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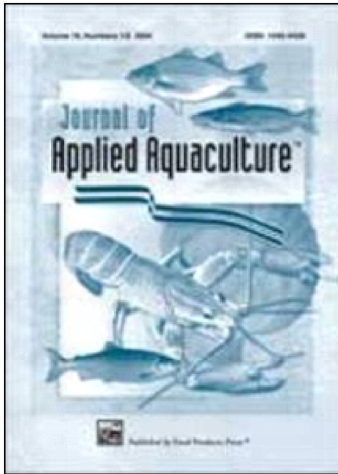

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Effect of Customized Probiotics on the Physiological and Immunological Responses of Juvenile Western King Prawns (*Penaeus latisulcatus* Kishinouye, 1896) Challenged with *Vibrio harveyi*

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Effect of Customized Probiotics on the Physiological and Immunological Responses of Juvenile Western King Prawns (*Penaeus latisulcatus* Kishinouye, 1896) Challenged with *Vibrio harveyi*

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Juvenile western king prawn P. latisulcatus *were fed 10^5 colony–forming units (CFU)/mL of two probiotics* Pseudomonas synxantha *and* P. aeruginosa *for 28 days.* P. latisulcatus *were then challenged with* V. harveyi *at 0 (control), 10^3, 10^5, and 10^7 CFU/mL. During the seven days of challenge, disease resistance of the probiotic-fed prawns was compared with that of prawns not fed probiotics. The immunological responses of the prawns did not improve during the challenge period in terms of total haemocyte count, hyalinocyte, semi-ganulocyte, granulocyte, clotting time, bacteraemia, and intestinal bacterial load. Overall, when prawns were challenged with* V. harveyi*, the LT_{50} values got shorter as* V. harveyi *concentration increased. LT_{50} values of prawns fed probiotics were significantly longer ($P < 0.05$) than those not fed probiotics. At a* V. harveyi *concentration of 10^3 CFU/mL, the 100%*

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survival of the prawns fed probiotics was three times more likely than those of the prawns not fed probiotics.

KEYWORDS Probiotics, Pseudomonas *spp.,* Vibrio harveyi*, Western king prawns,* Penaeus latisulcatus*, immune responses*

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INTRODUCTION

In recent decades, prawn culture has intensified rapidly leading to serious losses from diseases and environmental deterioration (Bondad-Reantaso et al. 2005; Rodríguez et al. 2007). *Vibrio harveyi* is considered to be one of the most harmful bacteria for prawns (Le Moullac et al. 1998; Austin & Austin 2005).

Substantial increases in the regular use of chemical additives and veterinary medicines as preventative and curative means against diseases has led to antimicrobial-resistant strains of pathogenic bacteria (Bachère 2000, 2003; Nomoto 2005). Alternatives to antibiotics and chemicals are desirable to improve the quality and sustainability of prawn production (Rengpipat et al. 1998; Meunpol, Lopinyosiri, & Menasveta 2003; Vaseeharan & Ramasamy 2003; Li et al. 2006), including the use of probiotics.

Previous applications of probiotics as water additives or as feed supplements (Moriarty 1998; Skjermo & Vadstein 1999) have improved growth, survival, and health of shrimps (Moriarty 1998; Skjermo & Vadstein 1999). Our previous studies have demonstrated that that customized probiotics *Pseudomonas synxantha* and *P. aeruginosa* are appropriate for use in the cultivation of *P. latisulcatus* (Hai, Fotedar, & Buller 2007; Hai, Buller, & Fotedar 2009; Hai & Fotedar 2009). However, a rational evaluation of the effectiveness of probiotics in aquaculture needs to be undertaken (Wang, Li, & Lin 2008). An immersion challenge of *P. latisulcatus* with *V. harveyi* was investigated in this study, in which *V. harveyi* isolated from Mahimahi (*Coryphaena hippurus*) was used to infect *P. latisulcatus* and then to investigate the effectiveness of the customized probiotics on *P. latisulcatus* by examining the physiological and immunological responses of the prawns.

MATERIALS AND METHODS

Juvenile western king prawns (4.57 ± 0.42 g) were collected from the Canning River, Western Australia (32°00′39 S; 115°51′15 E) and transferred to the Curtin Aquatic Research Laboratory. They were kept in 200 L composite tanks and fed twice a day with a formulated shrimp feed ST#1 (43% protein, 6% fat, and 2% fibre; Ridley Aqua-Feed, Ridley AgriProducts Pty. Ltd. Qld, Australia).

