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# Exposure to probiotics and β-1,3/1,6-glucans in larviculture modifies the immune response of *Penaeus vannamei* juveniles and both the survival to White Spot Syndrome Virus challenge and pond culture

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## Abstract

The effect of the inclusion of probiotics (*Vibrio alginolyticus*) and β-1,3/1,6-glucans in *Penaeus vannamei* larviculture was evaluated by measuring the immune response and survival of shrimp juveniles subjected to white spot syndrome virus (WSSV) challenge tests (*per os*) and pond culture. Treatments were designed to contrast the probiotic factor (inclusion *vs* non-inclusion) and β-1,3/1,6-glucans supplementation in various larvae stages; starting from early stage (Zoea II), to middle stage (PL 12), late stage (15 days pre-challenge) or non-supplemented. In larviculture, the highest survival was obtained in animals treated with β-1,3/1,6-glucans from Zoea II. The use of probiotics enhanced survival during the first 0–52 h post-WSSV challenge period. During 56–156 h post-WSSV challenge period, interactions were observed between β-1,3/1,6-glucans × time and β-1,3/1,6-glucans × time × probiotics. Significant differences in final survival rates between treatments were not observed. In the second WSSV challenge, immune parameters were analysed. Significant interaction between probiotics and β-1,3/1,6-glucans was observed for plasmatic protein (PP) concentration, super oxide anion ($O_2^-$) generation, antibacterial activity (AA), and total haemocyte count (THC). The use of probiotics in larviculture had a negative effect on the PP, but increased the AA and THC, while β-1,3/1,6-glucans had a negative effect on the $O_2^-$ generation. The most relevant results were obtained from treatments T2 (probiotics in larviculture, β-1,3/1,6-glucans from Z II) and T4 (probiotics in larviculture, β-1,3/1,6-glucans 15 days before challenge). Treatment T2 presented the highest survival rate in larviculture. After WSSV infection, the animals of this treatment displayed resistance to the virus, a strong AA and increase of THC. Treatment T4 increased the amount of PP, increased the $O_2^-$ generation and THC. Histological analysis showed that the animals of treatment T2 and T4 were able to limit the spread of the virus during the first hour after challenge with WSSV. The survivors from treatments T2 and T4 had a high THC, accompanied by a lack of white spot disease (WSD) injuries. A bioassay was carried out under farm conditions during the warm-rainy season using larvae from treatments T2 and T4. The animals were stocked at 18 animals/m² in earth ponds of 0.20 ha (three ponds/larvae kind). WSD outbreak was not detected, and the survival was significantly higher in ponds stocked with larvae from treatment T4 (70±3%) than in ponds stocked with larvae from treatment T2 (49±9%).
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## 1. Introduction

Shrimp culture in recent years has experienced serious losses due to the presence of pathogens, such as; protozoarians, fungi, bacteria and viruses (Aguirre Guzmán and Ascencio-Valle, 2000). The most devastating pathogen to shrimp are viruses, their list includes white spot syndrome virus (WSSV), hypodermic and haematopoietic infectious necrosis virus (IHHNV), Taura syndrome virus (TSV), and yellow head virus (YHV) (Aguirre Guzmán and Ascencio-Valle, 2000; Lightner, 1996). Bacterial pathogens: *Vibrio harveyi*, *Vibrio vulnificus*, and *Vibrio parahemolyticus*, have been frequently associated with infectious diseases in shrimp culture (Le Moullac et al., 1998) both in ponds and hatcheries. Viruses thus constitute the principal disease problem in ponds, while in hatcheries the mortalities are provoked mainly by bacteria.

Penaeid shrimp constitute the main crustacean species cultured in the world (FAO, 2004). In Ecuador the shrimp industry represents the third source of revenues after, oil and banana exports (CORPEI, 2007). The shrimp farming industry in Ecuador is trying to develop programs focused on shrimp resistance to specific and or non-specific pathogens. However, it has been reported that shrimp resistance to specific pathogens has been associated with animals with low growth rates (Gitterle et al., 2006). Because of the many difficulties in maintaining complex shrimp improvement programs, Ecuadorean shrimp farming groups have opted for more "hands on" approaches, which principally are based on animal health and pond management (CNA, 2007).

A promising and at present a very common strategy is the use of probiotics in managing shrimp health (Verschuere et al., 2000). These are considered as beneficial micro flora, which help to prevent colonization of pathogenic bacteria and may improve animal health through the induction of the immune system (Moriarty, 1999; Berger, 2000). In aquaculture the intestinal microbiota does not exist as entity by itself, but there is a constant interaction with the environment and the host functions (Verschuere et al., 2000). This intensive interaction between the culture environment and the host implies that a lot of probiotics are obtained from the culture environment (Verschuere et al., 2000).

The use of vibrio species as probiotics in aquaculture remains controversial, because certain genus used have been associated to pathogens. For example, in Ecuadorian shrimp hatcheries *Vibrio alginolyticus* is observed in both, healthy and non-healthy larvae cultures (Vandenberghe et al., 1999). Strains of this species of Vibrio have found their use as a probiotic in Ecuadorian hatcheries (Garriques and Arevalo, 1995; Vandenberghe et al., 1999). The Ili strain *of V. alginolyticus* isolated from healthy shrimp larviculture (Morales, unpublished) continues to be used in CENAIM's larvae production protocol. Ili strain is used from Nauplii 5 before the larvae open the mouth in order to allow the bacteria invade the animals (external surfaces and gut epithelium). Through its use, we have successfully mitigated what is commonly referred to as "bolitas" syndrome or Zoea 2 syndrome (Zhertmant et al., 1997; Vandenberghe et al., 1999).

