Kasetsart J. (Nat. Sci.) 39 : 262 - 273 (2005)

Probiotic Properties of *Bacillus pumilus*, *Bacillus sphaericus* and *Bacillus subtilis* in Black Tiger Shrimp (*Penaeus monodon* Fabricius) Culture

Watchariya Purivirojkul[1], Monchan Maketon[1] and Nontawith Areechon[2]

ABSTRACT

Three species of the genus *Bacillus* namely *B. pumilus*,*B. sphaericus*, and *B. subtilis* were isolated from the intestine of *Penaeus monodon* and tested for possible potential as a probiotic in black tiger shrimp rearing. The competition, colonization and inhibition activities of *Bacillus* spp. on *Vibrio harveyi*, a known pathogen in black tiger shrimp aquaculture were conducted. Transmission electron microscope observations showed the size of *V. harveyi*'s cell colonized by *B. pumilus, B. sphaericus* and *B. subtilis* to be smaller compare with its normal cell. This morphological deviation was permanently changed in every generation of *V. harveyi*. In addition, three species of the *Bacillus* could be grown in a wild range of conditions, these include salinity between 0-8 % NaCl, pH from 4 to 11 and temperature ranging of 25-50 °C except *B. sphaericus* which was not amenable to grow at 50 °C. Therefore, *B. pumilus, B. sphaericus* and *B. subtilis* showed promising potential to be used as a probiotic in black tiger shrimp.

As for probiotic properties in black tiger shrimp, the results shown that number of bacteria in intestinal tracts of the shrimps were increased in all treatments from 216 to 803% of the control group, and the amount of *V. harveyi*, were found reduced from 87.53 to 99.76% of the control, as also confirmed by scanning electron microscope observations. *B. subtilis*, the mixture of *B. sphaericus* + *B. subtilis*, and *B. pumilus* + *B. sphaericus* + *B. subtilis* in culture media showed immunostimulatory features measured by total hemocytes, phenol oxidase, superoxide anion, clearance ability and bactericidal activity which increased by 20.02-23.10, 26.02-39.43, 53.45-66.04, 44.68-59.57 and 50.00%, respectively.

Key words: *Bacillus pumilus, Bacillus sphaericus, Bacillus subtilis,* probiotic, *Penaeus monodon*

INTRODUCTION

Black tiger shrimp (*Penaeus monodon* Fabricius) has been one of the important export products of Thailand for more than a decade. However, in 2002, Thai shrimp production fell about 40 percent from 2001 to approximately 160,000 tons due to several diseases outbreak at the beginning of the year. Farmers utilized a large quantities of antibiotics in trying to solve this problem. Some antibiotic such as chloramphenicol was found as a residue in shrimps exported from China, Viet Nam, Indonesia and Thailand. Recently, the European communities are focusing in antibiotic residues in shrimp products by strictly inspection for all shrimp products imported from Asian countries.

The use of probiotic bacteria and some

[1] Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[2] Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand.

Received date : 03/11/04 Accepted date : 01/03/05

immunostimulant substances such as glucan and peptidoglycan have become popular methods developed for fighting againt diseases since the past decade (Fuller, 1992). Many genus of bacteria were used as probiotic such as *Vibrio* (Gullian *et al*., 2004) *Bacillus* spp. (Moriarty, 1998; Rengpipat *et al*., 2000; Gullian *et al*., 2004), especially those bacteria isolated from the intestine of *Penaeus monodon* (Rengpipat *et al*., 2000). There are several mechanisms of probiotic, these include production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, enhancement of the immune response and improvement of water quality (Verschuere *et al*., 2000)

In our studies, we conducted both *in vitro* and *in vivo* experiments by focusing on competitive and inhibitive capabilities of bacteria in the genus *Bacillus* in term of probiotic against *Vibrio harveyi*. Moreover, we continued study these bacteria on their effectiveness as probiotic properties in *P. monodon* in terms of growth and immune response indicated by total hemocytes, phenoloxidase activity, superoxide anion, bactericidal activity and clearance ability. Changing of bacterial community in shrimp intestines when feed with *Bacillus* spp. were also investigated by counting the number of *Bacillus* spp. and *Vibrio* spp., scanning electron microscope (SEM) was used for confirmation.

MATERIALS AND METHODS

This research was performed in both *in vitro* and *in vivo* systems.

Isolation and identification of *Bacillus* spp.