Previously tested probiotics—*P. synxantha* and *P. aeruginosa* (Hai, Fotedar, & Buller 2007)—were employed in this study. The method of isolation and identification of these probiotics is described by Hai, Fotedar, and Buller (2007). The probiotic solutions were prepared in normal saline after subculturing on marine salt agar (MSA) (Buller 2004) as in the previous work (Hai, Buller, & Fotedar 2009; Hai & Fotedar 2009). A known pathogenic bacterial strain of *V. harveyi* isolated from diseased Mahimahi and stored at −80°C at the Animal Health Laboratory, Department of Agriculture and Food, Western Australia, was used for this study. Both probiotics and *V. harveyi* solutions were cultured at 25°C for 24 h in tryptic soy broth (OXOID, Adelaide, SA, Australia) containing a final concentration of 2% NaCl (Buller 2004). The probiotic bacterial inoculum was prepared in 2% saline at a concentration of 10^5 CFU/mL (Moriarty 1998; Vijayan et al. 2006; Ziaei-Nejad et al. 2006). *V. harveyi* inoculates were prepared at concentrations of 10^3, 10^5, and 10^7 CFU/mL. The concentrations were obtained using an optical density reading of 560 nm (Spectrophotometer BOECO S-20, Hamburg, Germany) against a standard curve as described previously (Hai, Fotedar, & Buller 2007).

The entire experiment consisted of two phases. In phase 1, juvenile western king prawns were cultured for 28 days on a probiotic-laced diet and were subjected to *V. harveyi* exposure for another seven days during phase 2.

During phase 1, 100 juvenile western king prawns (4.12 ± 0.36 g) were fed the formulated diet supplemented with customized probiotics. A second control group of 100 juvenile prawns (4.13 ± 0.21 g) was fed the formulated diet without any supplementation of probiotics. Seawater ozonated at 700 mV by an ozone generator (ZOX, model AQ-2KP, Environplus, WA, Australia) was used as a culture medium. Each prawn group was split equally into four 70-L plastic tanks filled with 15 L of ozonated water and connected to independent recirculation/filtration units. Aeration was continuously supplied, and the water temperature was maintained at 25°C. Fifteen percent of water was exchanged weekly with ozonated seawater. Each kg of prawn formulated feed was sprayed with 20 mL of 2% saline solution containing 20 mL of prepared inoculum at equal proportions of *P. synxantha* and *P. aeruginosa* for the treatment group. For the control group, the feed was sprayed with the same volume of saline solution without probiotics (Hai, Buller, & Fotedar 2009; Hai & Fotedar 2009). The prawns were fed twice a day at 3–5% body weight.

During phase 2, both prawn groups were separately challenged with *V. harveyi* in a water bath placed in a laboratory hood so as to prevent the spread of contamination of *V. harveyi*. Twelve 2-L glass beakers were filled with 1.5 L of ozonised seawater at 35 parts per thousand. The beakers were set up as a random design in a water bath, in which an automatic heater and a pump were installed on one side of the water bath to maintain

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a temperature of 25°C. The beakers were also covered firmly by a net to prevent the prawns escaping from the beakers.

The juvenile prawns were stocked at 6 per beaker. The *V. harveyi* inoculum was added to the beakers in concentrations of 10^3, 10^5, or 10^7 CFU/mL against a control without *V. harveyi* and the challenge was run in triplicate.

During the challenge period, aeration was supplied continuously. The water was maintained at a level of 1.5 litres per beaker by syphoning uneaten feed and replacing with seawater containing the appropriate concentrations of *V. harveyi* suspension for each concentration. Dead prawns were removed from the beakers every 12 h. During the seven days of challenge, the prawns were fed with their respective formulated feeds, either with or without probiotics.

Survival and immunological responses were recorded at days 0, 14, and 28 during phase 1. During phase 2, the observable disease symptoms of the prawns challenged with *V. harveyi* were recorded every 12 h. The lethal time 50% (LT_{50}) was calculated as the time of exposure to *V. harveyi* at which 50% of the prawns were dead.

The total bacterial load in the intestine was determined by selecting one prawn per treatment, which was rinsed in distilled water, followed by a quick wash with 70% alcohol, and then rinsed again in distilled water to remove external bacteria. The prawn intestines were then removed and homogenized in a 1.5 mL microfuge tube using a micropestle. The homogenated samples were weighed, diluted serially with sterile 2% saline solution, and lawn inoculated onto MSA plates as above. The plates were incubated for 24 h at 25°C and the colonies counted to obtain the total bacterial count per gram.

Prawn haemolymph was taken from the pericardial cavity of individual prawns using a 1-mL syringe containing 0.2 mL of anticoagulant (1% glutaraldehyde in 0.2M sodium cacodylate, pH 7.0) and a 23-gauge needle to puncture the intersegmental membrane between the cephalothorax and the first abdominal segment. A 0.2 mL aliquot of haemolymph was dispensed into a 1.5 mL microfuge tube and kept on ice.

Total haemocyte count (THC) was determined using a haemocytometer at 100-fold magnification. Haemocyte cells were counted on both sides of the grids. THCs were calculated using the following equation:

$$\text{THC} = (\text{cells counted} \times \text{dilution factor} \times 1000)/\text{volume of grid } (0.1\,\text{mm}^3)$$

The differential haemocyte count (DHC) was measured by smearing together one drop of the mixture of anticoagulant and haemolymph onto a glass microscope slide. The smear was air dried and fixed in 70% methanol for 10 minutes. The fixed smears were stained in May-Grunwald and Giemsa stain for 10 minutes each (Bancroft & Stevens 1977). Three major haemocyte groups were identified as hyalinocyte (HC), semi-granulocyte (SGC) and

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granulocyte (GC) (Hose, Martin, & Gerard 1990; Sequeira et al. 1995; Jussila et al. 1997; Johansson et al. 2000). A total of 200 cells were counted on each slide. DHC was calculated using the following equation.

$$\text{DHC}\,(\%) = \frac{\text{Number of different haemocyte cell types}}{\text{Total haemocyte cells counted}} \times 100$$

To measure haemolymph clotting time, a 30 μL aliquot of haemolymph was quickly transferred and drawn into a capillary tube (Chase, Scientific Glass Inc. Rockwood, TN, USA). The time at which the haemolymph stopped moving back and forth was recorded. The haemolymph clotting time was ranked from 0 (0–10 s) to 9 (>90 s). If the clotting time lasted for longer than 90 s, 'no clot' was recorded for the haemolymph.