The β-1,3/1,6-glucans is considered as another alternative immune enhancer (Chang et al., 1999, 2003) currently in use in many shrimp culture systems. Studies have indicated, during a WSSV challenge when β-1,3/1,6-glucans were applied, shrimp survival was increased (Chang et al., 2003). Molecules involved in the recognition of β-1,3-glucans (β-glucan-binding protein) have been isolated in *Pacifastacus leniusculus* and in the Shrimp *Penaeus californiensis* (Vargas-Albores et al., 1996). In *P. leniusculus* (Duvic and Söderhäll, 1990) the association between the BGBP and β-1,3-glucans was demonstrated in the activation of granular haemocytes. However the use of β-1,3/1,6-glucans in production systems raises criticism due to the lack of validity of the published results (Smith et al., 2003). In addition, in several studies performed on the lobster *Homarus gamarus*, β-1,3/1,6-glucans failed to induce the expression of immune genes (Hauton et al., 2005, 2007) or improve survival when facing pathogens (Hauton et al., 2007). Our own experience however, has shown when β-1,3/1,6-glucans (extracted from the cell wall of baker's yeast-mannoprotein free) when applied in shrimp ponds increased survival to 70% (data available by the authors).

The aim of the present study was to evaluate the effect of the putative probiotic *V. alginolyticus* (Ili strain) and β-1,3/1,6-glucans applied during larviculture, on survival of post-larvae and the subsequent immune response of juvenile shrimp when challenged with WSSV. It is assumed that by enhancing the immune response of larvae, they will be better prepared to face present and future challenges. This work was carried out in three stages: 1) larviculture, which was performed following different protocols of immunostimulation; 2) survival and immune parameters of shrimp juveniles challenged with WSSV under laboratory conditions: 3) survival and growth of shrimp in experimental ponds.

## 2. Materials and methods

This study was conducted in two phases. Larviculture of *P. vannamei* nauplii under 6 different treatment protocols and 2

control protocols. The effect of the treatments was measured by survival at the end of larviculture, survival to a WSSV challenge, and effect over immune parameters during WSSV challenge. On the second phase, two groups of shrimp resulting from the treatment with best results in the larviculture in terms of survival and one control (treatment T4), where then reared in earthen ponds to commercial size.

## 2.1. Protocols of larviculture and immunostimulation

Shrimp larvae were reared at CENAIM's facilities, located on the Ecuadorian coast (170 km from Guayaquil). 1,425,000 nauplii were reared in tanks of 500 L at a density of 150 nauplii $L^{-1}$. Larviculture treatments were defined by the combination of factors and levels as described below.

Factor A  probiotics with two levels:
$a_1$        with Probiotics
$a_2$        without Probiotics
Factor B  β-1,3/1,6-glucans with three levels:
$b_1$        without β-1,3/1,6-glucans
$b_2$        β-1,3/1,6-glucans from zoeaII (ZII)
$b_3$        β-1,3/1,6-glucans from post-larvae (PL) 12

*V. alginolyticus* (Ili strain) was used as the probiotic. The stocks of this bacteria are maintained at −80 °C in Lennox L broth (LB broth; Sigma) containing 20% glycerol. The purity of the strain is currently controlled by colony blot using a monoclonal antibody against this bacterium (Donoso, 1996). Before use, the bacteria were streaked in Lennox L agar (LB-agar; Sigma) supplemented with 2% NaCl and incubated for 12 h. One colony was used to inoculate 10 ml of LB broth. Fourth, the volume required is obtained by successive inoculation in increased volumes of LB broth. The bacteria were added daily (10 ml/TM) to the larvae rearing water from nauplii 5 to PL 12 stage at a concentration of $10^{10}$ colony forming units (CFU $mL^{-1}$). Using this procedure, the final concentration of the probiotic in the larval rearing tanks was $10^5$ CFU $mL^{-1}$. At this concentration the animals exhibit resistance to pathogenic bacteria in challenge trials (Serrano, 1996). Microbiological analysis performed with samples from larviculture tanks showed that *V. alginolyticus* is the main bacteria present in the water and in the animals (90% UFC around).

The application of β-1,3/1,6-glucans to larvae in the early stage was performed using yeast *Saccharomyces cerevisae* bio-encapsulated in the rotifer *Brachionus plicatilis* (which are able to brake the yeast cell wall, allowing exposure of the β-1,3/1,6-glucans). From PL 4 the β-1,3/1,6-glucans were applied in the feed (150 mg of *S. cerevisae* wall fraction/kg of food). After 25 days of culture, the larvae were harvested (Pl 18) and survival rates were evaluated. Sixty seven animals per treatment were sampled to detect WSSV by nested polymerase chain reaction (PCR), considering a confidence level of 95%, an error margin of 12% and a possible prevalence of 50% (Des Clers, 1994). Nested PCR was performed using the primers of Kimura et al. (1996).

## 2.2. Challenge tests using WSSV

### 2.2.1. Infection method

Viral extract was prepared following the protocol described by Chou et al. (1998), using natural WSSV-infected *P. vannamei* with severe clinical signs of disease. This extract was used to infect *per os* shrimp juveniles. Dead shrimp, which were PCR positive for WSSV and displaying unequivocal signs of white spot disease (WSD), were macerated and used as infected material. This infected material was applied at 12 h intervals, at 10% of the biomass per experimental unit. After 1 h post-application, water exchange was performed in order to avoid deaths due to degradation of the water quality.

Nested PCR, (Kimura et al., 1996) was carried out as confirmation for detecting the presence of the virus in shrimp during the survival and immunity experiments.