Bacillus spp. were isolated from the intestine of *Penaeus monodon* harvested from shrimp farms in Chachoengsao province. Two hundred samples of shrimp were investigated. Intestine were dissolved in 5 ml of 1.5% NaCl per animal and heat shock on water bath at 80 °C for 20 min followed by cold shock with normal tap water. Then the solution was spreaded on plates using spread plate technique on Nutrient agar (NA) supplemented with 1.5% NaCl (w/v). These plates were incubated at 35 °C for 24 h. Isolates were purified by streaking on NA supplemented with 1.5% NaCl (w/v). Catalase test were used for identifying *Bacillus* species. Species identification were done by VITEK 32 *Bacillus* (Biomérieux).

1. Colonization and inhibition activities of three bacteria on *Vibrio harveyi in vitro*

V. harveyi was isolated from black tiger shrimp and streaked on TCBS (Thiosulfate Citrate Bile Sucrose) agar. *Bacillus* spp. were cultured on nutrient agar supplemented with 1.5% NaCl (w/v), and both were incubated at 35 °C for 24 h. Colonization activities tests were done on NA supplemented with 1.5% NaCl (w/v) by cross streak method. *V. harveyi* was streaked in the first line and then *Bacillus* spp. was streaked perpendicular to it. Each type of bacterium streaking was done in triplicate and they were incubated at room temperature for 24 h.

1.1 Morphological change of *V. harveyi* after colonization

V. harveyi was isolated from the colonization area, especially from the cross streaking point as well as from the control. All samples were cultured on TCBS agar and incubated at 35 °C for 24 h. Growth rates of the experimental *V. harveyi* were compared with the normal one. Single colony was used to determine morphological deviation by transmission electron microscope (TEM)

1.2 Investigation for the possibility of *V. harveyi* to return to its normal shape

All *V. harveyi* samples isolated from the cross streaking point were cultured on TCBS agar. These plates were incubated at 35 °C for 24 h and morphological change was determined. The samples were then subcultured every 24 h for three consecutive times. TEM was used to observe the change of shapes during the subculture times for investigation o the recovery.

1.3 *Bacillus* spp. growth in different conditions

Temperature, salinity and pH were tested for the effect on the growth of *Bacillus* spp. The bacteria were prepared on NA supplemented with 1.5% NaCl (w/v) and incubated at 35 °C for 24 hours. For temperature study, a single colony was selected from the plate and streaked on the NA supplemented with 1.5% NaCl (w/v) and incubated at 25 °C, 35 °C and 50 °C for 24 h. The single colony of *Bacillus* spp. from the plate was inoculated in NB supplemented with 1.5% NaCl (w/v). 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10% NaCl were added to the media for salinity study and the pH to 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 for pH study. Test materials were incubated at 35 °C for 24 hours.

2. Probiotic and immunology properties of three bacteria against *V. harveyi in vivo*

Penaeus monodon was obtained from shrimp farm in Chachoengsao Province, Thailand. Shrimp with a mean fresh weight of approximately 8-12 g per animal were used. They were acclimatised in an aerated aquarium system at 25 ppt and change water every week before testing.

2.1 Probiotic properties study

Three strains of *Bacillus*, they were *B. pumilus*, *B. sphaericus*, *B. subtilis*. Each bacterium was formulated at the concentration of 10^{11}-10^{12} cfu/g powder using clay as a filter. The experiment was designed as a CRD with 8 treatments and 3 replications each as shown in Table 1.

Each treatment was blended with the shrimp's feed, at the ratio of 5 g : 1 kg. feed and then fed at 3% of the body weight, three times daily. The growth rate were observed at 4 weeks after feeding. Scanning electron microscope (SEM) was used to confirm the present of *Bacillus* in shrimp intestine.

2.2 Immunology study

Preparation of hemolymph samples

0.5 ml of hemolymph from each sample was withdrawn from base of the third walking leg of the shrimp using a syrynge containing 1.5 ml anticoagulant (K-199 + 5% L-cysteine) at 4 weeks after feeding.

2.2.1 Total hemocytes

After collected hemolymph, hemocytes were counted using a hemocytometer and calculated as number of blood cells (total hemocytes per cubic millimeter)

2.2.2 Phenoloxidase activity assay

The method was modified from Supamattaya *et al*. (2000).After the blood was withdrawn, the hemocytes were washed three times with shrimp saline (1000 rpm. 4 °C 10 min). Hemocyte lysate (HLS) was prepared from hemocytes in a cacodylate buffer pH 7.4 by using

Table 1 Eight treatments of probiotic properties study.