The bacterial load in the haemolymph of the prawns was assessed on MSA. Individual drops of the haemolymph aliquot were placed onto separate MSA plates and lawn inoculated (5 drops of haemolymph were tested from each animal). The plates were incubated for 24 h at 25°C, and colony-forming units (CFU) were counted for each drop. CFU/mL for each sample was calculated on the basis of a total volume of 20 μL for each drop. The bacterial loads were ranked from 1 (0–250 CFU/mL) to 12 (2751–3000 CFU/mL), and the rank 13 was used for "too numerous for an accurate count."

The Statistical Package for the Social Sciences (SPSS) version 17.0 was used to conduct one-way ANOVA (analysis of variance). Least significant difference (LSD) post hoc tests were used to determine significant differences between the tested variables of the physiological and immune responses and survival of the prawns. The Games-Howell post hoc test was used, when variances were not homogeneous. All significance tests were performed at $P = 0.05$.

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RESULTS

At day 14, survival of the probiotic-fed prawns was significantly higher ($P < 0.05$) than those of the prawns not fed probiotic. Among the physiological and immunological responses measured, only the bacterial load in the prawn intestines differed among groups, being significantly higher ($P < 0.05$) in the prawns on the no-probiotics diet. By day 28, application of the probiotics resulted in significantly higher ($P < 0.05$) SGC, GC proportions, and significantly lower ($P < 0.05$) clotting time and bacterial load in the prawn intestines (Table 1).

Overall, when prawns were challenged with *V. harveyi* (phase 2), the LT_{50} values got shorter as *V. harveyi* concentration increased. LT_{50} values of prawns fed probiotics were significantly longer ($P < 0.05$) than those not fed probiotics (Table 2). At a *V. harveyi* concentration of 10^3 CFU/mL, the

TABLE 1 The Physiological and Immunological Responses (Mean ± SE) of Prawns Fed and not Fed Customized Probiotics (*in Brackets*) before Challenging with *V. Harveyi*

Parameter	Day 0	Day 14	Day 28
Survival (%)	$_{1}100.00 \pm 00^{a}$	$_{2}87.00 \pm 1.31^{a}$	$_{2}85.00 \pm 1.11^{a}$
	($_{1}100.00 \pm 00^{a}$)	($_{2}76 \pm 0.02^{b}$)	($_{2}73 \pm 0.00^{b}$)
THC ($\times 10^{6}$ Cells/mL)	$_{1}4.73 \pm 0.12^{a}$	$_{1}4.88 \pm 0.11^{a}$	$_{1}4.94 \pm 0.12^{a}$
	($_{1}4.73 \pm 0.12^{a}$)	($_{1}4.73 \pm 0.01^{a}$)	($_{1}4.76 \pm 0.03^{a}$)
Hyalinocyte (%)	$_{1}56.67 \pm 3.48^{a}$	$_{1}54.33 \pm 2.91^{a}$	$_{1}52.67 \pm 3.06^{a}$
	($_{1}56.67 \pm 3.48^{a}$)	($_{1}57.33 \pm 1.76^{a}$)	($_{1}57.67 \pm 0.65^{a}$)
Semi-Granulocyte (%)	$_{1}18.17 \pm 1.74^{a}$	$_{1}20.00 \pm 1.76^{a}$	$_{1}21.00 \pm 1.44^{a}$
	($_{1}18.17 \pm 1.74^{a}$)	($_{1}15.16 \pm 1.75^{a}$)	($_{1}14.18 \pm 1.48^{b}$)
Granulocyte (%)	$_{1}8.17 \pm 0.60^{a}$	$_{1}8.50 \pm 0.29^{a}$	$_{1}9.50 \pm 0.44^{a}$
	($_{1}8.17 \pm 0.60^{a}$)	($_{1}7.35 \pm 0.62^{a}$)	($_{1}7.26 \pm 0.45^{b}$)
Clotting time (rank)	$_{1}6.33 \pm 0.33^{a}$	$_{1}6.67 \pm 0.33^{a}$	$_{1}5.67 \pm 0.58^{a}$
	($_{1}6.33 \pm 0.33^{a}$)	($_{1}6.00 \pm 0.58^{a}$)	($_{1}7.00 \pm 0.33^{b}$)
Bacteraemia (rank)	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.33 \pm 0.33^{a}$	$_{1}3.67 \pm 0.33^{a}$
	($_{1}3.00 \pm 0.00^{a}$)	($_{1}2.67 \pm 0.33^{a}$)	($_{1}2.67 \pm 0.33^{a}$)
Bacterial load in	$_{1}0.34 \pm 0.009^{a}$	$_{1}0.45 \pm 0.006^{a}$	$_{1}0.56 \pm 0.004^{a}$
intestine ($\times 10^{3}$ CFU/g)	($_{1}0.34 \pm 0.009^{a}$)	($_{2}1.97 \pm 0.21^{a}$)	($_{3}3.74 \pm 0.41^{b}$)

Values in any one row not preceded by the same subscript numbers are significantly different at $P < 0.05$. Value in any column at the same parameters not followed by the same superscript letters are significantly different at $P < 0.05$.

