Probiotics in Aquaculture: A Case Study of Probiotics for Larvae of the Black Tiger Shrimp (*Penaeus monodon*)

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ABSTRACT: The trend of using probiotics in aquaculture is increasing due to research results indicating their ability to increase production and prevent disease in farm animals. The development of suitable probiotics for biocontrol in aquaculture would result in less reliance on chemicals and antibiotics and result in a better environment. In this investigation, a Thai *Bacillus* isolate (strain S11) was used as a probiotic bacterium by passage through *Artemia* sp. fed to the black tiger shrimp (*Penaeus monodon*). It was found that black tiger shrimp larvae reared using the *Bacillus*-fortified *Artemia* probiotic as a feed had significantly shorter development times and fewer disease problems than larvae reared without the probiotic.

Key words: *Penaeus monodon*, black tiger shrimp larvae, probiotics

INTRODUCTION

Microbes and aquaculture

Microbes play both direct and indirect roles in aquaculture. They are important causes of diseases which may readily spread through water to aquatic animal hosts. As flora in soil and water, they also influence the aquatic environment by involvement in C, N, S and P cycles important for ecological balances. Other microbes live in and on aquatic plants and animals. These may be specific for individual organisms and important to their health. An inbalance in the microbial flora in the water or in these organisms often leads to pathogenesis. For example, an inbalance in *Vibrio* species in the rearing water or in the GI tracts of shrimp and fish can lead to pathogenesis (Rengpipat 1996).

Marine culture of shrimp, crabs, fish, oysters and mussels in Thailand provides good income for producers and products that are popular because of their good taste and reasonable price. The diminishing seafood from capture fisheries has paved the way to industrial scale aquaculture. In Thailand, particularly for the black tiger shrimp (*Penaeus monodon*), most farmers build large ponds and raise shrimp intensively. Even though the production has been good for the past 10 years (Thai Department of Business Economics 1996), the trend for the last 2-3 years has been diminished production due to viral diseases such as yellow head virus and white spot syndrome virus (WSSV) (also called systemic ectodermal and mesodermal baculovirus or SEMBV) and to luminescent bacterial disease (Rengpipat 1996). These agents can cause sudden death on a massive scale.

Probiotic microbes in aquaculture

For more than 50 years, beneficial microbes defined as probiotics (Fuller, 1989, 1992 and 1997) have been used successfully for raising healthy and disease-tolerant farm animals like swine (Baird 1977; Pollman et al. 1980) and chickens (Dilworth & Day 1978; Miles et al.1981). These probiotics are now widely used for enhancing production of land animals and they have gained acceptance as being better, cheaper and more effective in promoting animal health than administration of antibiotics or chemical substances.

More recently (within the past 10 years) researchers have sought beneficial microbes for aquaculture by attempting to isolate from seawater, sediments and GI tracts those capable of producing antibiotics and/or antimicrobial substances that can inhibit pathogens *in vitro* (Table 1). Bacteria and unicellular algae capable of inhibiting pathogenic bacteria have been found (Munro et al. 1995; Austin and Day 1990). In setting criteria for the most suitable probiotics in aquaculture, one must be concerned with indirect effects on ecosystem cycles and food chains.

Douillet and Langdon (1994) used a commercial probiotic bacterium (CA2) as a larval feed supplement to increase production in oysters. Other researchers have found that probiotics prevent diseases in salmon (Austin et al. 1992 and 1995), larvae of scallop (Requelme et al. 1997) and black tiger shrimp (Rengpipat et al. 1998 and unpublished data). Austin et al. (1992) stressed that probiotics control diseases by prophylaxis, and that they are not meant to be used as

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Table 1. Reduction of pathogens by microorganisms that possess probiotic properties at *in vitro*.

Aquatic animal	Microorganisms	Pathogens	References
Fish	Antibiotic-producing marine bacteria (Obligate halophilic bacteria)	*Aeromonas hydrophila* B-32 *A. salmonicida* ATCC 14174	Dopazo et al. (1988)
Prawns	*Tetraselmis suecica*(microalgae)	*Vibrio alginolyticus* *V. anguillarum* *V. parahaemolyticus* *V. vulnificus*	Austin and Day (1990)
Fish	*Planococcus*	*Serratia liquefaciens*	Austin and Billaud (1990)
Fish	Gram -ve rod Gram +ve rod	*V. anguillarum* HI 11345 *A. salmonicida* *A. hydrophila*	Westerdahl et al. (1991)
Fish (Turbot larvae)	*Flavobacterium* sp. *V. fluvialis* *V. natvigens* *Vibrio* spp.	*Pavlova lutheri* (unicellular algae)	Munro et al. (1995)

Table 2. Probiotics and feed supplements used in aquaculture.

