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Effects of the *Vibrio alginolyticus* Probiotic, β-1,3/1,6-Glucans and Temperature on Shrimp Production

Jenny Rodríguez[1*] • Ricardo Cedeño[1] • Bonny Bayot[1] • Fabrizio Echeverría[1] • Jaime A. Teixeira da Silva[2] • Alejandra Valladares[3] • Daniel Aguayo[3] • Stanislaus Sonnenholzner[1] •

[1] Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM). Escuela Superior Politécnica del Litoral (ESPOL), Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador

[2] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan

[3] Facultad de Ingeniería Marítima y Ciencias del Mar. Escuela Superior Politécnica del Litoral. (ESPOL), Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O.Box 09-01-5863, Guayaquil, Ecuador

***Corresponding author*: * jrodrigu@cenaim.espol.edu.ec**

ABSTRACT

The main task of this research was to test certain health management strategies for cultured shrimp in order to improve overall production variables (yield, survival rate, weight and feed conversion ratio - FCR). To do so, four thorough studies were conducted with an emphasis on the use of three factors: the probiotic *Vibrio alginolyticus* (Ili strain), β-1,3/1,6-glucans and controlled water temperature. In the first experiment 45-day old postlarvae (PL45) were fed (or not) β-1,3/1,6-glucans, and the water temperature was kept at 24 or 31°C. As a consequence, less shrimp with white spot disease (WSD) injuries were detected when they were fed β-1,3/1,6-glucans and kept at 31°C. In the second experiment, the effect of administrating β-1,3/1,6-glucans from a nursery phase was tested. Larvae (Nauplius 5 - PL4) were reared with the probiotic. At the nursery and grow-out phases shrimp were separated into two groups: the first received β-1,3/1,6-glucans while the other did not. In the ponds where animals received immunostimulants, yield increased while the feed conversion ratio became lower. A third experiment was designed to determine the effect of applying the probiotic at an early stage of larviculture. Shrimp received (or not) the probiotic at early stages and were fed (or not) β-1,3/1,6-glucans in the grow-out phase. Shrimp yield and FCR increased and decreased significantly, respectively, if the larvae received only the probiotic. A fourth trial was designed based on the results of the first three experiments. Two management protocols were compared. In the first, shrimp received the probiotic in the early larviculture phase. In the second protocol, shrimp received the probiotic at the early larviculture stage and then were fed β-1,3/1,6-glucans during the nursery and grow-out stages. The resulting output was significantly improved using the second protocol.

Keywords: pond production, shrimp health management, WSD, WSSV

Abbreviations: CFU, colony forming unit; **DO**, dissolved oxygen; **FCR**, feed conversion ratio; **MT**, metric tons, **N**, nauplius; **PCR**, polymerase chain reaction; **PL**, postlarvae; **THC**, total haemocyte count; **WSD**, white spot disease; **WSSV**, white spot syndrome virus

INTRODUCTION

Despite improvement of Ecuadorian shrimp production after the industry's collapse caused by a white spot disease (WSD) epidemic, shrimp survival in ponds still averages only 50%. Common stocking densities in the country are 8-10 shrimps/m^2, which is a critical number given the fact that any further increment in stocking density would lead to higher mortality (Bayot and Rodríguez 2009). Such a low survival rate is the reason why the local shrimp industry has not exhibited any significant growth in the past few years (Bayot and Rodríguez 2009). Infectious diseases affect both larviculture and grow-out phases and WSD outbreaks still represents the main problem at the pond level. One of the strategies employed by shrimp farmers in order to increase the survival rate is the application of immunostimulants and probiotics in production systems. In recent years numerous articles have appeared on the use of additives in the shrimp industry. Several authors have pointed out the stimulatory effects of β-glucans coming from different sources, such as yeasts and fungi, on the shrimp immune system (Campa–Córdova *et al*. 2002; Chang *et al*. 2003; Burgents *et al*. 2004; Wang *et al*. 2008). However, the use of additives remains controversial, reflecting different opinions about their efficiency in enhancing disease resistance (see Smith *et al*. 2003; Dalmo and Bøgwald 2008). A variety of factors can influence the efficacy of β-glucans. For instance, the source (López *et al.* 2003; Wang *et al.* 2008), the type of β-glucan molecules (Ji *et al.* 2009; Van Hai and Fotedar 2009), dose (López *et al.* 2003; Sajeevan *et al.* 2008), administration schedule (Campa-Córdova 2002; López *et al.* 2003), experimental design (Campa-Córdova 2002; López *et al.* 2003), species (Chang *et al.* 1999; Wang *et al.* 2008), and size and age of the animals (Chang *et al.* 1999, 2000).

