

EVALUATION FOR SURVIVAL ABILITY AND THE EFFECT ON GROWTH PERFORMANCE OF *LITOPENAEUS VANNAMEI* WITH TWO CANDIDATE PROBIOTIC GROUPS

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Introduction

The white shrimp (*Litopenaeus vannamei*) were introduced in Thailand since 1998. In that period, black tiger shrimp cultured in Thailand were suffered with outbreaks, slow growth as well as low yield production. As a result, many Thai agriculturists decided to initial culture of white shrimp (Limsuwan, 1999). Currently, white shrimp are popularly cultured in Thailand because they are faster growth, more disease resistance and providing higher yield production, compared to black tiger shrimp (Laokiatsopon, 2006). The production system for white shrimp cultivation in Thailand is intensive because it produces the highest yield. However, this system leads to accumulation of organic waste and inorganic waste in culture pond resulting to the disease outbreaks, environmental deterioration and a wide disparity in the sizes of white shrimp at harvest time (Lin, 1995). So, antimicrobial drugs, disinfectants and pharmaceutical agents were applied in aquaculture for solving those problems and growth promotion of white shrimp but the abuse of those have been associated with the development of antibiotic resistance of pathogenic bacteria and concern of consumer safety (Esiobu et al., 2002).

Nowadays, probiotics which are environment-friendly sustainable approach are used increasingly in white shrimp aquaculture for reducing undesirable problems, prevention of disease and growth encouragement (Gatesoupe, 1999; Wang, 2007). Probiotics are microbial supplements that advantageously affect the host animals (Fuller, 1989). There are many beneficial bacteria isolated from indigenous and exogenous microbiota of aquatic animals which are used as probiotics including Gram-negative facultative anaerobic bacteria and Gram-negative facultative anaerobic bacteria such as *Lactobacillus* sp., *Roseobacter* sp., *Carnobacterium* sp., *Pseudomonas* sp., especially *Bacillus* (Balcazar et al., 2006; Kesarcodi-watson et al., 2008). *Bacillus* spp. have possessed the ability of adhesion, production of

bacteriocins and providing immunostimulation. Moreover, they have maintained in the spore form that lead to be prolonged shelf life. (Barbosa et al., 2005; Hong et al., 2005). For yeasts, they were used as probiotics based on immunostimulation ability, production of inhibitory compounds and providing protein. Until recently, there is a few studies that investigate the effect of combination of *Bacillus* and Yeasts on growth characteristic of white shrimp. Therefore, the purpose of this study was to evaluate for survival ability and the effect on growth performance of *Litopenaeus vannamei* with two candidate probiotic groups

Methods

1. Preparation of probiotics

Bacteria and yeasts probiotics were provided by Dr. Nimrat, Burapha University as previously mentioned by Nimrat et al. (2008). Bacteria probiotics comprised five strains of *Bacillus* including BUU 001, BUU 002, BUU 003, BUU 004 and BUU 005. Two strains of yeasts (BUU 01 and BUU 02) were used in this product. Each strain of *Bacillus* was separately inoculated into a 500-mL flask containing 200 mL of Trypticase Soy Broth (TSB; Difco, Detroit, MI, USA). Then, flasks were vigorously shaken at 150 rpm, 30 °C for 24 h. In case of yeasts preparation, a loopful of each yeasts species was separately distributed into a 500-mL flask containing 200 mL of Yeast Peptone Dextrose broth and shaken at 200 rpm, 25 °C for 24 h. Cells of all bacteria and yeasts probiotics were harvested by centrifugation (8,000 rpm, 4 °C, 5 minutes) and washed three-time with phosphate buffer solution. Then, cell suspension was adjusted to 1.5 A.U. (approximately 10^{10} CFU/mL) using spectrophotometer at 580 nm. Adjusted cell suspension was freshly prepared for further production as freeze-dried form following to protocol of Khaopong et al. (2010).

2. Shrimp pellets preparation

Experimental diets were divided into 4 groups including: T1: commercial shrimp pellets with freeze-dried *Bacillus* probiotics; T2: commercial shrimp pellets with combination of freeze-dried *Bacillus* probiotics and freeze-dried yeasts probiotics; T3: commercial shrimp pellets with freeze-dried yeasts and the control (only commercial shrimp pellets). All diets were stored in sterilized amber glass bottles at 4 °C for no longer than 1 month prior to use.

3. Experimental design

Pond bottom soil collected from shrimp pond was supplied to a depth of 7 cm in fiber glass tanks (0.8×1.2×0.6 m) in order to simulate the earthen pond and marine water with 5 ppt was transferred into all tanks. Thirty of *L. vannamei* (35-day old) was located into the experimental tanks. Nine tanks were set up for a treated group and a control group in triplicate (N=3) and studied for 120 days.