Aquatic animal	Probiotic strain	Challenge test with	Results	References
Salmon	*Tetraselmis suecica*	*A. salmonicida* *A. hydrophila* *Lactobacillus* spp. *S. liquefaciens* *V. anguillarum* *V. salmonicida* *Yersinia ruckeri* type I	-good control of diseases by Prophylaxis	Austin et al. (1992)
Oyster (larval culture)	CA2	N.D.	-better yield	Douillet and Langdon (1994)
Salmon	*V. alginolyticus*	*A. salmonicida* *V. anguillarum* *V. ordalii*	-good control of disease	Austin et al. (1995)
Salmon	*L. plantarum* (lyophilized form) could inhibit *V. anguillarum*	*A. salmonicida*	-*Lactobacillus* colonized intestinal wall -could not control disease	Gildberg et al. (1995)
Scallop (larval stage)	*Vibrio* spp. *Pseudomonas* sp.	*V. anguillarum* related (VAR)	- good control of disease	Riquelme et al. (1997)
Black tiger shrimp	*Bacillus* strain S11	*V. harveyi*	- better yield - good control of disease	Rengpipat et al. (1998)
Black tiger shrimp	*Lactobacillus* spp.	*V. harveyi*	- better yield - good control of disease	Rengpipat et al. (unpublished data)

therapeutics. For example, *Lactobacillus plantarum* inhibitory to the salmon pathogen *Vibrio anguillarum* could not be used to treat fish infected with the lethal pathogen, *A. salmonicida.* However, lyophilized *Lactobacillus plantarum* fed to salmon was shown to be able to survive in the GI tract (Gildberg et al, 1995).

Probiotics and black tiger shrimp culture

Rengpipat et al. (1998) isolated *Bacillus* strain S11 from the GI tract of *Penaeus monodon* broodstock caught in the Gulf of Thailand. It inhibited the luminescent disease bacterium, *Vibrio harveyi*, 100% and could promote better yields of black tiger shrimp. Mixed *Lactobacillus* species isolated

from the GI tract of local Thai chickens was also used as a feed supplement to black tiger shrimp (Rengpipat et al., unpublished data) and also resulted in higher shrimp production (Table 2).

Probiotics can be freshly prepared and mixed with the shrimp diet as described by Rengpipat et al. (1998). However, this study was carried out to determine the effectiveness of feeding the *Artemia* encapsulated probiotic *Bacillus* strain S11 for enhancing growth and survival of shrimp larvae.

MATERIALS AND METHODS

The present study was conducted at the aquaculture laboratory, Department of Marine Science and at the Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

Bacterial culture

Bacillus strain S11 (Rengpipat et al. 1998) was stocked on tryptic soy agar (TSA) (Difco) and cultured in tryptic soy broth (TSB) (Difco). Culture conditions were at 37°C in 2-L flasks for 24 h, after which the cells were centrifuged and washed in sterile normal saline solution (NSS) three times immediately before use.

Preparation of encapsulated probiotic *Artemia* sp.

Artemia cysts (Great Lake Artemia, Salt Lake City, Utah, U.S.A.) were hatched (1 g of cysts per liter gently aerated 30 ppt seawater) and harvested at 24 h. During harvesting the cysts and nauplii were seperated. The nauplii were then fed directly to the shrimp postlarvae or kept at 4°C for further use. For probiotic encapsulation, freshly prepared cell cultures of *Bacillus* strain S11 were added to *Artemia* cultures at the begining of the hatching process at a concentration of 10^4 cells/ml. The *Artemia* were subsequently harvested at 24 h and fed immediately to the shrimp postlarvae.

Experimental design

Penaeus monodon postlarvae-10 were bought from a backyard hatchery in Chonburi Province, Thailand. The postlarvae were from a single parent. After acclimatization at the laboratory for 5 days, uniform size postlarvae were selected for probiotic testing.

Experimental units for postlarvae rearing comprised 10-L cylindrical fiber glass tanks containing 7.5-L of 25 ppt seawater and an initital stocking of 50 postlarvae. Three replicates were used for a total of 150 postlarvae per treatment. Each rearing unit was a closed recirculating system with self-contained filtering unit consisting of sand and oyster shells. Water was continuously pumped through the filtering unit by an air lift system. A small amount of dechlorinated tap water was added every two days to compensate for evaporation and maintain a constant salinity.