As for probiotics, their use has become very extensive in productions systems in Ecuador. Approximately 100 products containing probiotics (mainly yeast and Gram$^+$ bacteria) are commercially available.

Nonetheless, it was established that temperature and stocking density are the main determinants triggering WSD outbreaks in ponds, strongly influencing shrimp production (Rodríguez *et al*. 2003; Bayot and Rodríguez 2009). Temperatures over 32°C reduce the pathogenicity of white spot syndrome virus (WSSV) (Vidal *et al*. 2001; Rahman *et al*. 2007). However, several studies suggested that the fitness temperature for *Penaeus vannamei* ranges between 27 and 31°C (Wyban *et al*. 1995; Hernández *et al*. 2006). In Ecuador, the sea and estuarine water temperature, which constitute the natural habitat of the species, never rises above 32°C. In addition, despite the fact that hyperthermia reduces WSSV pathogenicity, it appears to affect the prevalence only to a small degree (Sonnenholzner *et al*. 2002). The Ecuadorian coastal climate is highly influenced by oceanographic conditions. There are two seasons: a wet/warm sea-

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Table 1 Description of experiments performed in experimental station (pond size: 400 m^2). Three steps (early larviculture, nursery and grow-out) were examined using the probiotic *Vibrio alginolyticus* (Ili strain), fitness temperature (28 – 31°C) and β-1,3/1,6-glucans (β-G).

Experiment	Treatments	Early phase larviculture (N5 – PL4)	Nursery (PL4 to PL18 or PL 25)	Grow-out	Pond culture conditions
Experiment 2	First	Probiotic	28 – 31°C	-	Stocking density
Bioassay 1	Second	Probiotic	28 – 31°C+β-G	β-G	16 shrimp/m^2
					85 days of culture
					Wet/warm season
Bioassay 2	First	Probiotic	28 – 31°C	-	Stocking density
	Second	Probiotic	28 – 31°C+β-G	β-G	10 shrimp/m^2
					99 days of culture
					Dry/cold season
Experiment 3	First	-	-	-	Stocking density
	Second	Probiotic	-	-	8 shrimp/m^2
	Third	-	-	β-G	120 days of culture
	Fourth	Probiotic	-	β-G	Dry/cold season
Experiment 4	First	Probiotic	-	-	Stocking density
	Second	Probiotic	28 – 31°C+β-G	β-G	8 shrimp/m^2
					120 days of culture
					Dry/cold season

son from January to April and a dry/cold from June to November, with considerable changes in water temperature. The impact of variations in temperature is indirectly managed by farmers through the combination of pond stocking density and date. Producers stock the ponds at 10 and 8 shrimp/m^2 in the wet/warm and dry/cold seasons, respectively. Stocking is avoided during the climatic transition from the wet/warm to the dry/cold season (May) due to the large diurnal fluctuations in water temperature (Bayot and Rodríguez 2009).

In a previous study the authors observed that the administration of β-1,3/1,6-glucans and the probiotic *Vibrio alginolyticus* at different larviculture stages modified the immune response of shrimp (Rodríguez *et al*. 2007). Hence, juveniles exposed during early stages of larviculture (Nauplius 5 - N5 to 4-day old postlarvae - PL4) to the probiotic exhibited increased total haemocytes count (THC) and antibacterial activity of the plasma and superoxide (O_2^-) generation when exposed to WSSV. When β-1,3/1,6-glucans were combined with a probiotic early in larviculture, the THC and O_2^- of WSSV-challenged shrimp were depressed. However, a stimulatory effect of these immune parameters was observed when β-1,3/1,6-glucans were applied from the PL4 stage. Besides the immune response, other production variables were also altered, implying that the use of immunostimulants in shrimp larviculture is an effective means to increment shrimp survival and yield. In addition, a significant negative correlation between the prevalence of WSSV in ponds and shrimp survival was found at harvest, even if WSD outbreaks were not evident (Rodríguez *et al*. 2007).

The final step in the development of an effective disease control strategy is a thorough evaluation of its implementation at the production level. Some productivity variables at the shrimp pond level are yield (expressed as kg/ha), survival, harvest density, weight and size dispersion. The last two variables strongly influence the final price of shrimp, while a low feed conversion ratio (FCR < 1.5) implies an optimal investment in food.

This research focused on testing the effect of certain health management strategies (administration of the probiotic *V. alginolyticus* and β-1,3/1,6-glucans, and maintenance of fitness temperature) on production variables at harvest time. Our experiments showed that the application of the probiotic at the early stages of larviculture (N5-PL4), followed by administration of β-1,3/1,6-glucans during the nursery period (PL4-PL18/PL25) and grow-out phases, combined with controlled water temperature (28-31°C) during the nursery phase can significantly increase yield while simultaneously decreasing the FCR.

