ASSESSMENT OF TWO PROBIOTIC GROUPS ON MARINE BACTERIA AND *VIBRIO* SPP. PROFILES AND SURVIVAL RATE OF WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)

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Introduction

White shrimp (*Litopenaeus vannamei*) is considered as one of the most popularly cultivated shrimp in Thailand. However, L. vannamei in both hatchery and earthen-pond stages has usually suffered with pathogenic vibrios, especially *Vibrio harveyi*, *V. anguillarum*, *V. parahaemolyticus* and *V. vulnificus* (Mohney et al., 1994). Therefore, numerous practices have been carried out to enhancement of production levels and prevention of severely disease outbreaks such as application of antibiotics. However, the immoderate and unsuitable use of antibiotics has resulted in occurrence of multidrug-resistant bacteria and decrease in effitiveness of antibiotic treatment for human and animal illness (Moriarty 1997). Concern in antibiotic-resistant strain of pathogenic bacteria and increase in requirement of environmental-friendly product has led to application of probiotics (Moriarty, 1997). Probiotics are live microorganisms providing a health benefit for the animal host (Reid et al., 2003). Among probiotics bacteria, *Bacillus* possess so numerous benefit for use as probiotic in aquatic cultivation by several appearance (Nimrat et al., 2008). *Bacillus* produce many extracellular enzymes responsible for enhancement of feed assimilation and absorption, and water quality and they also decrease pathogenic vibrios in shrimp culture facilities (Dalmin et al., 2001). Yeast probiotics are also used in aquaculture because they have improved fish health by production of beneficially desirable growth factors (Tovar et al., 2002). Presently, application of bacteria and yeast incorporation as probiotics in white shrimp culture has been not well investigated. Therefore, the objective of this work was to study the efficiency of *Bacillus* spp. and yeast as freeze-dried form on the change in marine bacteria, *Vibrio* spp. and survival rate of *L. vannamei*

Methods

1. Preparation of probiotic

Probiotic product comprising five strains of *Bacillus* sp. and two types of yeast. Each *Bacillus* sp. was cultured in 500-ml flask containing 200 ml of Trypticase Soy Broth (Difco, Detroit,MI, USA) at 30 °C for 24 h under aerobic condition. Afterwards, cell were harvested by centrifuging at 8,000 rpm for 5 min and washed three-time with phosphate buffer solution. Cell suspensions were adjusted to a final concentration at an O.D.580 of 1.5 A.U. (approximately 10^{10} CFU/ml). In case of yeast, they were grown in 500-ml flask containing 200 ml of Yeast extract Peptone Dextrose Broth at 30 °C, 200 rpm for 24 h and prepared as previously described. Then, all probiotic suspensions were prepared for production as freeze-dried form as mentioned by Sangsong et al. (2010).

2. Diets

Experimental diets included 4 groups: T1: supplementation of freeze-dried *Bacillus* spp. to basal diet; T2: supplementation of freeze-dried *Bacillus* spp. and yeasts to basal diet; T3: supplementation of freeze-dried yeasts to basal diet and the control (no supplementation of probiotic). All diets were preserved in steriled bottles at 4 °C for no longer than 1 month prior to use.

3. Experimental setup

Twelve aquaria (0.8×1.2×0.6 m) were set for cultivation *L. vannamei* in triplicate of treatment and the control. Pond bottom soil collected from shrimp pond was added to a depth of 7 cm in aquaria and seawater with 5 ppt was transferred into all aquaria. Thirty healthy *L. vannamei* (PL-15) were distributed into each experimental aquarium. The experiment was accomplished for 120 days.

4. Bacterial counts

Shrimp samples were collected from each aquarium in order to examine the counts of marine bacteria and vibrios. Shrimp samples were washed with 70% alcohol and then rinsed again with sterilized distilled water to remove external bacteria. The intestine was dissected out using aseptic technique and homogenized with sterilized 0.85% (w/v) NaCl solution. All samples were diluted serially with same buffer and 0.1 ml of each dilution was transferred onto Marine agar and Thiosulfate-citrate-bile salt-sucrose agar (Difco, Detroit, MI, USA) for counting of marine bacteria and vibrios, respectively, according to Nimrat et al. (2008) method. After incubation for 24-48 h at 30 °C, colonies were counted and calculated as CFU/g. All the purified isolates were observed for biochemical tests following the criteria described by Holt et al. (1994) for identification to genus and species level.

5. Measurement of survival

Shrimp survival was determined by counting individuals in each aquarium of treatment and the control and calculated using the following formula:

$$(\%)\ \text{Survival rate} = \frac{\text{Number of shrimp at the end of the experiment}}{\text{Number of shrimp starting of the experiment}} \quad 100$$

6. Statistical analysis

Data were shown as mean±standard deviation (SD). Significant difference was tested by using a two-way analysis of variance (ANOVA). Duncan's New Multiple Range Test was used to isolate any significant differences detected in the ANOVA. All data were considered significant at $p<0.05$.