### 2.2.2. Experimental design

Two experimental challenges were designed as a factorial analysis ($2 \times 4$). Treatments were defined by the combination of factors and levels as described above for larviculture treatments. In this step two additional treatments were included. Treatment T4, a batch of animals sourced from treatment T1, which received β-1,3/1,6-glucans for 15 days before carrying out the challenge test, and treatment T8, a batch of animals sourced from treatment T5, which received β-1,3/1,6-glucans during 15 days before the challenge test. All the animals in the treatments received β-1,3/1,6-glucans 15 days prior the two challenge tests, with the exception of T1 and T5 treatments. Each combination was randomly assigned to experimental units. All the data is presented as the mean plus the standard deviation.

In the first challenge the survival to a WSSV challenge was evaluated. The bioassay was carried out at CENAIM's facilities for 15 days. The experimental units were glass containers of 2 L capacity. Each container held sixteen shrimp ($0.10 \pm 0.03$ g PL). Each treatment had 10 replicates ($n = 10$). The total animals per treatment were $16 \times 10 = 160$ shrimp.

Survival was evaluated in three phases. During each phase the number of dead shrimp per experimental unit was recorded. The first stage included individual observations per experimental unit every 2 h, during the 0 to 52 h period of the experiment. In the second phase, each observation was carried out every 4 h covering the period between 56 and 156 h. In the third phase, observations were made every 8 h until reaching the conclusion of the experiment (164 and 292 h).

In the second experimental challenge immune parameters were measured. This experiment was carried out at CENAIM's facilities for 15 days. The experimental units consisted of fiberglass tanks of 250 L capacity. Each tank held 30 shrimp ($2.64 \pm 0.93$ g juveniles). Each treatment had three replicates ($n = 3$). The total animals per treatment were $3 \times 30 = 90$ shrimp.

To obtain haemolymph samples, 10 shrimp were taken from each experimental unit, at three independent sampling intervals. First sampling $T_0$ (before the WSSV challenge). In these samples the base response was analyzed. Second sampling $T_{24}$ (24 h post-WSSV challenge). In these samples the response to infection was analyzed. Third sampling $T_{360}$

(360 h post-challenge). In these samples the response of survivors was analyzed.

### 2.3. Immunological techniques

Haemolymph was obtained from the ventral sinus. Samples were collected using a 23-gauge needle and 1 ml syringe containing 50 μl of pre-cooled (4 °C) 10% sodium citrate as an anticoagulant. Immune parameters measured were: haemograms, super oxide anion ($O_2^-$) production, plasma antibacterial activity and plasma protein concentration.

Total haemocyte count (THC) was performed as described by Muñoz et al. (2000). Super oxide generation ($O_2^-$) was measured by reduction of Nitro blue tetrazolium (NBT) according to the procedure described by Muñoz et al. (2000). Antibacterial activity (AA) in the plasma was detected following the procedure described by Gullian et al. (2004). Plasma protein concentration was measured using the method of Lowry et al. (1951) adapted to micro plates.

### 2.4. Histopathology analysis

The animals employed for sampling haemolymph were used for histopathology analysis (five from each treatment at each sampling time). Davidson AFA (alcohol, formalin, glacial acetic acid) fixative was used to preserve samples for histopathology. Shrimp tissue was processed according to the procedures outlined by Bell and Lightner (1988). Sections were cut at 5 μm and stained with Mayer Bennet haematoxylin and eosin (H & E). The tissues were examined for WSD lesions and haemocyte infiltrations.

Immunohistochemistry analysis against WSSV (DiagXotics) on tissue sections fixed on positively charged slides (Fisher Scientific) were performed following the procedure outlined by Destoumieux et al. (2000).

### 2.5. Farm culture

In the farm, larvae from treatments T2 and T4 were used. The experiment was carried out from January 21 to April 15 2003 in CENAIM's experimental station at Pesglasa shrimp farm, located on the Ecuadorian coastal zone (50 km from Guayaquil). Six ponds of 0.2 ha were stocked with 18 PL/m$^2$ (three ponds/treatment, $n = 3$). In all 6 ponds β-1,3/1,6-glucans were applied in the feed during spring tides, in order to synchronize their application with post-moult and inter-moult stages of shrimp (Molina and Cadena, 2001). The management procedure did not include water exchange. Every week, survival rates were estimated. On March 16 (week eight of culture), 30 animals per pond, 90 per treatment were sampled in order to determine WSSV prevalence.

### 2.6. Statistical analysis

Analysis of variance (ANOVA) using the linear model of two factors (probiotics & β-glucans) on a design completely randomised was applied to evaluate larvae survival at PL 18. Data was transformed by arcsine$\sqrt{y}$. When the ANOVA test showed significant differences, a multiple comparison procedure using Scheffé at 95% of confidence was performed in order to determine the differences between treatments (Zar, 1999).

The statistical analysis applied to the challenge test was ANOVA of repeated measures using the linear model of three factors (probiotics and β-glucans) (Rao, 1998). The third factor time, was evaluated on the same experimental unit within each treatment. All the data was tested in order to verify the model's assumptions. When lack of variance homogeneity was found a transformation took place using arcsine$\sqrt{y}$. When the ANOVA test showed significant differences, a multiple comparison procedure using Scheffé at 95% of confidence was performed in order to determine the differences between treatments (Zar, 1999).

In the second experiment, the results of every immune test were analysed using ANOVA of repeated measures following the same repeated measurement model explained for survival rates. Data from PP, THC and DHC, was transformed using [log $(x+1)$] function. Statistical analysis was performed using the Statistica® 4.1 program (1994–2000, Stat Soft, Oklahoma, USA).