MATERIALS AND METHODS

Four experiments were carried out in order to examine the effectiveness of the *V. alginolyticus* probiotic and β-1,3/1,6-glucans in two phases of larviculture (*early larviculture phase*: N5-PL4 and *nursery*: PL4-PL18/PL25) and the grow-out phase of *P. vannamei* in earthen ponds. Experiment 1 was performed in a laboratory in order to examine the effect that temperature and β-1,3/1,6-glucans could have in increasing shrimp resistance to natural WSSV infection. Experiments 2, 3 and 4 were conducted in 400 m^2 earthen ponds at the CENAIM experimental station located in Palmar 187 km NW of Guayaquil, Ecuador (2° 00′ 51.40″ S, 80° 43′ 21.17″ W). In experiment 2, the use of β-1,3/1,6-glucans in nursery and grow-out phases was evaluated. Experiment 3 was performed in order to isolate the effects of the probiotic from the administration of β-1,3/1,6-glucan; hence the only additive applied to the hatchery before pond stocking was the probiotic. In experiment 4, the best treatments, selected from experiments 2 and 3, were compared (probiotic *vs.* probiotic + β-1,3/1,6-glucans). A brief description of experiments and treatments is presented in **Table 1**.

Experiment 1

P. vannamei larvae were reared at the CENAIM's facilities (Ecuadorian coast, 170 Km from Guayaquil) following the procedure described in Rodríguez *et al*. (2007), which included the administration of a probiotic (*V. alginolyticus* - Ili strain) from N5 until the PL4 stage. The Ili strain of *V. alginolyticus*, isolated from healthy shrimp larviculture (Morales, unpublished), is used in CENAIM's larvae production protocol. By using *V. alginolyticus*, we successfully mitigated what is commonly referred to as "bolitas" syndrome or Zoea 2 syndrome (Vandenberghe *et al*. 1999). Bacteria were added daily (10 ml/MT) to the larvae rearing water at a concentration of 10^{10} colony forming units (CFU/mL). Using this procedure, the final concentration of the probiotic in the larval rearing tanks achieved 10^5 CFU/mL. Routine analysis performed with samples from the larviculture tanks showed that *V. alginolyticus* was the main bacterium present in the water and in the animals (approximately 90% CFU/mL). From PL4 until PL45, animals were maintained in an external tank (8 MT) at a water temperature of 28-31°C.

At the beginning of the experiment, 100 postlarvae (PL45) shrimp were collected from the external tank to determine the prevalence of WSSV using nested PCR analysis following the protocols described by Melena *et al*. (2006) using the primers designed by Kimura *et al*. (1996). The following WSSV-DNA fragments were amplified: one external 982 bp fragment (982F, 5′-ATCATG GCTGCTTCACAGAC-3′; 982R, 5′-GGCTGGAGAGGACAA GACAT-3′) and one internal 570-bp fragment (570F, 5′-TCTTCA TCAGATGCTACTGC-3′; 570R, 5′-TAACGCTATCCAGTATCA CG-3′). Nested PCR amplifications were carried out with a second

pair of primers (570F/570R) using the amplified product of the first pair (982F/982R) as a template. In both cases, PCR amplification was carried out in a 25 µL reaction mixture containing 1.5 µL of template DNA (100 ng), 1X PCR buffer, 1.5 µM of each deoxynucleotide triphosphate (Promega) and 1U of *Taq* DNA polymerase (Promega). The PCR protocol comprised one start cycle at 94°C for 2 min, 35 cycles at 94°C for 0.3 min, 55°C for 0.3 min and 72°C for 0.3 min with a final extension at 72°C for 5 min. For nested PCR, 2 µL of a one-step reaction mixture was added to the second PCR mixture. DNA from WSSV-negative shrimp was used as the negative control. PCR analyses were performed with a Mastercycle® (Eppendorf) thermocycler.

The experiment was performed in 250-L tanks equipped with an air-lift system for water recirculation. Water temperature (2 levels: 24 and 31°C) and β-1,3/1,6-glucans (*Saccharomyces cerevisae* wall fraction, mannoprotein-free) administration (2 levels: daily β-1,3/1,6-glucans administration at 150 mg/kg of food and no administration) were evaluated in order to obtain a crossed two-factors design. Four treatments, with 4 replicates by treatment and 38 shrimp (PL45) *per* tank were tested. After 20 days of treatment 33 shrimp per tank (132 per treatment, n=4) were sampled, preserved in 95% ethanol and analyzed by nested PCR for WSSV detection. Five shrimp *per* tank (20 per treatment, n=4) were preserved in Davidson fixative and processed for histological analysis according to procedures outlined by Bell and Lightner (1988). WSSV infection (PCR) and the presence of WSD injures (histology) data were used in order to perform two analyses of variance (ANOVAs) using the linear model of two factors (water temperature and β-1,3/1,6-glucans administration) in a completely randomised design using Data Desk 6.1. All data were checked for normality using the Kolmogorov-Smirnov test and variance homogeneity with the Bartlett test. Differences were considered significant when $P < 0.05$. All values shown are expressed as the mean ± standard deviation. Scheffé comparison method was used to compare means when interactions occurred. Prior to analysis, WSSV infection data were arcsin(√x) transformed to satisfy the assumption of variance homogeneity.