Results

1. Marine bacteria

Table 1 summarized the numbers of marine bacteria in the intestine of tested white shrimp in control, T1, T2 and T3 at the beginning of the experiment which were $4.90 \pm 1.38 \times 10^8$, $3.79 \pm 1.00 \times 10^8$, $4.13 \pm 1.67 \times 10^8$ and $4.58 \pm 1.94 \times 10^8$ CFU/g, respectively, which were not significant difference ($p> 0.05$). At the end of the experiment, numbers of marine bacteria in the intestine of T1 and T2 were $4.71 \pm 0.88 \times 10^8$ and $4.51 \pm 0.55 \times 10^8$ CFU/g, respectively, which were significantly higher ($p<0.05$) than those in the control and T3 ($3.53 \pm 0.43 \times 10^8$ and $3.61 \pm 1.46\times 10^8$ CFU/g, respectively).

Table 1 Numbers of marine bacteria in the intestine of tested white shrimps

Days	Marine bacteria CFU/g)			
	C	T1	T2	T3
0	$4.90 \pm 1.38 \times 10^{8\ (a,1)}$	$3.79 \pm 1.00 \times 10^{8\ (c,1)}$	$4.13 \pm 1.67 \times 10^{8\ (a,1)}$	$4.58 \pm 1.94 \times 10^{8\ (b,1)}$
30	$4.21\pm1.08 \times 10^{8\ (ab,1)}$	$5.49 \pm 2.17 \times 10^{8\ (b,1)}$	$5.12 \pm 2.14 \times 10^{8\ (a,1)}$	$4.97 \pm 1.38 \times 10^{8\ (b,1)}$
60	$7.57 \pm 1.05\times 10^{7\ (c,3)}$	$3.45 \pm 0.56\times 10^{8\ (c,2)}$	$1.12 \pm 0.12 \times 10^{8\ (b,3)}$	$9.27 \pm 1.17\times 10^{8\ (a,1)}$
90	$3.99 \pm 0.55 \times 10^{8\ (b,2)}$	$7.85 \pm 1.17 \times 10^{8\ (a,1)}$	$4.07 \pm 0.87 \times 10^{8\ (a,2)}$	$9.21 \pm 1.24 \times 10^{7(c,3)}$
120	$3.53 \pm 0.43 \times 10^{8\ (b,2)}$	$4.71 \pm 0.88 \times 10^{8\ (bc,1)}$	$4.51 \pm 0.55 \times 10^{8\ (a,1)}$	$3.61 \pm 1.46\times 10^{8\ (b,2)}$

[1,2] Different superscript numbers indicate significant difference ($p<0.05$) in the number of marine bacteria between a treatment and the control.

[a,b,c] Different superscript letters indicate significant difference ($p<0.05$) in the number of marine bacteria in different time.

2. *Vibrio* spp.

Vibrio spp. numbers in the intestine of tested *L. vannamei* all treatments and the control at the beginning were $7.67 \pm 2.32 \times 10^7$, $6.61 \pm 2.78 \times 10^7$ and $7.31 \pm 2.00 \times 10^7$ CFU/g, respectively (Table 2) with not significantly different $p>0.05$) ($(8.05 \pm 3.55) \times 10^7$ CFU/g as shown in Table 2. Then the numbers of *Vibrio* spp. in the supplemented with probiotics (T1, T2 and T3), decreased with time until the end of the experiment. At the end of the experiment, numbers of *Vibrio* spp. in the intestine of the tested animals in all treatments were $8.88 \pm 1.32 \times 10^4$, $3.33 \pm 0.58 \times 10^5$ and $1.62 \pm 0.41 \times 10^5$ CFU/g, respectively, which were significantly lowered than those in the controls $p<0.05$) which were $6.60 \pm 1.45 \times 10^7$ CFU/g.

Table 2 Numbers of *Vibrio* spp. in the intestine of white shrimp

Days	*Vibrio* sp. CFU/g)			
	C	T1	T2	T3
0	$8.05 \pm 3.55 \times 10^{7\ (b,1)}$	$7.67 \pm 2.32 \times 10^{7\ (a,1)}$	$6.61 \pm 2.78 \times 10^{7\ (a,1)}$	$7.31 \pm 2.00 \times 10^{7\ (a,1)}$
30	$4.59 \pm 1.83 \times 10^{7\ (c,1)}$	$4.48 \pm 0.47 \times 10^{6\ (b,2)}$	$7.59 \pm 1.84 \times 10^{6\ (b,2)}$	$5.24 \pm 1.83 \times 10^{6\ (b,2)}$
60	$7.71 \pm 1.55 \times 10^{7\ (b,1)}$	$4.33 \pm 1.21 \times 10^{6\ (b,2)}$	$9.32 \pm 0.75 \times 10^{5\ b,2)}$	$5.95 \pm 1.55 \times 10^{5\ (b,2)}$
90	$1.05 \pm 0.78 \times 10^{8\ (a,1)}$	$7.95 \pm 1.32 \times 10^{4\ (b,2)}$	$4.45 \pm 0.85 \times 10^{5\ (b,2)}$	$2.74 \pm 0.35 \times 10^{5\ (b,2)}$
120	$6.60 \pm 1.45 \times 10^{7\ (b,1)}$	$8.88 \pm 1.32 \times 10^{4\ (b,2)}$	$3.33 \pm 0.58 \times 10^{5\ (b,2)}$	$1.62 \pm 0.41 \times 10^{5\ (b,2)}$