TABLE 2 The Lethal Time 50% (LT_{50}) of Prawns Fed and not Fed Customized Probiotics and the Correlation Between the Prawn Mortalities (%) and Time (h)

	V. harveyi concentrations	Equation	R^2	LT_{50} (h)
Prawns not fed probiotics	10^3	y = 2.8241x – 5	$R^2 = 0.9877$	55
	10^5	y = 1.7063x + 5.291	$R^2 = 0.9790$	26
	10^7	y = 1.0101x – 5.6566	$R^2 = 0.9857$	20
Prawns fed probiotics	10^3	y = 0.6465x – 11.576	$R^2 = 0.9009$	95
	10^5	y = 0.9097x – 11.677	$R^2 = 0.9607$	68
	10^7	y = 1.6204x – 17.857	$R^2 = 0.9088$	42

*y and x presented the prawn mortalities and time, respectively.

100% survival of the prawns fed probiotics was three times more likely than those of the prawns not fed probiotics (Figure 1).

In the probiotic-fed prawn group, at 24 h onward, *V. harveyi* significantly altered ($P < 0.05$) the physiological and immunological responses of the prawns compared to the control. For the group fed probiotics for up to 24 h, challenge with *V. harveyi* at 10^3 and 10^5 CFU/mL had no significant influence on physiological and immunological parameters. By 36 h, GC proportions, bacteraemia, and bacterial load in the prawn intestines were significant higher ($P < 0.05$) at *V. harveyi* concentrations of 10^5 CFU/mL than at other *V. harveyi* concentrations (Table 3).

In the prawn group not fed probiotic at 60 h of challenge onward, challenge with *V. harveyi* significantly changed ($P < 0.05$) the physiological

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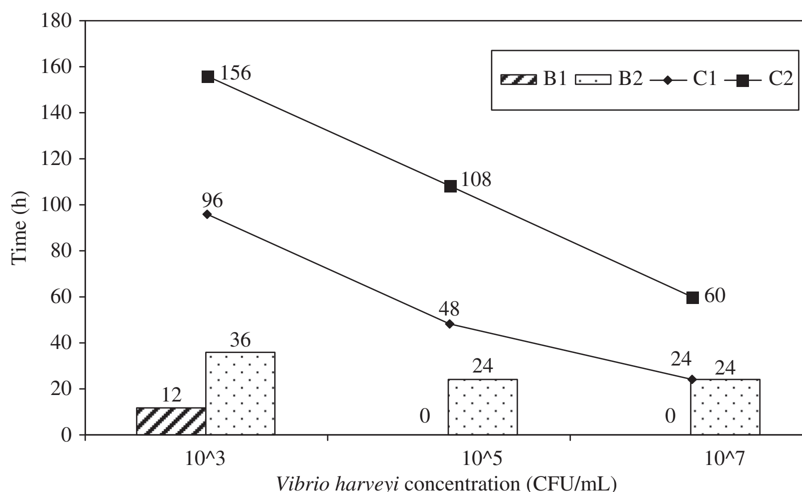

FIGURE 1 The 100% survival hours of the prawns not fed probiotics (B1) and probiotic-fed prawns (B2), the survival hours of the prawns not fed probiotics (C1), and probiotic-fed prawns (C2) challenged with *V. harveyi* at 0, 10^3, 10^5, and 10^7 CFU/mL.

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and immunological responses of the prawns. At 60 h of challenge, only *V. harveyi* at 10^7 CFU/mL significantly changed ($P < 0.05$) the physiological and immunological responses of the prawns compared to other *V. harveyi* concentrations. At 108 h of challenge, the GC proportions and bacterial load in the intestine of the prawns challenged with *V. harveyi* at 10^3 CFU/mL were significantly higher ($P < 0.05$) than those challenged with *V. harveyi* at 10^5 CFU/mL (Table 4).

