve digestive activity by synthesis of vitamins, cofactors or improve enzymatic activity (Fuller, 1989; Gatesoupe, 1999; Jory, 1998; Ziemer and Gibson, 1998). These properties could be the cause of the weight increase, improving digestion or nutrient absorption. At present, we ignore the mechanism by which probiotics operate and we also ignore which nutrients, or which enzymes may improve digestion. It is possible that this phenomenon operates by substitution of depressive microbial agents which hinder growth. Also, the growth promoter effect is conditioned to ambient factors; therefore, the results are subject to a high degree of variability. Consequently, the probiotics used as growth stimulant can yield different results under different culture conditions. The strains P62, P64 and Ili, should be considered as probiotics that improve the general shrimp health and not as growth promoters. Furthermore, the total number of haemocytes and the total plasmatic protein concentration in the three treatments remained within the normal values, indicating that its supplementation doesn't deteriorate shrimp health.

The plasma of the inoculated shrimp did not show antibacterial activity modifications, but the activation of the PO system and the changes observed in the differential haemocyte count suggests an immune alert. The animals stimulated with *Bacillus* P64 and *V. alginolyticus* (Ili), did not show significant total haemocyte count changes, but they did show a significant increase in the hyaline cell population. Tsing (1987) and Van de Braak et al. (2002) pointed out that an increase in circulation of young and immature haemocytes might be an indicator of an intense proliferating activity of haematopoietic tissue. The decrease of SG could be provoked by high infiltration of this cell type to connective tissue, stomach and gills, as occurs in the case of bacterial infections (Muñoz et al., 2002). The GR haemocyte number was not significantly different between treatments. On the other hand the PO activity values

for all treatments were significantly higher than the control, indicating that although the GR population remained constant, these cells were strongly stimulated.

The O_2^- generation was not significantly higher for the treatments compared to the control. This compares favourably with immunostimulation studies in vivo performed by Sung et al. (1996) in *P. monodon*, using *V. vulnificus* antigen, showed that 3 h after antigen immersion, oxygen radical generation increased until reaching significant levels at 6 h, but diminished to lower values than the control at 12 h. As a result the stimulation rate did not differ significantly from the control 12 h after the last inoculums. A probable explanation could be the expression of antioxidants such as super oxide dismutase, glutathione peroxidase or catalase that neutralise reactive oxygen metabolites to avoid self-damage (Smith et al., in press).

The immune index evaluation demonstrated that *Bacillus* P64 and *V. alginolyticus* were effective than at stimulating the shrimp immune response while Vibrio P62 was not. The results obtained with *Bacillus* P64 correspond to those obtained by Rengpipat et al. (2000) with *Bacillus* S11 strain in *P. monodon*. However, the immune values cannot be compared because the techniques used for the phagocyte determination, phenoloxidase and antibacterial activities are different to those used in this work. These authors pointed out that *Bacillus* S11 provided protection against diseases, activating the immune system and performing competitive exclusion mechanism in shrimp intestine. In the case of *Vibrio* P62, its colonization capacity could be associated to avoiding the shrimp cellular and humoral defence barriers as no evidence of immune response activation was observed. Tizard (1988) pointed out that anti-phagocyte capsules and intracellular parasitism, together with phagocyte depressor factors, are mechanisms that bacteria use to avoid the immune response in vertebrates. These mechanisms are unknown for marine bacteria, however we cannot discard their existence.

The results demonstrate that *Vibrio* P62 and *Bacillus* P64 could establish themselves as probiotics in the prevention of *P. vannamei* diseases by competitive exclusion or by stimulation of a defence reaction in the host. This research is a first stage that clears the way toward a better understanding of the beneficial bacteria associated with shrimp and their interaction with other micro flora. The main objective in the use of these probiotic strains would be to exploit their benefits by limiting the appearance of pathogenic bacteria in shrimp culture systems.

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