[1,2] Different superscript numbers indicate significant difference (p<0.05) in the number of *Vibrio* spp. between a treatment and the control.

[a,b] Different superscript letters indicate significant difference (p<0.05) in the number of *Vibrio* spp. in different time.

3. Survival rate

Table 3 demonstrated the survival rate of tested shrimps in all treated treatments (T1, T2 and T3) which were 79.45%, 75.00% and 78.89%, respectively, with no significant difference (p>0.05) among those groups, but significantly higher p<0.05) than those in the control (66.67%).

Table 3 Survival rate of white shrimp

Treatment	Survival rate(SR; %)
C	66.67^{b}
T1	79.45^{a}
T2	75.00^{a}
T3	78.89^{a}

[a,b]Different superscript letters indicate significant difference (p<0.05) of survival rate in white shrimp between a treatment and the control.

Discussion

Numbers of marine bacteria in control, T1, T2 and T3 ranged from $7.57 \pm 1.05 \times 10^7$- $4.90 \pm 1.38 \times 10^8$, $3.45 \pm 0.56\times 10^8$ - $7.85 \pm 1.17 \times 10^8$, $1.12 \pm 0.12 \times 10^8$ - $5.12 \pm 2.14 \times 10^8$ and $9.21 \pm 1.24 \times 10^7$- $9.27 \pm 1.17\times 10^8$ CFU/g, respectively. These results were in accordance with Ruangpan et al. (1998) who reported that marine bacteria in the intestine of *P. monodon* cultured in intensive system were in range from 5.1×10^5 to 2.7×10^8 CFU/g. Moreover, the obtained result in present study found that the majority of bacteria in the intestine of white shrimp in T1 and T2 were *Bacillus* sp. while there was a minority of *Bacillus* sp. in the control and T3. Similarly, Rengpipat et al. (1998) and Boonthai et al. (2007) revealed that

Bacillus sp. in digestive tract of *P. monodon* significantly increased when shrimp were fed the diet supplemented with *Bacillus* sp. as probiotics.

Numbers of *Vibrio* spp. in the intestine of white shrimp of control were relatively constant along the course of experiment while numbers of *Vibrio* spp. in the supplemented with probiotics (T1, T2 and T3) decreased with time until the end of the experiment. Results suggested supplementation of probiotics into diet reducing numbers of vibrios which was in agreement with several reports (Boonthai et al., 2007; Nimrat et al., 2008). Rengpipat et al. (1998) informed that *Vibrio* spp. numbers in the intestine of juvenile *P. monodon* fed diet with *Bacillus* S11 for 100 days were higher than that of control group. In addition, inoculation of *Bacillus* sp. in the pellets for *P. monodon* postlarvae cultivation and found *Vibrio* spp. numbers in the intestine of *P. monodon* supplemented with probiotics significantly decreasing while *Vibrio* spp. in those of untreated group substantially increased (Boonthai et al., 2007). The reduction of vibrios numbers in the intestine of the present study may be associated with competition abilities of *Bacillus* for nutrients and spaces contributing to exclusion of other harmful bacteria. Additionally, *Bacillus* also secretes either many exoenzymes or metabolites that degrade slime layers and/or biofilms of pathogenic vibrios that lead to penetration of inhibitory substances such as polymyxin, bacitracin and gramicidin into the cells (Moriarty, 1997; Rhodehamel and Harmon, 1998). Cell wall of *Bacillus* and its metabolites also perform as immunogen for shrimp by stimulating phagocytic activity of granulocytes (Itami et al., 1998).

Several previous studies have demonstrated that yeasts can biosynthesize microcins, the biological substances possessing inhibitory effect on growth of pathogenic bacteria (Golubev & Boekhout, 1992). The cell wall of yeasts consisting of glucans, mannoprotein and chitin is able to enhance disease resistance through either stimulation of nonspecific components of immune defense or presentation of antigens during specific adaptive immune defense resulting to inducing antimicrobial activity in plasma and enhancement of phagocytosis activity, cell adhesion and superoxide production in hemocytes (Song and Hsieh, 1994; Scholz et al., 1999).

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