DISCUSSION

The application of probiotics improves the survival, growth, and food conversion ratio of various prawns such as *L. vannamei* (Garriques & Arevalo 1995; Balcázar, Rojas-Luna, & Cunningham 2007; Wang 2007; Zhou, Wang, & Li 2009; Guo et al. in press), *P. monodon* (Maeda & Liao 1992), and *Fenenpenaeus indicus* (Ziaei-Nejad et al. 2006). The survival of probiotic-fed *P. monodon* challenged with *V. harveyi* was 100%, whereas, *P. monodon* reared without probiotics showed only 26% survival (Rengpipat et al. 1998). In agreement with these studies and our previous studies (Hai, Buller, & Fotedar 2009; Hai & Fotedar 2009), this study showed that the probiotic-fed prawns had higher survival and growth rates than those not fed probiotics. Probiotics can increase the innate immune system of fish and prawns (Sakai 1999) and disease resistance of the host through stimulation of non-specific defense mechanisms (Skjermo et al. 2006). In our studies, the probiotic-fed prawns were healthier, indicated by higher SGC,

TABLE 3 The Physiological and Immunological Responses (Mean ± SE) of the Probiotic-Fed Prawns During the Challenge with *V. harveyi* at 0, 10^3, 10^5, and 10^7 CFU/mL

	V. harveyi (CFU/mL)			
Time (h)	0	10^{3}	10^{5}	0^{7}
Total Haemocyte Count (× 10^6 Cells/mL)				
0	$_{1}4.89 \pm 0.15^{a}$	$_{1}4.89 \pm 0.15^{a}$	$_{1}4.89 \pm 0.15^{a}$	$_{1}4.89 \pm 0.15^{a}$
24	$_{1}4.73 \pm 0.12^{a}$	$_{2}2.73 \pm 0.13^{b}$	$_{2}2.77 \pm 0.37^{b}$	$_{3}1.83 \pm 0.15^{b}$
36	$_{1}4.7 \pm 0.06^{a}$	$_{2}2.33 \pm 0.09^{b}$	$_{2}2.00 \pm 0.15^{c}$	
96	$_{1}4.6 \pm 0.27^{a}$	$_{2}1.73 \pm 0.07^{b}$		
Hyalinocyte (%)				
0	$_{1}52.00 \pm 3.69^{a}$	$_{1}52.00 \pm 3.69^{a}$	$_{1}52.00 \pm 3.69^{a}$	$_{1}52.00 \pm 3.69^{a}$
24	$_{1}52.33 \pm 3.28^{a}$	$_{2}62.33 \pm 2.33^{b}$	$_{2}61.67 \pm 0.67^{b}$	$_{3}73.00 \pm 2.31^{b}$
36	$_{1}51.67 \pm 3.38^{a}$	$_{2}68.33 \pm 1.76^{b}$	$_{2}66.00 \pm 1.53^{b}$	
96	$_{1}51.67 \pm 3.33^{a}$	$_{2}70.67 \pm 2.96^{b}$		
Semi-Granulocyte (%)				
0	$_{1}21.00 \pm 1.44^{a}$	$_{1}21.00 \pm 1.44^{a}$	$_{1}21.00 \pm 1.44^{a}$	$_{1}21.00 \pm 1.44^{a}$
24	$_{1}21.67 \pm 0.88^{a}$	$_{2}14.33 \pm 0.33^{b}$	$_{2}13.33 \pm 2.40^{b}$	$_{3}8.33 \pm 0.88^{b}$
36	$_{1}21.33 \pm 1.45^{a}$	$_{2}10.33 \pm 1.20^{c}$	$_{2}8.67 \pm 0.33^{c}$	
96	$_{1}21.33 \pm 1.20^{a}$	$_{2}8.33 \pm 0.88^{c}$		
Granulocyte (%)				
0	$_{1}8.50 \pm 0.50^{a}$	$_{1}8.50 \pm 0.50^{a}$	$_{1}8.50 \pm 0.50^{a}$	$_{1}8.50 \pm 0.50^{a}$
24	$_{1}8.33 \pm 0.33^{a}$	$_{2}6.33 \pm 0.33^{b}$	$_{2}6.00 \pm 0.58^{b}$	$_{3}3.00 \pm 0.58^{b}$
36	$_{1}8.83 \pm 0.44^{a}$	$_{2}4.67 \pm 0.33^{c}$	$_{3}3.33 \pm 0.33^{c}$	
96	$_{1}8.67 \pm 0.33^{a}$	$_{2}3.33 \pm 0.33^{d}$		
Clotting time (rank)				
0	$_{1}5.67 \pm 0.33^{a}$	$_{1}5.67 \pm 0.33^{a}$	$_{1}5.67 \pm 0.33^{a}$	$_{1}5.67 \pm 0.33^{a}$
24	$_{1}6.00 \pm 0.00^{a}$	$_{1}6.33 \pm 0.33^{ab}$	$_{1}6.67 \pm 0.67^{ab}$	$_{2}8.67 \pm 0.33^{b}$
36	$_{1}5.67 \pm 0.33^{a}$	$_{2}7.00 \pm 0.58^{bc}$	$_{2}7.33 \pm 0.33^{b}$	
96	$_{1}5.67 \pm 0.33^{a}$	$_{2}7.67 \pm 0.33^{c}$		
Bacteraemia (rank)				
0	$_{1}2.33 \pm 0.33^{a}$	$_{1}2.33 \pm 0.33^{a}$	$_{1}2.33 \pm 0.33^{a}$	$_{1}2.33 \pm 0.33^{a}$
24	$_{1}2.33 \pm 0.33^{a}$	$_{2}5.33 \pm 0.88^{b}$	$_{2}5.67 \pm 0.33^{b}$	$_{3}8.33 \pm 0.33^{b}$
36	$_{1}2.00 \pm 0.00^{a}$	$_{2}6.33 \pm 0.88^{bc}$	$_{3}8.00 \pm 0.58^{c}$	
96	$_{1}2.00 \pm 0.00^{a}$	$_{2}7.33 \pm 0.33^{c}$		
Bacterial load in intestine (× 10^3 CFU/g)				
0	$_{1}3.68 \pm 0.43^{a}$	$_{1}3.68 \pm 0.43^{a}$	$_{1}3.68 \pm 0.43^{a}$	$_{1}3.68 \pm 0.43^{a}$
24	$_{1}3.43 \pm 0.44^{a}$	$_{2}5.11 \pm 0.25^{b}$	$_{2}5.53 \pm 0.24^{b}$	$_{3}7.82 \pm 0.23^{b}$
36	$_{1}3.35 \pm 0.58^{a}$	$_{2}5.69 \pm 0.30^{b}$	$_{3}7.36 \pm 0.31^{c}$	
96	$_{1}3.33 \pm 0.51^{a}$	$_{2}7.36 \pm 0.32^{c}$		