At pond harvest with the data of all yield indicators (survival, weight, shrimp/m$^2$, feed conversion rate (FCR) and yield production) a *t*-test (Data Desk 6.1) was applied in order to determine significant differences among the means of production indicators of the two kinds of larvae used to stock the ponds. A correlation analysis (Data Desk 6.1) was performed on the PCR and survival data.

## 3. Results

### 3.1. Larviculture survival

The survival mean of the larviculture evaluated at PL 18 was 72.95%. A significant effect of β-1,3/1,6-glucans was found ($p = 0.014$). The highest survival was obtained with the larviculture procedures, which used β-1,3/1,6-glucans at early stage T2 (87.47%) and T6 (80.60). PCR analysis did not prove animals positive to WSSV in the treatments tested.

### 3.2. Survival experiment

In the first challenge test, mortality started 12 h after WSSV infection, observing an accumulative mortality of 50%, 36 h after infection. Nested PCR results of moribund shrimp taken between 24 and 48 h post-infection revealed an index of infection of 3 (on a scale from 0 to 3), thus, indicating a strong process of viral replication and transmission. Non-significant differences between treatments in final survival rates were observed, but the statistical analysis by ANOVA of repeated measures, allowed us to analyse the behaviour of mortality. During the period 0–52 h significant differences were observed for the probiotics factor ($p < 0.01$). In this period, survival was

Table 1
ANOVA results of survival in the first WSSV challenge

| Source | Probability, Time 0–52 | Probability, Time 56–156 | Probability, Time 164–292 |
|---|---|---|---|
| Probiotics | <0.01 | 0.83 | 0.50 |
| β-1,3/1,6-glucans | 0.05 | 0.83 | 0.33 |
| Time | <0.01 | <0.01 | <0.01 |
| Probiotics × β-1,3/1,6-glucans | 0.82 | 0.65 | 0.68 |
| Probiotics × time | 0.91 | 0.67 | 0.99 |
| β-1,3/1,6-glucans × time | 0.32 | <0.01 | 0.99 |
| Probiotics × β-1,3/1,6-glucans × time | <0.01 | <0.01 | <0.01 |

higher ($p<0.01$) in the treatments using probiotics. There was also a significant interaction ($p<0.01$) between probiotics, β-1,3/1,6-glucans and time (Table 1).

During the period 56–156 h; interactions were observed between β-1,3/1,6-glucans–time ($p<0.01$) and β-1,3/1,6-glucans–time–probiotics ($p<0.01$) (Table 1). For this period the treatments of early and late immunostimulation without probiotics had the highest survival rate ($p<0.001$).

During the last challenge period (164–292 h) there was a significant interaction between probiotics-β-1,3/1,6-glucans–time ($p<0.01$) (Table 1), observing the best survival in treatments of early and late immunostimulation without probiotics. Mortalities ended at 180 h of sampling. After this period the percentage of survivors remained constant until the end of the experiment.

### 3.3. Immune parameters experiment

In the four immune parameters evaluated in this study, the statistic analysis detected a significant interaction between probiotics and β-1,3/1,6-glucans ($p<0.05$), observing signif-
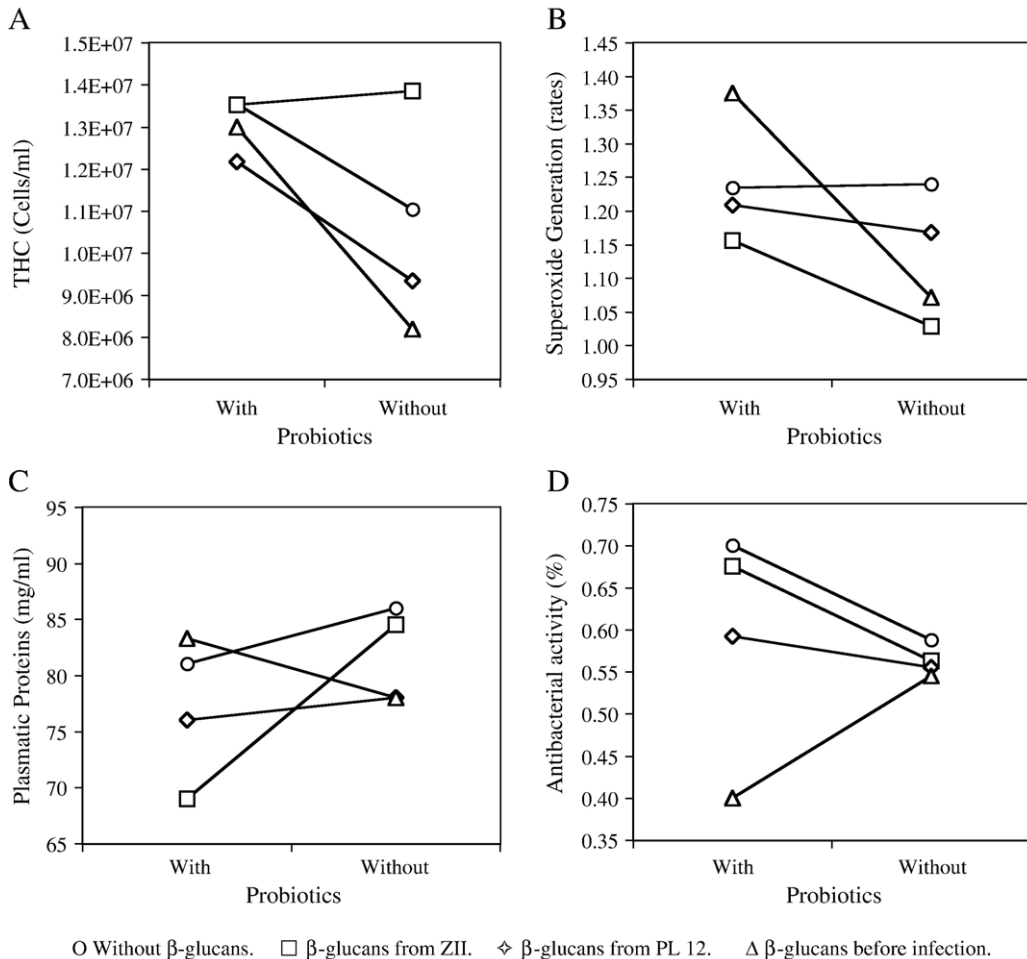


O Without β-glucans.     □ β-glucans from ZII.     ◇ β-glucans from PL 12.     △ β-glucans before infection.