Treatment	Species of *Bacillus* spp.
1	*B. pumilus*
2	*B. sphaericus*
3	*B. subtilis*
4	*B. pumilus* + *B. sphaericus* (1:1)
5	*B. pumilus* + *B. subtilis* (1:1)
6	*B. sphaericus* + *B. subtilis* (1:1)
7	*B. pumilus* + *B. sphaericus* + *B. subtilis* (1:1:1)
8	No *Bacillus* (control)

the sonicator at 30 amplitute for 5 second and the suspension was then centrifuged at 10,000 rpm., 4°C for 20 min. The supernatant was collected as HLS. Then 200 µl of trypsin 0.1% in cacodylate buffer was mixed to the 200 µl HLS followed by 200 µl of L-dihydroxyphenylalanine (L-DOPA) 4 mg/ml as the substrate. Enzyme activity was measured as the absorbance of dopachrome at 490 nm wavelength. Measurement of protein content in HLS was made by using the method of Lowry *et al.* (1951). The phenoloxidase activity was calculated as the increasing of optimum density (OD) per minute per mg of protein as :

1 unit of phenoloxidase = Δ OD_{490} / min/ mg protein

2.2.3 Superoxide anion (O_2^-)

The method was modified from Supamattaya *et al.* (2000). The O_2^- was detected by reduction of redox dye, nitroblue tetrazolium (NBT). By this method, hemocytes were washed 3 times in K-199 solution. Living cells were separated by using trypan blue solution and adjusted to 1×10^7 cell/ml suspension. 200 µl of the cell suspension of each sample was dropped into well of a 96 microwell sterile plate. The plate was left for 45 min at room temperature for incubation period. Unattached cells were washed out by K-199 solution. 100 µl of the reaction mixture (0.5 mg zymozan in 0.5 ml serum + 20 mg NBT in 1 ml DMSO + K-199) was added to each well and the reaction mixture incubated at 25 °C 60 min. NBT was reduced by O_2^- during incubation period into a water insoluable blue formozan. The reaction was inhibited by putting 70% methanol into the samples for 3 min and the samples were then allowed to air dry. 120 µl of 2M NaOH and 140 µl of dimethyl sulfoxide (DMSO) were added to each well in order to dissolve the formozan. The concentration of the prussian-blue-colored solution was measured at 620 nm, KOH/DMSO was used as a blank control. The amount of O_2^- was indicated by the increasing in absorbance at 620 nm of 0.001 from control.

2.2.4 Clearance ability

The method of study the clearance ability was modified from Martin *et al.* (1993). The bacterial pathogen *V. harveyi* was subcultured in TSA (Tryptic Soy Agar) with 1.5% NaCl and incubated at 35°C for 24 hours. A single colony of *V. harveyi* was solved in the 1.5% NaCl sterile water. The suspension with the OD of 0.13 (2.45×10^8 cfu/ml) measured by absorbance value at 640 nm was used to count for the number of bacteria after cultured on TCBS agar by spread plate technique. Then 0.1 ml of the bacterial suspension with the counted number was injected to each tested shrimp while the control was injected with saline water. Three hours after injections, 0.5 ml of the blood from each shrimp was withdrawn for counting the number of bacteria by spread plate technique and calculated for the difference.

2.2.5 Bactericidal activity

Serum was separated from hemolymph of each shrimp sample and diluted by 2.6% NaCl in the proportion of 1:2, 1:4, 1:8, 1:16 and 1:32 serum to NaCl. The 0.5 ml of each serum dilution and 0.5 ml of NaCl as the control were used in the study. 0.5 ml of bacterial suspension, *V. harveyi*, prepared from the method as in number 2.2.4 was put into each serum dilution including the control. The treatments were incubated at room temperature for 3 hours before counting the number of bacteria made by a spread plate technique. Recording of the results were made for the dilution that could decrease 50% *V. harveyi* compared to the control.

2.3 Study for the bacterial concentration in shrimps intestine

The average concentration of both probiotic bacteria and *V. harveyi* were determined after 4 weeks of feeding. Scanning Electron Microscope (SEM) was used for confirmation the results.

RESULTS

Isolation and identification of *Bacillus* spp.

Out of twenty isolates from shrimp

intestines, there were only three species belong to the genus *Bacillus* and were identified as *B. pumilus*, *B. sphaericus* and *B. subtilis*. The percentage of each species were shown in Table 2

1. Study on the colonization and inhibition activities of three bacteria on *Vibrio harveyi in vitro*

The results showed that only 12 hours after the tested, *B. pumilus* had inhibition effect against *V. harveyi* as shown in Figure 1.1. On the test plate, some clear zone area were existed and more colonization areas were observed after 24 hours as shown in Figure 1.2. On the other hand, the clear zone did not appear in the colonized plates of both *B. sphaericus* and *B. subtilis*.