Values in any one row not preceded by the same subscript numbers are significantly different at $P < 0.05$. Values in any one column not followed by the same superscript letters are significantly different at $P < 0.05$.

GC proportions, significantly lower clotting time, and intestinal bacterial load. Therefore, the customized probiotics not only improved the prawn physiological responses, but also accelerated the immune responses.

A number of infection routes by bacteria can be involved where *V. harveyi* caused significant mortalities of *P. monodon* larvae and postlarvae in an immersion challenge (Lavilla-Pitogo et al. 1990). An immersion

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TABLE 4 The Physiological and Immunological Responses (Mean ± SE) of Prawns not Fed Probiotics during the Challenge with *V. harveyi* at 0, 10^3, 10^5, and 10^7 CFU/mL

	V. harveyi (CFU/mL)			
Time (h)	0	10^3	10^5	10^7
Total Haemocyte Count ($\times 10^6$ Cells/mL)				
0	$_{1}4.92 \pm 0.12^{a}$	$_{1}4.92 \pm 0.12^{a}$	$_{1}4.92 \pm 0.12^{a}$	$_{1}4.92 \pm 0.12^{a}$
60	$_{1}4.93 \pm 0.12^{a}$	$_{2}2.97 \pm 0.27^{b}$	$_{2}2.83 \pm 0.34^{b}$	$_{3}2.07 \pm 0.15^{b}$
108	$_{1}4.69 \pm 0.12^{a}$	$_{2}2.53 \pm 0.12^{bc}$	$_{2}2.20 \pm 0.27^{c}$	
156	$_{1}4.78 \pm 0.12^{a}$	$_{2}2.03 \pm 0.12^{c}$		
Hyalinocyte (%)				
0	$_{1}52.67 \pm 3.06^{a}$	$_{1}52.67 \pm 3.06^{a}$	$_{1}52.67 \pm 3.06^{a}$	$_{1}52.67 \pm 3.06^{a}$
60	$_{1}52.45 \pm 3.06^{a}$	$_{2}61.67 \pm 2.73^{bc}$	$_{2}60.67 \pm 1.45^{b}$	$_{3}72.33 \pm 1.76^{b}$
108	$_{1}52.08 \pm 3.06^{a}$	$_{2}66.67 \pm 2.33^{c}$	$_{2}66.00 \pm 1.53^{b}$	
156	$_{1}51.87 \pm 3.06^{a}$	$_{2}70.67 \pm 2.96^{cd}$		
Semi-Granulocyte (%)				
0	$_{1}21.00 \pm 1.44^{a}$	$_{1}21.00 \pm 1.44^{a}$	$_{1}21.00 \pm 1.44^{a}$	$_{1}21.00 \pm 1.44^{a}$
60	$_{1}21.78 \pm 0.25^{a}$	$_{2}15.00 \pm 0.58^{b}$	$_{2}14.33 \pm 1.86^{b}$	$_{3}8.67 \pm 0.67^{b}$
108	$_{1}22.06 \pm 0.58^{a}$	$_{2}10.67 \pm 0.88^{c}$	$_{2}9.00 \pm 0.58^{b}$	
156	$_{1}22.45 \pm 0.64^{a}$	$_{2}9.33 \pm 0.67^{c}$		
Granulocyte (%)				
0	$_{1}8.83 \pm 0.44^{a}$	$_{1}8.83 \pm 0.44^{a}$	$_{1}8.83 \pm 0.44^{a}$	$_{1}8.83 \pm 0.44^{a}$
60	$_{1}8.88 \pm 0.56^{a}$	$_{1}6.33 \pm 0.33^{b}$	$_{1}6.00 \pm 0.58^{b}$	$_{2}3.33 \pm 0.33^{b}$
108	$_{1}8.78 \pm 0.25^{a}$	$_{2}5.00 \pm 0.00^{c}$	$_{3}3.67 \pm 0.33^{c}$	
156	$_{1}9.67 \pm 0.04^{a}$	$_{2}3.67 \pm 0.33^{d}$		
Clotting time (rank)				
0	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$
60	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.33 \pm 0.33^{ab}$	$_{1}6.67 \pm 0.67^{a}$	$_{2}8.33 \pm 0.33^{b}$
108	$_{1}5.67 \pm 0.88^{a}$	$_{2}7.00 \pm 0.58^{ab}$	$_{2}7.33 \pm 0.33^{a}$	
156	$_{1}5.67 \pm 0.88^{a}$	$_{2}7.67 \pm 0.33^{b}$		
Bacteraemia (rank)				
0	$_{1}2.67 \pm 0.33^{a}$	$_{1}2.67 \pm 0.33^{a}$	$_{1}2.67 \pm 0.33^{a}$	$_{1}2.67 \pm 0.33^{a}$
60	$_{1}2.33 \pm 0.33^{a}$	$_{2}5.00 \pm 0.58^{b}$	$_{2}5.00 \pm 0.58^{b}$	$_{3}8.33 \pm 0.67^{b}$
108	$_{1}2.33 \pm 0.33^{a}$	$_{2}6.00 \pm 0.58^{bc}$	$_{2}7.33 \pm 0.33^{c}$	
156	$_{1}2.00 \pm 0.00^{a}$	$_{2}7.00 \pm 0.58^{c}$		
Bacterial load in intestine ($\times 10^3$ CFU/g)				
0	$_{1}3.74 \pm 0.41^{a}$	$_{1}3.74 \pm 0.41^{a}$	$_{1}3.74 \pm 0.41^{a}$	$_{1}3.74 \pm 0.41^{a}$
60	$_{1}3.81 \pm 0.28^{a}$	$_{1}4.77 \pm 0.13^{b}$	$_{1}4.87 \pm 0.28^{b}$	$_{2}7.49 \pm 0.41^{b}$
108	$_{1}4.02 \pm 0.18^{a}$	$_{1}5.35 \pm 0.10^{b}$	$_{2}7.02 \pm 0.16^{c}$	
156	$_{1}4.25 \pm 0.32^{a}$	$_{2}7.03 \pm 0.12^{c}$		