Statistical comparisons of the control group (fed *Artemia* only) and the treated group (fed probiotic *Artemia*) were carried out using a student t-test. The experiment was monitored for two weeks. Shrimp were fed three times daily at 9:00, 13:00 and 18:00 h with an excess of *Artemia* nauplii. All materials used for each experimental unit were separated to avoid any cross contamination.

Lengths and weights of 15 randomly selected shrimp from each tank were recorded weekly. Shrimp survival was also determined in each tank at the end of the first and second weeks. Weekly water samples of 100 ml were collected from the center of each tank for two weeks. Water quality was monitored weekly and included the parameters of temperature, pH, salinity, dissolved oxygen, ammonium ion and phosphate ion measured using techniques described by Strickland and Parsons (1972).

Pathogen challenge test

After feeding for two weeks, shrimp were challenged with the luminous bacterium *V. harveyi* D331 which had been cultured and maintained using thiosulphate citrate bile salt TCBS broth and agar (Difco). Shrimp in the control and treated groups (85 shrimp per treatment) were immersed in a suspension of *V. harveyi* D 331 at ~ 10^7 CFUml^{-1} according to Austin et al. (1995). Shrimp survival was determined after 4 days of challenge. At the same time, three shrimp from each treatment were randomly sampled. Each whole shrimp was cut into small pieces using sterile surgical sciccors and transferred to a sterile tube. Bacterial determinations were then made using serial dilutions in NSS, followed by plating on TSA and TCBS agar. After 24-48 h of incubation at 37°C, colonies were counted and recorded. Microbial strains from TSA were re-examined using Gram staining, spore staining and selected biochemical tests as described by Sneath (1986). *V. harveyi* cultures isolated from shrimp were purified and identified using Gram staining, an oxidase test and motility test and they were compared with the original *V. harveyi* D331 culture. *V. harveyi* D331 culture was kindly provided by the Shrimp Culture Research Center, Charoen Pokphand Feedmill Co. Ltd., Samutsakorn, Thailand. We reconfirmed the identity of *V. harveyi* by following procedures described by Holt et al. (1986). Shrimp survival was determined for each treatment after 4 days of the challenge test.

RESULTS AND DISCUSSION

Bacillus strain S11 showed no inhibitory effect on *Artemia* hatching when compared to *Artemia* alone or *Artemia* fed with *Saccharomyces cerevisiae*. *Artemia* nauplii at ~$1.84x10^5$ g^{-1} were counted after hatching for 24 h. *Bacillus* strain S11 on *Artemia* nauplii were found to be ~$2x10^2$, ~ $6.4x10^4$ and ~$1x10^2$ CFU g^{-1} (wet weight) at 0, 4 and 8 h, respectively, after hatching.

The raising of black tiger shrimp postlarvae using *Artemia* encapsulated *Bacillus* strain S11 showed an increase in body weight and length (Table 3). No obvious effects of *Bacillus* strain S11 on water quality were found (Table 4). During the first week, however, ammonium increased to 1.67 mgL^{-1} in one control group, but later decreased to near zero. At two weeks, *Penaeus monodon* survival was significantly different between the control group (85%) and the treated group (89%)(Figure 1).

Table 3. Average live weight and length of *Penaeus monodon* cultured for 2 weeks in two feed treatments

Parameters	Control	Probiotics
Weight (mg)	26.0*	43.8*
Length (cm)	$1.71^{b} \pm 0.20$	$1.83^{a} \pm 0.31$

Control: shrimp with artemia; Probiotics: shrimp with *Bacillus* strain S11- fed artemia; *Total weight divided by a number of shrimp (43 shrimp); ^{b,a} Different superscripts in the same row significantly different

Table 4. Range of water quality values in shrimp culture water during 2 weeks of probiotic trial.