1.1 Study on the morphological changed of *V. harveyi* after colonization

The cross streaking point between *V. harveyi* and each colonizing *Bacillus* spp. on the TCBS agar was isolated for *V. harveyi*. Results showed that *V. harveyi* colonized by *B. pumilus* had slower growth compare with the control but the others two *V. harveyi* colonized by *B. sphaericus* and *B. subtilis* had normal growth with slightly change as shown by TEM (Figure 2)

In addition, the cell of *V. harveyi* colonized by *B. pumilus*, was almost destroyed as shown in Figure 2.2, this might occurred from some metabolites produced from *B. pumilus*. The size of normal *V. harveyi* and those colonized by *B. pumilus*, *B. sphaericus* and *B. subtilis* were about 0.71 × 1.54 µm, 0.50 × 0.78 µm, 0.68 × 0.96 µm and 0.68 × 1.07 µm as shown in Figure 2.1, 2.2, 2.3 and 2.5 respectively.

1.2 Investigation for the possibility of *V. harveyi* to return to its normal shape

After three consecutive subcultures those deviated *V. harveyi* colonized from each bacterium on the TCBS agar at every 24 hours confirmed that their morphologies were permanently changed

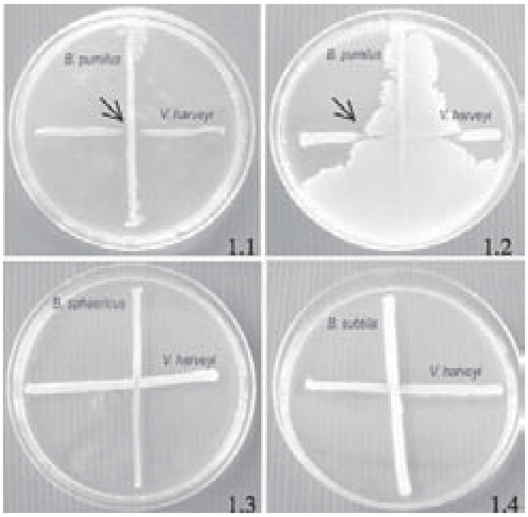

Figure 1 Colonization activities of *Bacillus* spp. 3 species on *V. harveyi in vitro*.
1.1 inhibition effect of *B. pumilus* against *Vibrio harveyi* after 12 hours.
1.2 inhibition effect of *B. pumilus* against *Vibrio harveyi* after 24 hours.
1.3 colonization activities of *B. sphaericus* against *Vibrio harveyi* after 24 hours.
1.4 colonization activities of *B. subtilis* against *Vibrio harveyi* after 24 hours.

Table 2 The result of isolate *Bacillus* spp. from shrimp intestine by VITEK 32.

Species of *Bacillus* spp.	Number of isolated	Percent
Bacillus pumilus	1	5
Bacillus sphaericus	2	10
Bacillus subtilis	3	15
Non Bacillus species	14	70
Total	20	100

compare with the control. Thus, it might not possible for the colonized *V. harveyi* to return to its regular size and shape again as shown in Figure 2.3, 2.5 and 2.7

1.3 *Bacillus* spp. growth in different conditions

Table 3 showed that *B. pumilus* and *B. subtilis* could grow at 25-50 °C but *B. sphaericus* could not grow at 50 °C. All three *Bacillus* species could grow in the salinity ranging between 0-10% and pH ranging from 4 to 11.

2. Study on the probiotic and immunology properties of three bacteria against *V. harveyi in vivo*

2.1 Probiotic properties study

Percent mean weight increasing of shrimps after 4 weeks fed by the mixture of *B. sphaericus* + *B. subtilis* (55.72 ± 24.43) is significantly higher ($P<0.05$) than the single formulation of *B. pumilus*, *B. sphaericus*, *B. subtilis* and the mixture of *B. pumilus* + *B. sphaericus*, the mixture of *B. pumilus* + *B. subtilis*, the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* and control which percent mean weight increasing were 9.99 ± 5.71, 19.44 ± 9.62, 24.17 ± 23.46, 19.94 ± 4.25, 13.63 ± 7.27, 18.77 ± 7.64 and 12.46 ± 3.22, respectively as shown in Figure 3.

2.2 Immunology study

2.2.1 Total hemocytes

Mean of total hemocytes from shrimp hemolymph after cultured with 8 feeds for 4 weeks