Fig. 1. Effect of the interaction between probiotics and β-1,3/1,6-glucans over the total haemocyte count ($10^6$ cell/mL) (A), the generation of superoxide anion (B), the protein plasma concentration (C) and the plasma antibacterial activity (D) of *Penaeus vannamei* juveniles (from 8 larviculture treatments) challenged with WSSV.

Table 2
Total haemocyte count ($10^6$ cell/mL) of *P. vannamei* juveniles (from 8 larviculture treatments) challenged with WSSV

| Treatments | Sampling time | | |
|---|---|---|---|
| | 0 h | 24 h | 360 h |
| T1 | 7.03±3.10a* | 13.54±6.45b** | 16.62±7.82c** |
| T2 | 5.85±2.07a* | 12.41±6.11b** | 20.23±8.39c*** |
| T3 | 6.37±1.87a* | 10.09±5.05b** | 23.13±2.70c*** |
| T4 | 10.92±3.12b** | 6.55±2.82a* | 19.67±7.49c*** |
| T5 | 9.45±3.76b** | 7.25±2.67b** | 16.40±7.86c* |
| T6 | 11.33±4.10a** | 10.02±5.23a** | 16.88±3.05b** |
| T7 | 11.14±4.25b** | 5.11±2.49a* | 14.16±4.98c** |
| T8 | 8.51±3.69b* | 6.66±3.67a* | 11.20±4.32c* |

Data in the same row with different letters show significant differences (Scheffé 95%).
Data in the same columns with different number of (*) show significant differences (Scheffé 95%).

icant differences between the treatments at time 0 (before infection) as well as after the challenge and in survival.

### 3.3.1. Total haemocytes counts (THC)

A high THC was observed with middle (PL 12), late phase (15 days) and without immunostimulation when probiotics were used in larviculture (Fig. 1A). With early immunostimulation the THC was high with or without probiotics in larviculture (Fig. 1A). Before infection the THC was greater in treatments without probiotics. In response to infection (24 h) a significant increase of the THC was observed in treatments using probiotics, with the exception of treatment T4. Survivors (360 h post-infection) increased their THC in all treatments, observing the highest concentrations in animals that combined probiotics and β-1,3/1,6-glucans (Table 2).

### 3.3.2. Super oxide anion ($O_2^-$) generation

When probiotics were applied in larviculture, $O_2^-$ generation was high if β-1,3/1,6-glucans were used 15 days before

the challenge (Fig. 1B). Without the application of β-1,3/1,6-glucans super oxide generation was not affected by the probiotic. At time 0, $O_2^-$ production was significantly lower when the application of β-1,3/1,6-glucans was performed without probiotics (treatments T6 and T8). 24 h after infection, non-significant differences between treatments were found. In survivors (360 h after infection), $O_2^-$ production was low in all treatments, except in treatment T4 ($p < 0.01$) (Table 3).

### 3.3.3. Plasmatic protein concentrations

The probiotics had a depressive effect over the protein plasma concentration. The level of this effect was related to the larval stage at which time the β-1,3/1,6-glucans were administered. Lower protein values were observed in animals that received β-1,3/1,6-glucans from ZII stage (T2). This effect was not observed in animals reared with probiotics in larviculture, but receiving β-1,3/1,6-glucans only 15 days before WSSV challenge (T4) (Fig. 1C). Before the experimental infection, non-significant differences between treatments were found, but

Table 4
Plasma protein concentration in *P. vannamei* juveniles (from 8 larviculture treatments) challenged with the WSSV

| Treatments | Sampling time | | |
|---|---|---|---|
| | 0 h | 24 h | 360 h |
| T1 | 76.2±16.3b* | 67.0±18.2a* | 95.0±16.9c* |
| T2 | 88.9±27.4b* | 61.9±13.8a* | 89.7±17.0b* |
| T3 | 76.0±26.8a* | 67.4±17.1a* | 71.6±28.8a* |
| T4 | 65.5±16.9a* | 92.3±15.5b** | 92.0±23.1b* |
| T5 | 67.9±13.4a* | 98.6±18.0b** | 117.4±19.9c** |
| T6 | 75.5±19.0a* | 93.0±19.8b** | 91.9±21.5b* |
| T7 | 84.4±15.7a* | 84.4±19.9a** | 81.5±22.4a* |
| T8 | 71.2±14.4a* | 88.3±17.7b** | 83.7±11.4b* |

Data in the same row with different letters show significant differences (Scheffé 95%).
Data in the same columns with different number of (*) show significant differences (Scheffé 95%).