	Range of water quality values	
Parameter	Control	Probiotics
Temperature (°C)	29.5	29.5
pH	7.79 - 8.23	7.78 - 8.22
Salinity (ppt)	25	25
Dissolved oxygen (mg L^{-1})	7.9 - 8.1	8.0 - 8.1
Ammonium (mg L^{-1})	0 - 1.67	0 - 0.5
Phosphate (mg L^{-1})	3	3

Control : shrimp with artemia
Probiotics : shrimp with *Bacillus* strain S11- fed artemia

When challenged with the luminous disease bacterium *Vibrio harveyi*, the shrimp treated with probiotics showed a higher survival (13%) when compared to the control group (4%) (Figure 2). *Vibrio harveyi* was more virulent to younger stages of shrimp. High numbers of *Vibrio harveyi* were present in both the rearing water and the shrimp themselves on the fourth day of this experiment (Table 5). However, *Bacillus* strain S11 was also detected in significant numbers in the rearing water and the shrimp, clearly showing that *Artemia* was an effective probiotic carrier.

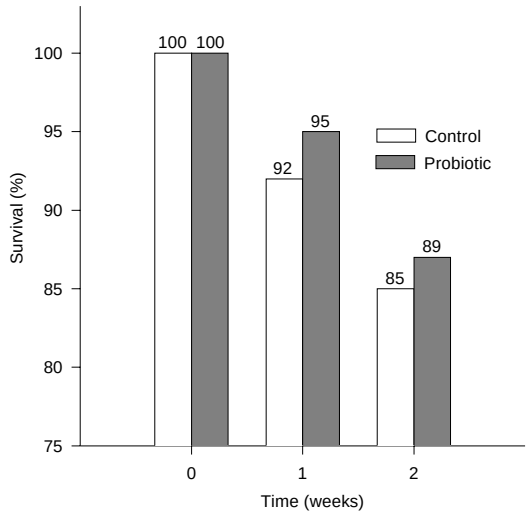

Figure 1. *Penaeus monodon* survival after culture for 2 weeks on control and probiotic feeds.

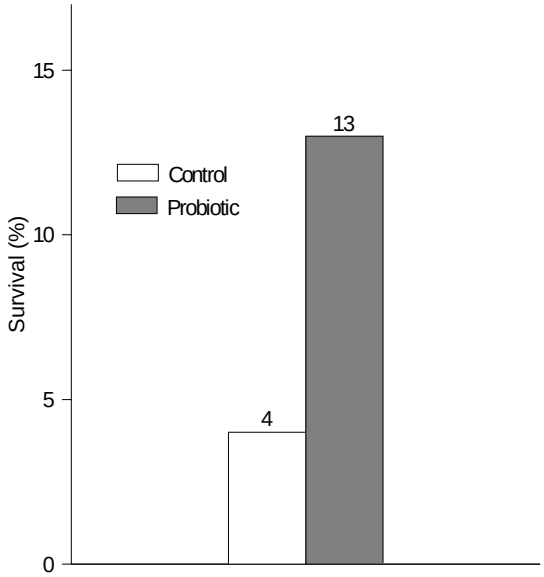

Figure 2. Percentage survival of *Penaeus monodon* after challenge with *Vibrio harveyi* D331.

Bacillus strain S11 is considered a saprophytic strain which is environmental-friendly and has been proven before as a probiotic bacterium for black tiger shrimp (Rengpipat et al., 1998) when mixed with shrimp feed. Therefore, this investigation supported the previous work and also showed that probiotics could be passed through *Artemia* which are routinely used to feed shrimp larvae. Our method may prove beneficial as an enhancement for hatchery postlarvae or for improvement of young shrimp survival at the initial stages of earthen pond culture.

Mechanisms of probiotic action in the host are not fully understood. However, a user may select strains that are suitable or specific for a particular host and environmentally safe. The purpose of their use in aquaculture is to reduce the dependence on antibiotics and chemicals, thus improving environmental safety. Use of local isolates is recommended for biosafety reasons and to avoid sudden changes in the microbial flora of the ecosystem.

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Table 5. Average bacterial counts in water and whole shrimp during challenged with *V. harveyi* D331 by immersion

		V. harveyi (CFU ml^{-1} or g^{-1})		*Bacillus* strain S11 (CFU ml^{-1} or g^{-1})	
		Control	Probiotics	Control	Probiotics
Water	- day 1	$1.28x10^7$	$1.31x10^7$	0	75
	- day 4	$1.07x10^6$	$3.64x10^5$	0	50
Shrimp	- day 1	$6.36x10^6$	$6.09x10^6$	0	$1.20x10^3$
	- day 4	TNTC	TNTC	0	$2.76x10^2$

Control : shrimp with artemia; Probiotics : shrimp with *Bacillus* strain S11- fed artemia
TNTC : Too numerous to count

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