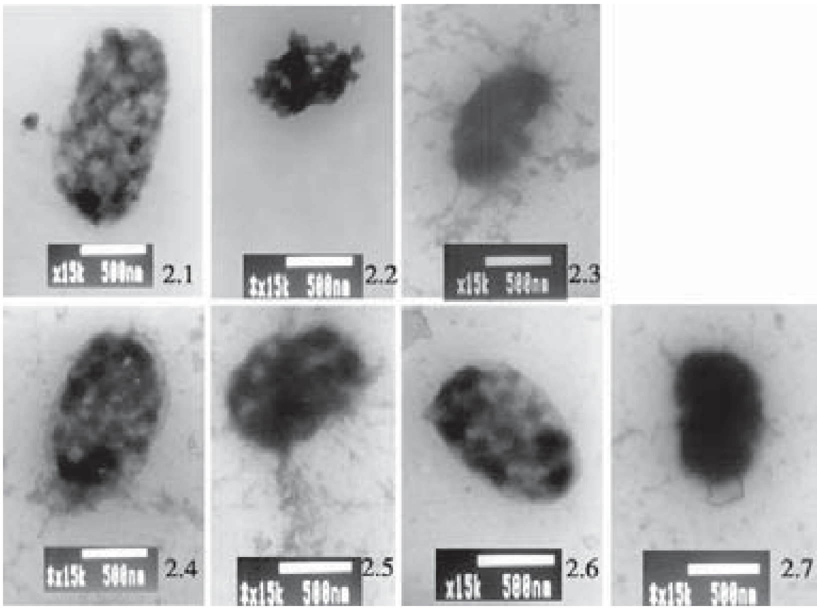

Figure 2 Morphological structure of normal *V. harveyi* compared with those deviated from colonization by TEM.

2.1 normal *V. harveyi*
2.2 *V. harveyi* colonized by *B. pumilus*
2.3 *V. harveyi* colonized by *B. pumilus* after three consecutive subcultures
2.4 *V. harveyi* colonized by *B. sphaericus*
2.5 *V. harveyi* colonized by *B. sphaericus* after three consecutive subcultures
2.6 *V. harveyi* colonized by *B. subtilis*
2.7 *V. harveyi* colonized by *B. subtilis* after three consecutive subcultures

showed that *B. subtilis* (11.03 ± 1.51 × 10^6), the mixture of *B. sphaericus* + *B. subtilis* (11.28 ± 1.88 ×10^6), and the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* (11.31 ± 1.46 × 10^6) were significantly higher (P<0.05) than those fed with *B. pumilus*, *B. sphaericus*, the mixture of *B. pumilus* + *B. sphaericus*, the mixture of *B. pumilus* + *B. subtilis* and control which total hemocytes were 9.78 ± 0.82, 10.02 ± 1.35, 10.70 ± 1.74, 10.41 ± 1.65 × 10^6 and 9.19 ± 0.81 × 10^6 cell/ml., respectively, as shown in Figure 4.

2.2.2 Phenoloxidase

Mean of phenoloxidase from shrimp hemocytes after fed with *B. subtilis* (297.04 ± 20.69), the mixture of *B. pumilus* + *B. subtilis* (307.56 ± 15.31) and the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* (310.58 ± 29.58) for 4 weeks were significantly higher (P<0.05) than fed with *B. pumilus*, *B. sphaericus*, the mixture of *B. pumilus* + *B. sphaericus*, the mixture of *B. sphaericus* + *B. subtilis* and control which phenoloxidase were 278.53 ± 56.47, 253.79 ±

Table 3 Growth of *Bacillus* spp. in NB at 25, 35 and 50 °C; NaCl 0-10% and pH ranging from 3-12.

	B. pumilus	*B. sphaericus*	*B. subtilis*
Temperature			
25 °C	+	+	+
35 °C	+	+	+
50 °C	+	-	+
Salinity			
0% NaCl	+	+	+
1% NaCl	+	+	+
2% NaCl	+	+	+
3% NaCl	+	+	+
4% NaCl	+	+	+
5% NaCl	+	+	+
6% NaCl	+	+	+
7% NaCl	+	+	+
8% NaCl	+	+	+
9% NaCl	+	-	+
10% NaCl	+	-	+
pH			
3	-	-	-
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	-	-	-

28.92, 234.52 ± 26.77, 280.70 ± 30.64 and 222.75 ± 15.34, respectively, as shown in Figure 5.

2.2.3 Superoxide anion

Mean of superoxide anion from shrimp hemocytes after fed with *B. subtilis* (8.13 ± 2.16), the mixture of *B. pumilus* + *B. sphaericus* (8.33 ± 1.66), the mixture of *B. sphaericus* + *B. subtilis* (8.40 ± 1.41) and the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* (8.80 ± 1.31) for 4 weeks were significantly higher ($P<0.05$) from *B. pumilus*, *B. sphaericus*, the mixture of *B. pumilus* + *B. subtilis* and control which superoxide anion were 5.63 ± 1.22, 4.60 ± 1.35, 7.63 ± 0.91 and 5.30 ± 0.92, respectively, as shown in Figure 6.