Experiment 2

As the first step in the process of evaluating the use of β-1,3/1,6-glucans in shrimp nursery and grow-out phases, two bioassays were performed at the CENAIM experimental station during both Ecuadorian climatic seasons (wet/warm and dry/cold) (**Table 1**). Shrimp larvae were reared at the CENAIM's facilities and the probiotic *V. alginolyticus* was used in the early larviculture phase (N5-PL4), following the procedure previously described in Experiment 1. Afterwards, larvae were transferred to external tanks (8 MT), where the water temperature was kept between 28 and 31°C (PL4-PL18/PL25) (first treatment). During the second treatment, shrimp were treated in the same way as described above but, in addition, during the entire nursery period, they received β-1,3/1,6-glucans (150 mg/kg of artificial food). Shrimp of both treatments received an artificial diet containing 50% protein. The food was administered following a regime standardized by Lourdes Cobo (pers. comm.), which started at PL4 with 76 g/million of larvae and 25 *Artemia nauplii* (instar 2) *per* larva and ended with shrimp at PL25 with 363 g/million of larvae and 83 *A. nauplii* (instar 2) per larva. Animals from the first treatment did not receive any additive during the nursery phase.

In the bioassay performed in the wet/warm season (Bioassay 1) animals were stocked in ponds at 16 PL18/m^2 while in the bioassay performed in the dry/cold season (Bioassay 2) animals were stocked at 10 PL25/m^2. Eight ponds were employed in each bioassay. Four ponds were stocked with animals of the first treatment, while the other four ponds were stocked with animals of the second treatment; the latter ponds, in addition, received β-1,3/1,6-glucans during the grow-out phase. The dosage and timing of β-1,3/1,6-glucans application were decided on the basis of results reported by Rodríguez *et al.* (2000), which indicated that the shrimp immune system was enhanced when a low dose (50, 100, 150 mg of β-1,3/1,6-glucans/kg of food) was employed, in particular O_2^- generation and fenoloxidase activity. In the present study, the dose was increased considering that cultured shrimp in semi-intensive earthen ponds dispose of other food sources (natural production). The feeding rate started with 12% shrimp biomass and decreased to 2.5% when animals reached 13 g. Hence, the dose began with 150 mg/kg of food, but when the feeding rate decreased to 5% or less of pond biomass, the dose of β-1,3/1,6-glucans was increased to 300 mg/kg of food. As for the time of application we took advantage of the relative synchronization between moulting and lunar cycles. During spring tides the major percentages of shrimp population are in post- and intermoult stages (Molina 2003); these stages are related to an increase of feed consumption. Therefore β-1,3/1,6-glucans were added to food 7-8 days around spring tides (around 15 days each month).

At harvest (85 and 99 days for the wet/warm and dry/cold bioassays, respectively) data of shrimp biomass (kg) was collected for each replicate. A randomized sample of 100 shrimp was collected during harvest for each replicate; individual weights were Registered and averaged in order to estimate the mean weight of shrimp for each replicate. The total quantity of food administered (kg) during the entire production cycle was also registered for each replicate. Using this information four other production data sets were calculated: (1) yield, calculated as shrimp biomass divided by the pond area and expressed as kg/ha; (2) survival, calculated as shrimp number at harvest (shrimp biomass divided by mean weight of shrimp at harvest) divided by the stocked number of shrimp and expressed as a percentage; (3) harvest density, calculated as shrimp number at harvest divided by the pond area, expressed as number of shrimp/m^2 and (4) FCR, calculated as total food administered during all production cycles divided by the shrimp biomass. Data of yield, survival, shrimp weight, harvest density and FCR for both treatments were compared in each bioassay using a *t*-test (95% confidence level) (Data Desk 6.1).