Table 3
Superoxide production in *P. vannamei* juveniles (from 8 larviculture treatments) challenged with WSSV

| Treatments | Sampling time | | |
|---|---|---|---|
| | 0 h | 24 h | 360 h |
| T1 | 1.282±0.148a* | 1.336±0.190a** | 1.085±0.225a* |
| T2 | 1.228±0.059b* | 1.438±0.161b** | 0.801±0.307a* |
| T3 | 1.256±0.142a* | 1.179±0.061a** | 1.191±0.389a* |
| T4 | 1.346±0.143b* | 1.056±0.050a* | 1.722±0.093c* |
| T5 | 1.359±0.140b* | 1.181±0.166a** | 1.179±0.169a* |
| T6 | 1.025±0.036a** | 1.105±0.062a** | 0.954±0.321a* |
| T7 | 1.239±0.221a* | 1.135±0.012a** | 1.131±0.143a* |
| T8 | 1.103±0.075a** | 1.128±0.070a** | 0.984±0.038a* |

Data in the same row with different letters show significant differences (Scheffé 95%).
Data in the same columns with different number of (*) show significant differences (Scheffé 95%).

Table 5
Plasma antibacterial activity of *P. vannamei* juveniles (from 8 larviculture treatments) challenged with the WSSV

| Treatments | Sampling time | | |
|---|---|---|---|
| | 0 h | 24 h | 360 h |
| T1 | 0.410±0.241a*** | 0.921±0.089b** | 0.847±0.122b** |
| T2 | 0.090±0.120a* | 0.921±0.114b** | 0.952±0.055b*** |
| T3 | 0.088±0.142a* | 0.764±0.146b* | 0.985±0.018c*** |
| T4 | 0.178±0.151a** | 0.574±0.201b* | 0.581±0.186b* |
| T5 | 0.329±0.256a*** | 0.650±0.141b* | 0.784±0.140b** |
| T6 | 0.197±0.255a** | 0.915±0.105b** | 0.606±0.161c* |
| T7 | 0.280±0.173a*** | 0.769±0.158b* | 0.581±0.168c* |
| T8 | 0.238±0.219a** | 0.745±0.165b* | 0.784±0.069b** |

Data in the same row with different letters show significant differences (Scheffé 95%).
Data in the same columns with different number of (*) show significant differences (Scheffé 95%).

Table 6
Data of WSSV (PCR) prevalence and final survival of ponds stocked with larvae from treatments T2 and T4

| Treatments | Pond | Prevalence % (PCR) | Prevalence CI | Survival |
|---|---|---|---|---|
| T2 | 5 | 13 | 12 | 49 |
| T2 | 7 | 17 | 13 | 39 |
| T2 | 9 | 10 | 11 | 58 |
| T4 | 6 | 7 | 9 | 66 |
| T4 | 8 | 3 | 6 | 69 |
| T4 | 10 | 23 | 15 | 73 |

CI: Confidence interval.

24 h after WSSV challenge, animals, which received only probiotics or probiotics and β-1,3/1,6-glucans at early stage (T1 and T2), exhibited a decrease of protein concentration in their plasmas. Protein concentrations were restored in survivors. The two treatments of middle immunostimulation (T3 and T7) did not show modifications after infection, nor did the survivors. 360 h post-infection the highest protein concentration was found in animals, which did not receive any immunostimulant (T5) (Table 4).

### 3.3.4. Plasma antibacterial activity

The use of probiotics in larviculture appeared to have a positive effect over antibacterial activity of the plasma. This effect was modified in different ways by the levels of β-1,3/1,6-glucans, expressing a decreasing pattern when β-1,3/1,6-glucans were added. This reduction was greater in treatments using late immunostimulation (15 days) (Fig. 1D). Before infection, antibacterial activity of the plasma was significantly lower in animals from treatments that received probiotics and β-1,3/1,6-glucans at early and medium stages (T2, T3). In response to infection (24 h) there was a significant increase of the AA in all treatments; the highest percentage of bacterial inhibition occurred in the treatments with probiotics T1 (without β-1,3/1,6-glucans), and in the treatments that received β-1,3/1,6-glucans at early stage (T2, T6). The plasma antibacterial activity 360 h after infection was high in treatments with probiotics (Table 5).

### 3.4. Histological analysis

From histology and immunohistochemistry analysis, three observations can be noted. The first was the presence of WSD lesions in all treatments before the first WSSV challenge test.

Histological analysis showed nuclear hypertrophy with eosinophilic to basophilic inclusions (WSD injuries) in animals from treatments T1, T6, T8. These injuries were very clear at 72 h post-challenge, observing an increase in WSD lesions for all treatments. Infected cells were observed in the general epithelium, connective tissue, heart, stomach epithelium and antennal gland. The immunohistochemistry showed labelling for WSSV in animals in all treatments. Second, a low infection in shrimp from treatments T2 and T4, which exhibited WSSV labelling only in tegumental glands (TG). In addition, the survivors from these treatments did not present infected cells. The third observation noted, was the increase of infiltrating haemocytes after infection mainly in animals, which received probiotics in larviculture. Survivors of all treatments presented high infiltration indexes.

### 3.5. Pond culture

The temperature was optimal for the culture of *P. vannamei* juveniles, 29 °C in the morning (Wyban et al., 1995). No external pathological signs or mortality were observed, although the results of PCR analysis showed the presence of WSSV in all ponds (Table 6). The correlation analysis performed for the data of prevalence and survival showed a significant and direct association ($r = 0.96$; $p < 0.01$; $n = 5$). The data of pond 10 (treatment T4) was not taken in account because this pond had the highest confidence interval for the prevalence value (Table 6). After 85 days of culture the animals reached commercial size. The instantaneous growth rate was 0.9 g/week for animals from treatment T4 and 1.0 g/week for animals from treatment T2. At harvest, the survival means of 70%±3% was obtained with the larvae of treatment T4, which was significantly higher than the survival mean of 49%±9% obtained with the larvae of treatment T2 (Table 7).