2.2.4 Clearance ability

Clearance ability of shrimp blood circulation system after fed with 8 feeds for 4 weeks and then injected with *V. harveyi* found that shrimp fed with *B. subtilis* (346.67 ± 70.95), the mixture of *B. sphaericus* + *B. subtilis* (253.33 ± 122.20) and the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* (320.00 ± 98.49) could reduce *V. harveyi* in hemolymph significantly different ($P<0.05$) from shrimp fed with *B. pumilus*, *B. sphaericus*, the mixture of *B. pumilus* + *B. sphaericus*, the mixture of *B. pumilus* + *B. subtilis* and control which amount of *V. harveyi* cells were 456.67 ± 85.05, 660.00 ± 105.36, 483.33 ± 201.08, 420.00 ± 40.00 and 626.67 ± 192.96 cfu/ml, respectively, as shown in Figure 7.

2.2.5 Bactericidal activity

Bactericidal activity from shrimp serum at 4 weeks after fed with *B. sphaericus*, the mixture of *B. pumilus* + *B. subtilis* , the mixture of *B. sphaericus* + *B. subtilis* and the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* (8.80 ± 1.31) at the dilution ratio of 1:8 serum to brine could killed 50 percent of *V. harveyi*. While, shrimp fed

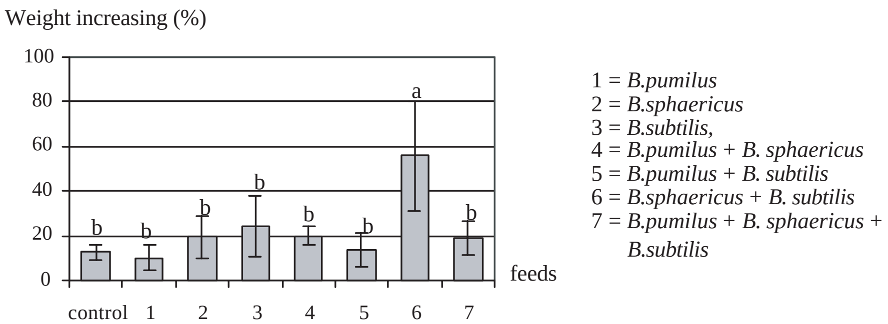

Figure 3 Percent weight increase of the *P. monodon* after 4 weeks cultured with 8 feeds.

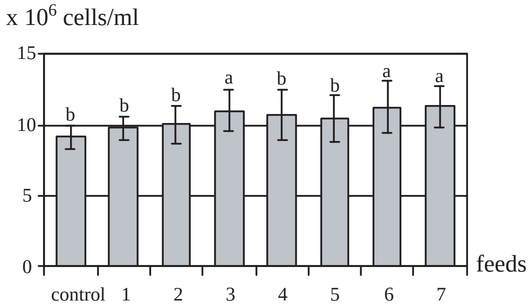

Figure 4 Average of the *P. monodon* hemocytes after 4 weeks cultured with 8 feeds.

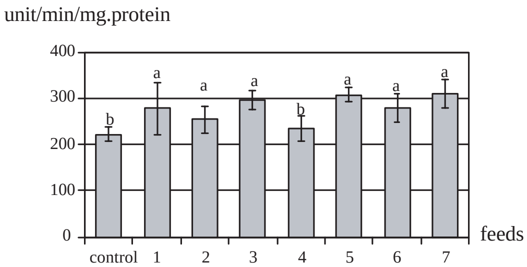

Figure 5 Average of the *P. monodon* phenoloxidase after 4 weeks cultured with 8 feeds.

with *B. pumilus*, *B. subtilis*, the mixture of *B. pumilus* + *B. sphaericus* and control killed 50 percent of *V. harveyi* at the dilution ratio of 1:4, as shown in Figure 8.

2.3 Study for the bacterial concentration in shrimps intestine

2.3.1 *Bacillus* spp.

The number of *Bacillus* spp. in shrimp intestine after fed with *B. pumilus* (803.33 ± 395.01), *B. sphaericus* (536.67 ± 310.86), *B. subtilis* (359.00 ± 270.63), the mixture of *B. pumilus* + *B. sphaericus* (373.33 ± 156.31), the mixture of *B. pumilus* + *B. subtilis* (216.33 ± 94.87), the mixture of *B. sphaericus* + *B. subtilis* (493.33 ± 187.17) and the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* (476.00 ± 234.03) for 4 weeks were significantly different ($P<0.05$) from control which number of *Bacillus* spp. average was $1.00 \pm 1.73 \times 10^4$ cfu/g, as shown in Figure 9.

2.3.2 *Vibrio* spp.