Experiment 3

Animals were reared at CENAIM facilities during the dry/cold season. First, shrimp were divided into two groups, which accordingly received (or not) the probiotic at the early phase of larviculture. Then each group was split in two again and accordingly was fed (or not) β-1,3/1,6-glucans in the grow-out stage. All four treatments had 4 replicates (ponds). No treatment was applied at the nursery phase. Animals (PL22) were stocked in the ponds at a density of 8 shrimp/m^2. The harvest was carried out after 120 days of culture. Production data (survival, yield, weight, harvest density and FCR) were analyzed with one-way ANOVA (Data Desk 6.1).

Experiment 4

Two treatments were compared during the dry/cold season (probiotic *vs* probiotic + β-1,3/1,6-glucans). The first treatment was based on the administration of the probiotic as a unique treatment at the early stage. The second one combined the use of the probiotic in the larviculture phase with administration of β-1,3/1,6-glucans in nursery (PL4-PL18) and grow-out stages. Shrimp larvae were reared at CENAIM's facilities, using the probiotic, as mentioned above. For shrimp receiving the probiotic and β-1,3/1,6-glucans, the water temperature during the nursery (PL4 until PL18) was kept between 28 and 31°C. Four ponds *per* treatment were employed in this experiment. The stocking density was 8 shrimp/m^2 and the culture lasted 120 days. The incremental cost by using probiotic and β-1,3/1,6-glucans in 1 ha pond during one production cycle was also calculated. Production data (survival, yield, weight, harvest density and FCR) were analyzed with a *t*-test (95% confidence level) (Data Desk 6.1)

RESULTS

Experiment 1

Before the start of the experiment all shrimp were found to be WSSV-negative (0/100) using nested PCR analysis. After 20 days, WSSV-positive animals were detected by PCR and histology. No significant differences in WSSV infection were detected with PCR data. A significant interaction ($P < 0.05$) between temperature and β-1,3/1,6-glucans was observed when the data of animals exhibiting WSD injuries

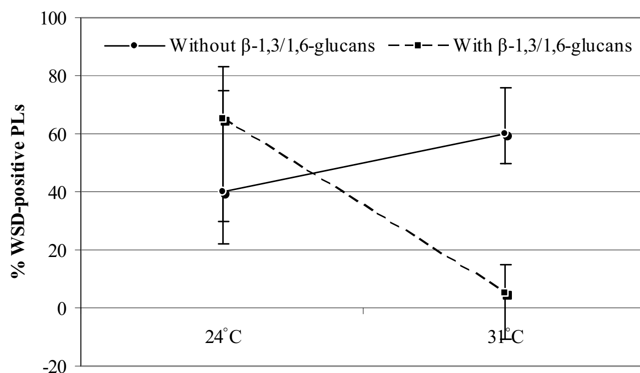

Fig. 1 Effect of interaction between water temperature (24 and 31°C) and β-1,3/1,6-glucans over WSD presence (histology) in *Penaeus vannamei* PL66 (%). Experiment 1. Four treatments were tested: PL fed with β-1,3/1,6-glucans at 24°C; PL fed with β-1,3/1,6-glucans at 31°C; PL fed without β-1,3/1,6-glucans supplementation at 24°C; PL fed without β-1,3/1,6-glucans supplementation at 31°C. Values represent means ± standard deviation.

Table 2 Survival (%) at the end of nursery period of PL used in experiments 2 and 4.

	Survival (%)	
	With β-1,3/1,6-glucans (28 – 31°C)	**Without β-1,3/1,6-glucans**
Experiment 2. Bioassay 1 (PL 18)	94	82
Experiment 2. Bioassay 2 (PL 25)	73	63
Experiment 4 (PL 18)	64	43

(histology) were analyzed (**Fig. 1**). Hence, few shrimp receiving β-1,3/1,6-glucans at 31°C exhibited WSD lesions.

Results of experiments performed in pond

Table 2 shows PL survival at the end of the nursery period for experiments 2 and 4. In all cases survival was higher when shrimp were administrated β-1,3/1,6-glucans at 28-31°C.

Survival of both bioassays of experiment 2 was between 53 and 74%, the yield was above 500 kg/ha, and the density at harvest of animals treated with β-1,3/1,6-glucans was high (11 and 7 shrimp/m^2) (**Table 3**). Despite the fact that ponds were administred with β-1,3/1,6-glucans, resulting in higher yields and lower FCR, only in the wet/warm season bioassay were these results significantly different ($P < 0.05$) to those of ponds stocked with PL to which only the probiotic was added. In the wet/warm season two stress factors affected all ponds; after day 40 until the harvest, an algae bloom provoked a drop of dissolved oxygen (DO, 1-2 mg O_2/ml). In order to increase the oxygen concentration, strong and repeated water exchanges (40-50% of water) were performed. WSD outbreaks (confirmed by PCR and histology, data not shown) were observed in all ponds starting at the 7th week until the 9th week.