## 4. Discussion

Ecuadorian shrimp producers in meeting the challenges of disease have for the most part reduced their stocking densities from 6 to 8 animals per m$^2$. In mitigating their potential exposure to diseases, the use of immune enhancers has gained popularity. The industry is recovering with a record volume in 2006 (CNA, 2007) passing the last reported record achieved prior to WSD

Table 7
Production indicators for ponds stocked with larvae reared under treatment protocols T2 and T4

| Treatments | Survival % | FCR | Weight (g) | Yield (kg/ha) | Density (ind./m$^2$) | |
|---|---|---|---|---|---|---|
| | | | | | Initial | Final |
| T2 | 49±9* | 1.6±0.32* | 11.63* | 1072±83* | 19±0.6* | 9±2* |
| T4 | 70±3** | 1.1±0.06* | 10.84* | 1330±599* | 18±1.5* | 12±1* |

FCR: Feed conversion ratio.
Data in the same columns with different number of (*) show significant differences (*t*-test 95%).

arrival. There is no doubt that many factors have contributed to this recovery, of significant importance, has been the contribution made by implanting improved health management practices.

The practice of good health management must begin from the start i.e. larviculture. Common practices in many hatcheries have included the use of water sterilization and antibiotic. These practices may produce deleterious effects, in that they potentially expose the larvae to opportunistic resistant bacteria. In performing larviculture under conditions, which will induce an immune response, and reduce bacterial pathologies, we applied as immune enhancers two components, the bacteria *V. algynoliticus* (Ili strain) and β-1,3/1,6-glucans. These immune enhancers were combined in 6 larviculture protocols following a factorial design. Two controls were added before the experiments (Treatments T4 and T8). The response variables tested included larvae survival, the values of several immune parameters and finally survival in the face of a WSSV challenge under laboratory conditions as well as in ponds.

In larviculture the best survival rates were obtained under treatments T2 and T6 indicating a positive effect of early application of β-1,3/1,6-glucans. In the first WSSV challenge, the viral attack was very aggressive. Nested PCR results revealed a strong viral load. Several factors could have influenced these results, the method of infection employed (*per os*), and re-infection by means of cannibalism or waterborne transmission.

Non-significant differences between treatments in final survival rates were observed, but the statistical analysis showed differences in mortality behaviour. During the initial hours post-infection, the animals of treatments that received probiotics showed a slower mortality rate. After the first initial hours, resistance decreased. The increased mortality observed from mid-experiment and onwards in animals from treatments that showed a higher resistance at the beginning of bioassay, could be related to a strong viral load in the medium due to higher shrimp density in the aquaria caused by a low initial removal of infected animals. From 52 h after infection and until the end of experimental challenge, treatments T6 and T7 expressed the lowest mortality rate. Both treatments included β-1,3/1,6-glucans, suggesting a positive effect of this additive, which would increase the resistance of surviving animals.

Considering immune parameters, the results showed that the larviculture conditions influence the future immune response of juveniles. In addition, a strong interaction between the factors: β-1,3/1,6-glucans and probiotics, was observed producing a large number of possible responses. However some patterns related to the use of probiotics and β-1,3/1,6-glucans, as well as WSSV infection (time), were observed. The use of probiotics in larviculture stimulated the generation of $O_2^-$, counteracting the negative effect of β-1,3/1,6-glucans over this parameter. The negative effects of β-1,3/1,6-glucans on $O_2^-$ generation appeared mainly with early application. The decrease of $O_2^-$ generation in survivors of treatments using β-1,3/1,6-glucans could be attributed to the action of the anti-oxidative mechanisms such as superoxide dismutase (SOD) induced by an early and simultaneous exposure to two kinds of microbial interventions (i.e. probiotics and β-1,3/1,6-glucans). This mechanism of cell protection has been previously reported by Campa-Córdova et al. (2002). In their study they obtained a SOD 1.5 times greater in *P. vannamei* juveniles when treated with β-1,3/1,6-glucans when compared to the control. When probiotics and β-1,3/1,6-glucans were not applied simultaneously (animals of treatment T4) a surge in this parameter was observed.

Anti-microbial activity has been detected in numerous decapods including; lobster *Panulirus cygnus* (Tsvetnenko et al., 2001), blue crab *Callinectes sapidus* (Noga et al., 1994), hermit crab *Pagurus bernhardus*, spider crab *Hyas araneus*, king crab *Paralithodes camtschatica* (Haug et al., 2002), shrimp *Penaeus setiferus* and *P. vannamei* (Alabi et al., 2000). Our results show the highest anti-microbial activity when animals were early exposure to β-1,3/1,6-glucans and probiotics. This might be the reason for the high survival in the larviculture stage, which is typically characterized by various bacterial problems.

The animals cultivated with probiotics in larviculture lost their protein plasma content after the WSSV challenge. Haemocyanin constitutes the mayor protein of shrimp plasma, reaching values of 90 to 95% of total protein (Khayat et al., 1995). The primary function of haemocyanin is to transport $O_2$. However, products of haemocyanin proteolysis can perform other functions such as, anti-microbial activity (Destoumieux et al., 2001). The animals from treatment T4 did not decline the plasma protein concentration and the antibacterial activity was lower than that observed in the other treatments including probiotics in larviculture. There is a need to investigate if the increase of antibacterial activity is related to the drop of plasma protein concentration.