The number of *Vibrio* spp. in shrimp intestine at 4 weeks after fed with normal feed (control) (970.00 ± 285.13 cfu/g) was significantly different ($P<0.05$) from shrimp fed with *B. pumilus*,

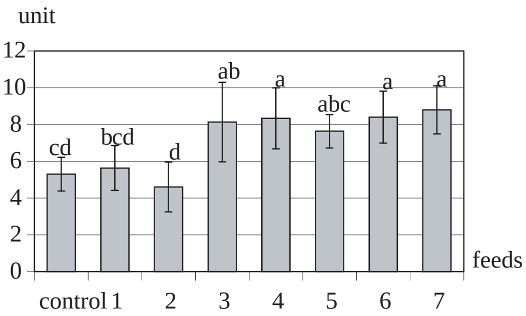

Figure 6 Average of the *P. monodon* superoxide anion after 4 weeks cultured with 8 feeds.

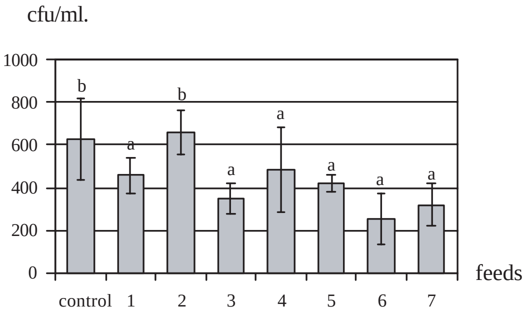

Figure 7 Cells of *V. harveyi* from *P. monodon* hemolymph after infected with *V. harveyi* 3 hours after 4 weeks cultured with 8 feeds.

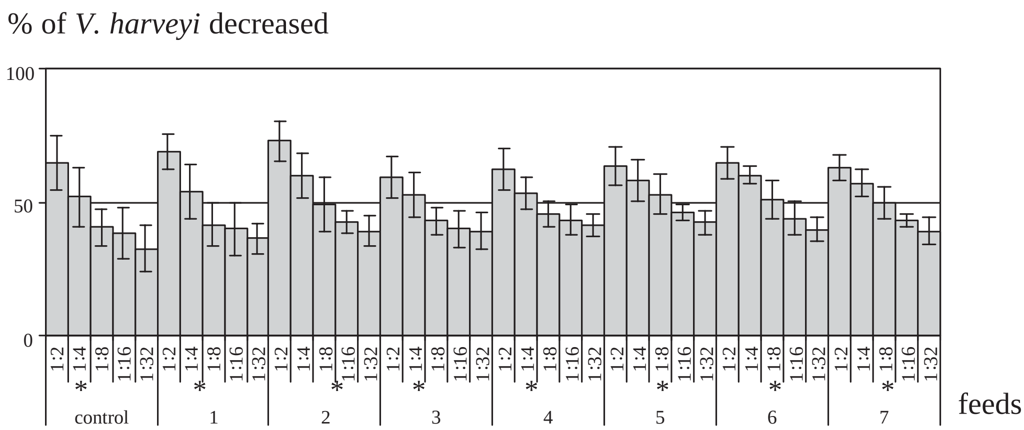

Figure 8 Value of diluted serum to kill *V. harveyi* 50% (*) of *P. monodon* after 4 weeks of culture when provided with 8 feeds (1 = *B. pumilus*, 2 = *B. sphaericus*, 3 = *B. subtilis*, 4 = *B. pumilus* + *B. sphaericus*, 5 = *B. pumilus* + *B. subtilis*, 6 = *B. sphaericus* + *B. subtilis*, 7 = *B. pumilus* + *B. sphaericus* + *B. subtilis*).

B. sphaericus, *B. subtilis*, the mixture of *B. pumilus* + *B. sphaericus*, the mixture of *B. pumilus* + *B. subtilis*, the mixture of *B. sphaericus* + *B. subtilis* and the mixture of *B. pumilus* + *B. sphaericus* + *B. subtilis* which the number of *Vibrio* spp. in shrimp intestine were 11.33 ± 1.16, 121.67 ± 11.50, 8.00 ± 1.00, 26.00 ± 3.00, 32.00 ± 3.61, 10.00 ± 3.00, 2.33 ± 1.53 cfu/g, respectively, as shown in Figure 10.

2.3.3 Scanning electron microscope

SEM revealed that *Bacillus* spp. could survived in shrimp intestine after treated with either individual isolation or mixtures of two and three of *B. pumilus*, *B. sphaericus* and *B. subtilis* as shown in Figure 11.1. While, in control group, only *Vibrio* spp. was observed in shrimp intestine as shown in Figure 11.2.