4. Microbiological enumeration

Three shrimp samples were collected from each tank every 30-days for microbiological determination starting from the first day of the feeding trials. Then, shrimp were dissected using sterilized surgical scissors and intestines were removed for microbial enumeration and identification. Intestine samples were homogenized using sterile 0.85% (w/v) NaCl solution and serially diluted for viable plate counts. A hundred microliter of each dilution was pipetted on agar using spread plating. Total heterotrophic bacterial (THB) and *Bacillus* counts were determined using Plate Count Agar (PCA; Difco, Detroit, MI, USA) and inoculated plates were incubated at 30 °C for 24–48 h (Nimrat et al., 2008). Yeasts probiotics were enumerated using Yeast peptone dextrose agar (YPD agar), at 25 °C for 48 h. Then, all colonies were counted and express as CFU/g. The representative colonies were identified based on morphological and biochemical characteristics according to method of Krieg and Holt (1984)

5. Weight gain analysis

The weights of shrimp in each treatment and control were measured at the beginning and the end of experiment for calculation of Daily Weight Gain following:

$$\frac{\text{Final weight (g)} - \text{Initial Weight}}{120 \text{ days}} \times 100$$

6. Statistical analysis

Data were showed as mean \pm standard deviation. Analysis of variance (ANOVA) and Duncan's multiple Range Test were used to find any significant difference between various parameters of the treatment and control groups. A significance level of $p < 0.05$ was used.

Results

The numbers of *Bacillus* spp. as probiotic bacteria in the intestine of T1 and T2 at the beginning of experiment (day 0) were $6.61 \pm 0.39 \times 10^4$ and $6.69 \pm 0.40 \times 10^4$ CFU/g, respectively, which were not significant difference ($p > 0.05$) between these group (Table 1). However, They were significantly higher ($p < 0.05$) than those in T3 ($3.80 \pm 0.82 \times 10^3$) and the control ($3.20 \pm 0.19 \times 10^3$). Then, the number of *Bacillus* of T1 and T2 sharply increased at day 30 of the experiment ($1.50 \pm 0.14 \times 10^8$ and $1.40 \pm 0.46 \times 10^8$) while *Bacillus* number of the control and T3 were slightly increased and were significantly different ($p < 0.05$), compared T1 and T2. Afterwards, *Bacillus* number in T1 and T2 were slightly increased until the end of the experiment. At the end of the experiment, the number of *Bacillus* in T1 ($2.16 \pm 0.36 \times 10^8$ CFU/g) and T2 ($1.94 \pm 0.47 \times 10^8$ CFU/g) were significantly ($p < 0.05$) higher than those in T3 and the control.

Table 1 the number of *Bacillus* in the intestine of white shrimp

Day	<i>Bacillus</i> (CFU/g)			
	C	T1	T2	T3
0	3.20±0.19x10 ³ (1,a)	6.61±0.39x10 ⁴ (1,b)	6.69±0.40x10 ⁴ (1,b)	3.80±0.82x10 ³ (1,a)
30	3.27±0.21x10 ³ (1,a)	1.50±0.14x10 ⁸ (2,b)	1.40±0.46x10 ⁸ (2,b)	4.58±1.59x10 ³ (2,a)
60	5.93±1.02x10 ³ (3,a)	1.73±0.58x10 ⁸ (2,b)	1.42±0.71x10 ⁸ (2,b)	7.07 ± 1.71x10 ³ (2,a)
90	4.33±.42x10 ³ (2,a)	1.79±0.09x10 ⁸ (2,b)	1.79±0.09x10 ⁸ (2,b)	6.19 ± 1.89x10 ³ (1,a)
120	3.66±0.51x10 ³ (12,a)	2.16±0.36x10 ⁸ (3,b)	1.94±0.47x10 ⁸ (3,b)	4.07 ± 1.11x10 ³ (1.,a)

C = Control, T1 = Bacteria, T2 = Bacteria+Yeasts, T3 = Yeasts

^{a,b} Different superscript numbers indicate significant difference (p<0.05) in the number of *Bacillus* between a treatment and the control.

^{1, 2, 3} Different superscript letters indicate significant difference (p<0.05) in the number of *Bacillus* in different time.

Mean±SD

At the commencement of culture period, yeasts numbers in the intestine of T2 and T3 were 8.82±1.42x10³ and 8.62±1.50x10³ CFU/g, respectively (Table 3), which were not significantly difference (p>0.05) between these groups, but significantly higher (p<0.05) than those in T1 (<10 CFU/g) and the control (<10 CFU/g). The numbers of yeasts in T2 and T3 significantly increased when supplemented diets were provided for 60 days. At the end of the experiment, the number of yeasts in T2 and T3 were 4.85±1.01x10⁵ and 5.04±0.90x 10⁵ CFU/g, respectively, which were not significant difference (p>0.05) between these group, but significantly higher (p<0.05) than those in T1(<10 CFU/g) and the control (<10 CFU/g).