Values in any one row not preceded by the same subscript numbers are significantly different at $P < 0.05$. Values in any one column not followed by the same superscript letters are significantly different at $P < 0.05$.

challenge with *V. carchariae* resulted in an increase in mortality of brown-spotted grouper (*Epinephelus tauvina*) (Saeed 1995). In contrast, an immersion challenge with *Vibrio campbellii* did not affect the *L. vannamei* survival (Phuoc et al. 2009). Most outbreaks of prawn vibriosis happen either in combination with physiological stress factors or following primary infections with other pathogens (Sung et al. 2001). In our study, the

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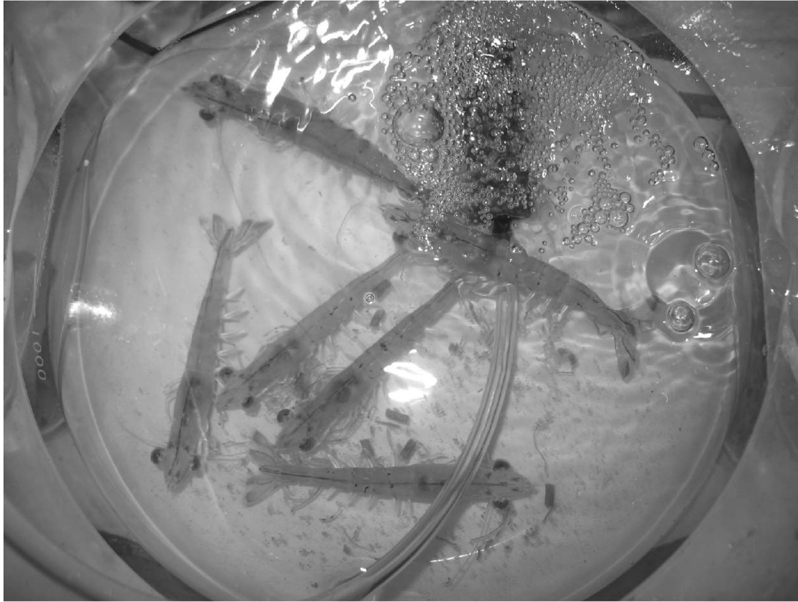

FIGURE 2 The healthy prawns after 36 h of exposure to *V. harveyi*.

FIGURE 3 The moribund prawns with the cross disease symptom after 36 h of challenge onwards.

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probiotics helped prawns to improve their disease resistance against *V. harveyi* infection, resulting in longer survival. *V. harveyi* infection has caused the appendages to erode as well as black gills and black spots along the abdominal segments, whereas bacterial infections in *P. monodon* displayed red disease syndrome (Alapide-Tendencia & Dureza 1997).