The haemocytes constitute the principal effectors of the immune response of crustaceans. Their function involves cellular mediation mechanisms, consisting of reactions such as phagocytosis, encapsulation, citotoxicity, etc. and humoral factors such as coagulation's

proteins, hydrolytic agglutinins (lectins), and anti-microbial peptides, such as peneidins, which are produced by cellular factors (Destoumieux et al., 2000). Other reports have indicated a depletion in circulating haemocytes accompanied by infiltration in the tissues after a bacterial challenge with a posterior recovery of haemocyte count (Muñoz et al., 2002). In our study increases in haemocyte infiltration were observed in all treatments 24 h after WSSV challenge. In contrast the treatments T5, T6, T7 and T8 lost circulating haemocytes, the animals treated in combination with probiotics — β-1,3/1,6-glucans in larviculture, showed an increase of the THC after infection or the survivors exhibited the highest THC concentration. The increases of both THC and haemocyte infiltration should indicate a strong haemocyte proliferation. In contrast, other researchers have pointed out a decrease of THC during the first 24 h post-challenge with WSSV, both in non-immunostimulated animals and when animals were only stimulated with β-1,3/1,6-glucans, (Kim et al., 1999; Chang et al., 2003). In the same way in our study when β-1,3/1,6-glucans were used alone, they seemed to fail to induce an efficient increase of haemocytes.

This study did not attempt to find a correlation between immune parameters and resistance to WSSV. Our aim was to determine if the larviculture conditions influence the way shrimp immune defences react to a posterior infection challenge. However, by observing the response of survivors to the WSSV challenge, two aspects seem to be related to survival, haemocytic count (circulatory and infiltrating) and the antibacterial activity in the plasma. Futures studies must be undertaken in order to confirm the antiviral interventions of haemocytes and it to would be advisable determine if ALF expression is involved in the increase of antibacterial activity. In this context, several works have been performed and two proteins at least seem to be related with antiviral activity; pmAV (Luo et al., 2003) and the antilipopolysaccharide factor (ALF), which interferes with WSSV replication (Liu et al., 2006). In addition, Robalino et al. (2005) showed that double-stranded RNA (dsRNA) induces antiviral protection in *P. vannamei* shrimp by gene silencing, as it occurs in vertebrates.

The use of *V. alginolyticus* (Ili) in our trials, were found to increase, haemocytic count (circulatory and infiltrating) and the antibacterial activity in the plasma. This supports our findings of a beneficial effect of the probiotic factor over survival, particularly after the first hours post-WSSV infection.

From the PCR data we see that the animals remained negative to WSSV until stage PL 18. However, the histological analysis and immunohistochemistry indicate the presence of WSSV in all treatments specifically in the TG cells before the second challenge. The WSSV presence is not surprising, since this virus is endemic to Ecuador and in this study we used local animals, cultured under a normal procedure, which included the holding of animals in external tanks during the nursery phase. On the other hand, this observation suggests horizontal transmission in a waterborne manner, as mortality was not an issue, because the shrimp were asymptomatic. Whereas we found WSSV positive cells in TG as well as other tissues for most of the treatments, we noted an exception in treatments T2 and T4, with only TG cells expressing positively for WSSV and negatively for all other tissues. This observation suggests that animals of treatments T2 and T4 were the most efficient in limiting the spreading of the virus into their tissues.

The results of treatments T2 and T4 are obviously the most interesting. In the first hours post-infection, they expressed a high resistance to WSSV, displaying a strong increase in THC and antibacterial activity. In addition, the animals of treatment T2 did not express WSD injuries before infection. The animals of treatment T4 had a different reaction from the other treatments that combined probiotics and β-1,3/1,6-glucans. In fact, in this treatment, the two factors; probiotics and β-1,3/1,6-glucans were not applied simultaneously. Larviculture was carried out only with probiotics and the β-1,3/1,6-glucans were applied just 15 days before the challenges. This combination increased the amount of plasmatic protein concentration and the $O_2^-$ generation. Inducing in addition, a high number of initial and final THC.

The animals from T2 and T4 treatments were also used for farm grow-out trails. Both treated groups produced results, which are considerably higher compared to the national average of 154 kg/ha for that time frame (Bayot personal communication). Comparing the production results obtained from treatments T2 and T4, we found that during the larval stage, survival was higher in treatment T2, but in ponds the best yield at harvest was obtained with the animals from treatment T4. The animals of treatment T2 expressed a high capacity to increase the antibacterial activity after the microbial challenge. This capacity could be responsible for the high survival in the larviculture stage, which is typically characterized by various bacterial problems. In ponds, the best survival of animals from treatment T4 could be related to a lower viral load. The WSSV prevalence in ponds stocked with animals from this treatment was lower than ponds stocked with animals from treatment T2. Future studies should be designed in order to

determine if this result could be related with phagocytosis efficiency (high $O_2^-$ generation). Other strategies could be considered in order to induce the decrease of viral load of post-larvae before pond stocking. WSD outbreaks were not observed, even though PCR analysis showed the presence of WSSV in the shrimp populations, due to possibly to high water temperature observed during this study. Taking in to account these last observations, pond experiments should be performed during the cold-drier season, when WSD outbreaks are more common and severe. The high THC, accompanied by the lack of WSD injuries in survivors of WSSV challenge from treatments T2 and T4, suggest a low risk of a second outbreak in ponds stocked with larvae from these treatments.

The results of the present study indicate that exposure to different combinations of probiotics and β-1,3/1,6-glucans in larviculture, will modify the immune response of *P. vannamei* juveniles, influencing WSSV prevalence and survival in ponds. It is also necessary to evaluate in future studies how the presence of other microbial communities (e.g.. vibrios) or the use of other commercial additives during the rearing process of shrimp larvae influence the resistance of shrimp to pathogens in grow-out ponds.

## Acknowledgements

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