Table 2 The concentrations of yeasts in the intestine of white shrimp

Day	Yeasts (CFU/g)			
	C	T1	T2	T3
0	<10 (1,a)	<10 (1,a)	8.82±1.42x10 ³ (1,b)	8.62±1.50x10 ³ (1,b)
30	<10 (1,a)	<10 (1,a)	5.39±0.67x10 ⁴ (12,b)	5.63±0.60x10 ⁴ (1,b)
60	<10 (1,a)	<10 (1,a)	1.06±0.09x10 ⁵ (2,b)	1.26±0.13x10 ⁵ (2,c)
90	<10 (1,a)	<10 (1,a)	3.91±0.80x10 ⁵ (3,b)	3.65±0.72x10 ⁵ (3,b)
120	<10 (1,a)	<10 (1,a)	4.85±1.01 x10 ⁵ (4,b)	5.04±0.90x10 ⁵ (4,b)

C = Control, T1 = Bacteria, T2 = Bacteria+Yeasts, T3 = Yeasts

^{a,b} Different superscript numbers indicate significant difference (p<0.05) in the number of *Bacillus* between a treatment and the control.

^{1, 2, 3} Different superscript letters indicate significant difference (p<0.05) in the number of *Bacillus* in different time.

Mean±SD

Weight gain

Percentages of weight gain in T1, T2 T3 and the control were $99.35\pm 6.20\%$, $75.21\pm 5.69\%$, $85.59\pm 3.18\%$ and $55.73\pm 2.09\%$, respectively (Table 3). The values of weight gain in all treated groups were significantly higher ($p>0.05$) than those of the Control.

Table 3 Percentage of weight gain in treated and control treatments at a culture period of 120 days

Treatment	Weight gain (%)
C	55.73 ± 2.09^a
T1	99.35 ± 6.20^d
T2	75.21 ± 5.69^b
T3	85.59 ± 3.18^c

C = Control, T1 = Bacteria, T2 = Bacteria+Yeasts, T3 = Yeasts

^{a,b} Different superscript numbers indicate significant difference ($p<0.05$) in the number of *Bacillus* between a treatment and the control.

^{1, 2, 3} Different superscript letters indicate significant difference ($p<0.05$) in the number of *Bacillus* in different time.

Mean \pm SD

Discussion

The numbers of *Bacillus* in the intestine in T1 and T2 at a culture period of 120 days were significantly higher ($p<0.05$) than T3 and control. These results were according to Rengpipat et al. (1998) who found that *Bacillus* S11 numbers in black tiger shrimp (*Penaeus monodon*) intestine markedly increased when probiotics were added in black tiger shrimp diet for 100 days. In addition, *Bacillus* S11 numbers in guts of black tiger shrimp in probiotic-treated groups increased more than 50%, compared to those in the control (Rengpipat et al., 2000). Increase in *Bacillus* numbers in treated groups perhaps resulted from replacement of *Bacillus* in digestive tract. *Bacillus* are accepted as putative probiotics capable of competing for adhesion sites and nutrients through production of inhibitory substances, and active proliferating in digestive tract of aquatic animals (Keracodi-watson et al., 2008). Yeasts concentrations in shrimp intestine in T2 and T3 at a culture period of 120 days were significantly higher ($p<0.05$) than T1 and control. In recent years, there are many reports about the application of yeasts as probiotics in mariculture. Tovar-Ramirez et al. (2002, 2004) found yeasts concentrations in intestine of sea bass (*Dicentrarchus labrax*) larvae increasing when administration of *Debaryomyces hansenii* HF1 as probiotics into sea bass diets. It is indicated that yeasts can adhere and survive in the intestine of aquatic biota. Significantly enhancement of percentage of weight gain in treated groups in this study suggested that *Bacillus* and yeasts authorized as probiotics were appropriate for cultivation of

L. vannamei. This phenomena has been attributed from biosynthesis of extracellular enzymes responsible for digestion and assimilation processes in shrimp intestine, such as proteases, carbohydrases and lipases as well as providing necessary growth factors (Arellano and Olmos, 2002; Ochoa and Olmos, 2006). Generally, yeasts can secrete polyamines, the ubiquitously natural substances, that play a integral role in proliferation and differentiation of cell, advocate the growth and metabolism of aquatic animal (Peulen et al., 2002) as well as adhesion capacity to intestinal mucus that are in turn explain increase in number of yeasts in shrimp intestine.

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