Vibrio harveyi is regarded as an opportunistic pathogen (Saeed 1995), as *V. harveyi* from broodstock, water, *Artemia,* or bacterial biofilms on the

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surface of plastic or cement tanks (Karunasagar, Otta, & Karunasagar 1996; Abraham & Palaniappan 2004) may lead to a disease outbreak under stressful conditions (Thaithongnum et al. 2006). Therefore, the prior application of probiotics into a new environment and culture conditions is recommended for preventing the effect of infectious bacteria on the host. As the prawns in our study were fed probiotics for 28 days before they were challenged with *V. harveyi*, the effects of challenge were reduced. The probiotic-fed prawns were stronger with higher resistance against the pathogen *V. harveyi* due to longer survival hours and LT_{50} values.

The bactericity of *V. harveyi* on the hosts depends on the concentration of *V. harveyi* used for the challenge. A challenge of *V. harveyi* at 10^2–10^4 CFU/mL caused significant mortalities of *P. monodon* larvae and postlarvae (Lavilla-Pitogo et al. 1990; Karunasagar et al. 1994; Prayitno & Latchford 1995). In our study, *V. harveyi* at 10^7 CFU/mL were more harmful than the lower concentrations shown by shorter survival hours. The 5-day LC_{50} values for 164-g silvery black porgy (*Acanthopagrus cuvieri*) and 156-g *E. tauvina* were 10^7 and 10^9 CFU/fish, respectively (Saeed 1995). Other studies have also showed that the disease outbreaks can occur with concentrations of *V. harveyi* at 10^2–10^3 CFU/mL (Lavilla-Pitogo et al. 1990). In our study, the probiotic-fed prawns resisted *V. harveyi* at 10^3 CFU/mL as the 100% survival of these prawns was three times more likely than those of the prawns not fed probiotics. *V. harveyi* challenge at 10^3 CFU/mL for 48 h resulted in only 51.50% survival of *P. monodon* (Prayitno & Latchford 1995), while the LT_{50} value of *P. latisulcatus* fed probiotics were at 90 h in our study. Hence, the probiotics at 10^5 CFU/mL provided a positive effect on the *P. latisulcatus* survival.

Adding an artificial dominant bacterial strain is the main factor used to manipulate indigenous microbiota communities (Balcázar et al. 2006). Competitive exclusion is one of the ecological processes so that the small changes affecting the growth and mortality of one species may lead to a change of species dominance in the community (Moriarty 1998). In our previous studies, the probiotics showed a high inhibition test against *V. harveyi* (Hai, Fotedar, & Buller 2007), and the probiotic-fed prawns showed healthier signs than those fed without probiotics (Hai, Buller, & Fotedar 2009; Hai & Fotedar 2009). In our current study, the probiotic-fed prawns died after 36 h of challenge with *V. harveyi* at 10^3 CFU/mL. It is possible that 28 days of feeding with probiotics did not provide enough time for probiotics to be dominant over *V. harveyi* even at low concentration of 10^3 CFU/mL.

THC and DHC are the health assessors for aquatic animals (Jussila et al. 1997). The THC decreased when *Litopenaeus stylirostris* (Le Moullac & Haffner 2000), *L. vannamei* (Cheng, Wang, & Chen 2005), and *P. monodon* (Wang & Chen 2006) were under temperature stressor; when both bacterial-injected *L. vannamei* (Hsu & Chen 2007) and *Marsupenaeus japonicus* (Cheng, Hsu, & Chen 2007) were exposed to sulfide; and when

Panulirus cygnus was exposed to the air (Fotedar, Tsvetnenko, & Evans 2001). THC decreased in lobsters (*Homarus americanus*) (Stewart, Cornick, & Dingle 1967) and in the blue crab (*Callinectes sapidus*) (Johnson 1976) when harmful bacteria were present in the rearing media. THC was also lower in the moribund *P. cygnus* than in the healthy ones (Jussila et al. 1997). The similar results were achieved in our study when *P. latisulcatus* was exposed to *V. harveyi*. The HCs are the most numerous of haemocytes (Sequeira et al. 1995) and are chiefly involved in phagocytosis (Johansson et al. 2000). In our study, the higher HC were produced in *P. latisulcatus* when they were exposed to *V. harveyi* infections. In agreement with a study conducted by Rowley and Powell (2007), the elevated levels of phagocytosis are a partial explanation for enhancing immune response of prawns.

In addition, during the challenge period, the decreases in THC, SGC, and GC, and the increases in clotting time, bacteraemia, and intestinal bacterial load may be consequences of immune degradation due to exposure to *V. harveyi*, thus bacterial infectious symptoms viz. erosion of appendages and black gills appeared. Therefore, the probiotics could not counteract the effects of the *V. harveyi* infection even at low a concentration of 10^3 CFU/mL.

High concentration of *V. harveyi* at 10^7 CFU/mL was more harmful than lower concentrations. The resistibility of the probiotic-fed prawns to *V. harveyi* at 10^3 CFU/mL within 36 h of challenge has proved that *P. synxantha* and *P. aeruginosa* can be used as appropriate probiotics for the cultivation of juvenile *P. latisulcatus*. Although the probiotic-fed prawns were healthier than the prawns not fed probiotics, the immunological responses of the prawns subjected to *V. harveyi* challenge were not improved, while only the survival of the probiotic-fed prawns was longer than those not fed probiotics. The customized probiotics could not counteract the effects of *V. harveyi* in the improvement of immunological responses of the prawns.

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