In experiment 3, a significant ($P < 0.05$) effect of the probiotic was observed on yield and FCR (**Table 4**). The use of a probiotic in the early larviculture phase caused a 20% increase in survival while the yield was 208 kg/ha higher than the control (without any additives). In addition, the FCR was significantly reduced to 1.1. β-1,3/1,6-glucans applied only in grow-out ponds did not improve the results obtained with the probiotic (**Table 4**).

The second treatment (probiotic + β-1,3/1,6-glucans at nursery and grow-out phases) of experiment 4 showed a significant higher ($P < 0.05$) yield than the first treatment (probiotic only) (**Table 5**). The harvest density was 7 shrimp/m^2, and the FCA decreased to 1 being significantly lower than the first treatment.

DISCUSSION

This study was conducted to formulate a health management strategy based on the administration of *the V. alginolyticus* probiotic (early larviculture phase: N5-PL4), β-1,3/1,6-glucans (nursery: PL4-PL18/PL25 and/or grow-out phases) and maintenance of fitness temperature (nursery). The effect of these factors on production data at shrimp pond harvest was evaluated. Few studies have been performed at this study level (Rengpipat *et al.* 2000; Castex *et al.* 2008).

In the past, we carried out another experiment (Rodríguez *et al.* 2007), which combined the probiotic in early larviculture with β-1,3/1,6-glucans in the grow-out phase. In that experiment we observed a significant negative correlation between the prevalence of WSSV in ponds and shrimp survival at harvest, even if WSD outbreaks were not evident. For this reason, the present study incorporates experiments where PL were immunostimulated at the nursery phase with a dietary supplement of β-1,3/1,6-glucans, keeping the water temperature between 28 and 31°C. We hypothesized that by applying this method the immune system of PL can be enhanced, so they could survive WSD and other diseases.

Two short bioassays (experiment 2), which included a nursery phase, were performed over two seasons (wet/warm and dry/cold). In both bioassays the yield was highest in ponds to which β-1,3/1,6-glucans was added, but these results were significant only during the wet/warm season (experiment 2, Bioassay 1). In this experiment the environmental conditions were very unstable with a drop in DO and several strong water exchanges. In addition, an outbreak of WSD was observed in all ponds. In *Penaeus stilyrostris*, hypoxia (1 mg O_2/ml) produced a significant decrease of THC (Le Moullac *et al.* 1998) while in *Penaeus monodon* the phagocytic activity of haemocytes was less efficient in oxygen-depleted shrimp (Direkbusarakom and Danayadol 1998).

Shrimp exposed to stress conditions in bioassay 1 could have reduced their capacity to mount an immune response and hence would have been more susceptible to WSSV when the health management strategy was limited to the administration of the probiotic in larviculture. On the contrary, animals receiving the probiotic and β-1,3/1,6-glucans demonstrated greater resilience to the WSD outbreak under stress conditions, which was later reflected in a high yield during harvest.

Experiment 3 was performed in order to observe the effects of the probiotic application during the early larviculture phase from β-1,3/1,6-glucan administered during the grow-out phase. Hence, two batches of PL were stocked: animals exposed solely to the probiotic and animals without any treatment in larviculture (control). Our results showed that the use of the probiotic alone was sufficient to increase the survival and yield by 20% and 200 kg/ha, respectively. Furthermore, the experimental design we employed allowed us to conclude that the administration of β-1,3/1,6-glucans in the grow-out phase did not produce better results than the control. This fact confirmed indirectly that the nursery treatment should be a key step in the health management of ponds.

Experiment 4 compiled all possible strategies in one treatment during larviculture (e.g. administration of probiotic in early larviculture and β-1,3/1,6-glucans during PL4-PL18/PL25 at 28-31°C and administration of β-1,3/1,6-glucans in the grow-out phase), which was compared with a treatment based only on the use of the probiotic in early larviculture. Yields were higher in ponds treated with β-1,3/1,6-glucans than in ponds to which β-1,3/1,6-glucans were not added. In addition the FCR decreased to 1 in ponds administered with β-1,3/1,6-glucans.

In fact, in all three experiments (2, 3 and 4) performed in ponds, treatments producing the highest yields also exhibited a significantly lower FCR. We hypothesize that healthy shrimp in ponds with the highest yield should have a better feeding assimilation. Animals receiving β-1,3/1,6-

Table 3 Production results obtained from experiment 2 (Bioassay 1: wet/warm season and Bioassay 2: dry/cold season). Two treatments were tested 1) Probiotic: The stocked larvae were treated with the probiotic *Vibrio alginolyticus* (N5-PL4). 2) Probiotic+β-G: The stocked larvae were treated with *V. alginolyticus* (N5-PL4) and received β-1,3/1,6-glucans in nursery and grow-out pond.

	Treatments	Stocking (shrimp/m^2)	Harvest (shrimp/m^2)	Survival (%)	Yield (kg/ha)	Weight (g)	Feed conversion ratio
Bioassay 1	First: Probiotic	16	8.5 ± 2.3 a	53.3 ± 15.4 a	699.9 ± 188.0 a	7.8 ± 0.4 a	2.4 ± 0.7 a
Wet/warm season	Second: Probiotic + β-G	16	11.4 ± 2.0 a	71.4 ± 12.6 a	1036.9 ± 157.3 b	8.6 ± 0.5 a	1.5 ± 0.2 b
Bioassay 2	First: Probiotic	10	6.4 ± 1.6 a	64.0 ± 16.2 a	544.0 ± 133.8 a	8.5 ± 0.6 a	1.8 ± 0.5 a
Dry/cold season	Second: Probiotic + β-G	10	7.4 ± 0.9 a	74.5 ± 8.7 a	674.7 ± 87.9 a	9.1 ± 0.3 a	1.4 ± 0.2 a

In each bioassay means in each column indicated with different letter are significantly different at $p < 0.05$ based on the *t*-test.

Table 4 Production results obtained of experiment 3 (dry/cold season). Four treatments were tested, 1) animals received any treatment (control), 2) animals were supplemented with the probiotic *Vibrio algnolyticus* (Ili strain) during larviculture, 3) animals received β-1,3/1,6-glucans at grow-out, 4) animals were supplemented with the probiotic at larviculture and received β-1,3/1,6-glucans in grow-out pond. Any treatment was applied during nursery.

Treatments	Stocking (shrimp/m^2)	Harvest (shrimp/m^2)	Survival (%)	Yield (kg/ha)	Weight (g)	Feed conversion ratio
First: Not one (control)	8	4.9 ± 1.3 a	60.8 ± 16.2 a	477.3 ± 73.6 a	10.1 ± 1.4 a	1.7 ± 0.3 a
Second: Probiotic	8	6.5 ± 0.8 a	80.8 ± 9.8 a	684.7 ± 10.9 b	10.7 ± 1.3 a	1.1 ± 0.0 b
Third: β-G	8	5.7 ± 1.8 a	71.1 ± 22.2 a	563.9 ± 126.8 ab	10.2 ± 1.4 a	1.4 ± 0.3 ab
Fourth: Probiotic + β-G	8	5.9 ± 1.0 a	73.8 ± 12.9 a	625.0 ± 89.5 ab	10.7 ± 0.8 a	1.2 ± 0.2 ab

Data in the same columns with different letter show significant differences, Scheffé (95% confidence level).

Table 5 Production results of tested treatments of experiment 4 (performed at dry/cold season). Treatment 1: the stocked larvae were exposed to the probiotic *Vibrio alginolyticus* (Ili strain) (N5-PL4). Treatment 2: the stocked larvae were exposed to the probiotic and received β-1,3/1,6-glucans in nursery and grow-out ponds.

Treatments	Stocking (shrimp/m^2)	Harvest (shrimp/m^2)	Survival (%)	Yield (kg/ha)	Weight (g)	Feed conversion ratio
First: Probiotic	8	5.9 ± 0.8 a	73.8 ± 10.0 a	650.6 ± 37.5 a	11.2 ± 1.6 a	1.2 ± 0.1 a
Second: Probiotic + β-G	8	6.5 ± 0.6 a	80.7 ± 7.2 a	750.0 ± 38.3 b	11.7 ± 0.5 a	1.0 ± 0.1 b

Means in each column indicated with different letter are significantly different at $p < 0.05$ based on the *t*-test.

glucans in nursery and grow-out phases also exhibited a lower dispersion size (**Tables 3, 5**). These results indicate that the probiotic and β-1,3/1,6-glucans might contribute to reducing feeding costs and the final price of production.

The dose of β-1,3/1,6-glucans employed in this study (150-300 mg/kg of food), was lower than that reported in the literature. López *et al*. (2003) employed 2 g of of β-glucans/kg of food while Wang *et al*. (2008) administered 2 and 10 g of β-glucans/kg of food. Still, doses used in our experiments were sufficient to significantly increase pond yield. With an additional cost of only US$ 8/ha/cycle spent on 169 g of β-1,3/1,6-glucans, 750 kg/ha of shrimp can be harvested. To perform the calculation we considered a FCR of 1.5, the highest dose of β-1,3/1,6-glucans (300 mg/kg of food) and the application of the immunostimulant only during spring tides (15 days/month).

Concerning the probiotic, with US$ 4 we can produce the bacteria culture applied during the early larviculture phase of 100,000 PL (shrimp amount required to stock 1–ha of earthen ponds). The temperature of external nursery tanks was kept at 28-31°C using greenhouse plastic, and water exchanges.

The most important production variable is yield (kg/ha). In 2009, the shrimp Ecuadorian production reached 136,094 MT (Cámara Nacional de Acuicultura del Ecuador 2010) with 162,240 ha of ponds (Dirección Nacional de los Espacios Acuáticos 2010). In order to obtain yield higher than 500 kg/ha Ecuadorian producers need to harvest at least 4 animals/m^2 with a shrimp weight higher than 12 g each. As others tropical countries, such as Brazil or Thailand, Ecuador should be able to produce shrimp over the entire year, using a semi-intensive system (10-30 PL/m^2). However, Ecuadorian shrimp production is strongly influenced by the Pacific oceanographic conditions. During the dry/cold season, when Humboldt Current (cold conditions) appears at the Ecuadorian coastal the water temperature of ponds can decrease to 21-24° and the shrimp production decrease. Before WSD, producers employed a semi-intensive system (10-20 PL/m^2). Since WSD, producers have decreased the stocking density in order to mitigate the impact of this disease over the survival. In 2009 farmers employed 8 and 10 PL/m^2 in the cold/dry and wet-warm seasons, respectively. They avoid to stock during the climatic transition (May) from the wet/warm to the dry/cold season (Bayot and Rodríguez 2009). Although, the pond management continue to be performed according with a semi-intensive system (employing cultured larvae, artificial food supply to complement natural pond food, and monitoring environmental parameters), according the description available from FAO (2010) the average of stocking density employed by Ecuadorian producers corresponds to an extensive system.

Table 6 Penaeid shrimp production of selected countries of Asian and America in 2008.

Country	Shrimp production (Metric tons)	Production system according the pond stocking conditions
China	1,268,074	Semi-intensive/extensive/intensive
Thailand	507,500	Semi-intensive/intensive
Vietnam	381,300	Semi-intensive/intensive
Indonesia	408,246	Semi-intensive/intensive/extensive
Ecuador	150,000	Extensive/Semi-intensive
Mexico	130,201	Semi-intensive
Brazil	65,000	Semi-intensive/intensive

Source: www.fao.org/fishery/statistic/global.aquaculture

Low stocking densities have allowed Ecuadorian farmers to obtain a survival rate of 50% (Bayot and Rodríguez 2009). Until now several trials performed by producers in order to increase stocking densities have provoked drops in survival (mainly due to WSD outbreaks). For this reason since 2004 the stocking density used in Ecuador was stabilized around 8 PL/m^2. Both bioassays of experiment 2 were performed at relatively higher stocking densities (10-16 shrimp/m^2). The first goal was to test the treatments under the density conditions capable of triggering WSD outbreaks. The second goal was to find a health management strategy that would enable the increment of Ecuadorian yield to the levels of other tropical countries (**Table 6**). Countries as Vietnam and Brazil are able to stock 20 PL/m^2, obtaining productions of 2-4 MT/ha (Mole and Bunge 2002; Kiet and Sumalde 2008). However, the difficulties related to high stocking densities have induced local producers to exploit a semi-intensive system of shrimp culture. Some Ecuadorian

shrimp producers shifted their focus to the production of organic shrimp (Naturland certification, for example) cultivated without chemotherapeutic agents. Using less artificial food, the environmental impact is attenuated, because low organic material is discharged to effluents (Nunes *et al.* 1996). Other producers take advantage from low culture shrimp densities, reducing the production cost from \$US 700 to \$US 500 (Roberto Retamales pers. comm.) by mean of a low investment in seed (PL) and food, which in a semi-intensive system can represent 60 % of the production costs (Akiyama *et al.* 1992). In extensive systems, contribution to the nutrition of shrimp from the naturally biota is increased. In addition, water exchanges and personal are also reduced. Taking in account the real situation in the Ecuadorian industry, during experiments 3 and 4 we employed 8 animals/m^2, which is a low-risk stocking density. In these conditions our treatments would have a positive effect only if the harvest density remains higher than that obtained by farmers. In both bioassays we obtained 6-7 animals/m^2 as survival was 80%. Both harvest density and survival can be considered satisfactory (as a reference Ecuadorian shrimp farmers obtain around 4 animals/m^2).

CONCLUDING REMARKS

We present evidence supporting the fact that a health management strategy based on the administration of the probiotic *V. alginolyticus*, β-1,3/1,6-glucans and temperature control can significantly improve the output of shrimp ponds investing an additional cost of only US\$ 12/ha/production cycle. The results reported here offer new insight into the health management procedures in shrimp ponds.

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