DISCUSSION

Isolation of *Bacillus* spp. from shrimp intestine found three species namely *B. pumilus*, *B. sphaericus* and *B. subtilis*. Original of those bacteria might came from water, soil, food or normal flora in the intestine. Bonde (1981) reported that in seawater dominated by *B. licheniformis* followed by *B. subtilis* and *B. pumilus*. Other species encountered in low numbers include *B.*

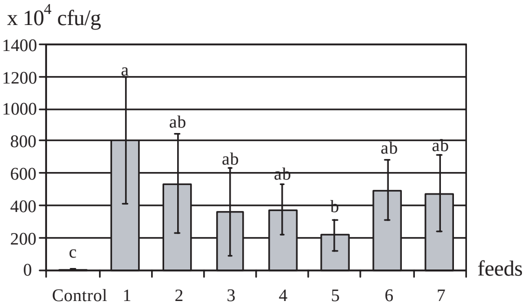

Figure 9 The number of *Bacillus* spp. in *P. monodon* intestine after 4 weeks of culture when provided with 8 feeds.

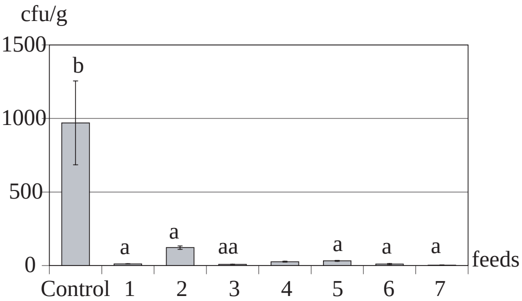

Figure 10 The number of *Vibrio* spp. in *P. monodon* intestine after 4 weeks of culture when provided with 8 feeds.

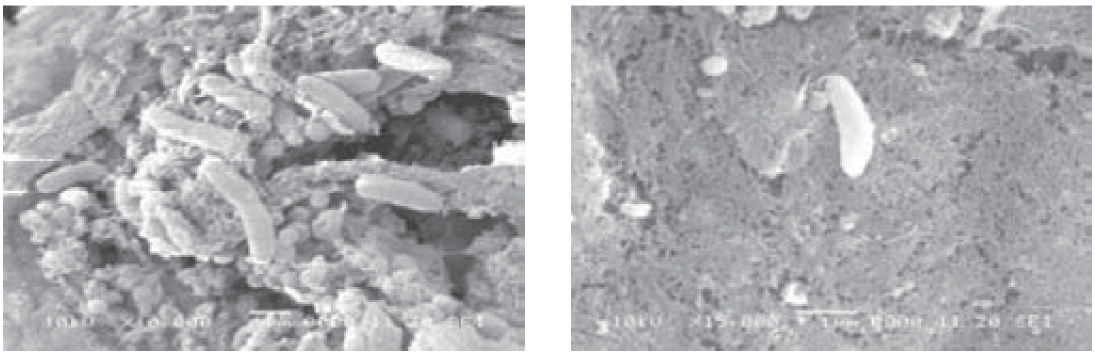

Figure 11 Scanning Electron Microscope picture.
11.1 Scanning Electron Microscope picture of *Bacillus* spp. in shrimp intestine after fed with mixture of *B. pumilus*, *B. sphaericus* and *B. subtilis* (× 10,000)
11.2 Scanning Electron Microscope picture of *Vibrio* spp. in shrimp intestine (control group) (× 15,000)

brevis, *B. firmus* and *B. sphaericus*, largely in nonpolluted areas. In a numerical study of North Sea sediments, Boeyé and Herts (1976) found that *B. subtilis*, *B. licheniformis* and *B. firmus* strains predominated. So, it was possible for *B. pumilus*, *B. sphaericus* and *B. subtilis* to contaminate in intestinal of shrimp by sea water.

In vitro production of inhibitory compounds toward known pathogens for the considered species has often been used in the selection of putative probiotic strains (Verschuere *et al*., 2000). In this study we demonstrated that the isolated *Bacillus* spp. from intestinal of black tiger shrimp are potential competitors for *V. harveyi*, the results showed colonization effect of each *Bacillus* spp. to *V. harveyi* in 24 hours. Special results we received from this experiment, the effective of *B. pumilus* could produce some substance and effective to destroy *V. harveyi* confirm this result by clear zone area in the test plates. The inhibition of *B. pumilus* to *V. harveyi* observed clear zone of *V. harveyi* colony in 12 hours and eradicate all of *V. harveyi* colony in 48-72 hours. Although *B. sphaericus* and *B. subtilis* did not show colonization effect but they showed some inhibition effect to *V. harveyi*, which confirmed by distorted shape of *V. harveyi* by TEM the shape of *V. harveyi* had smaller size and some area of cell wall was destroyed. The abnormal shape of *V. harveyi* were permanently changes confirmed with three consecutive subcultures. *Bacillus* spp. might produced some metabolites for instance antibiotic (Williams and Vickers, 1986) or enzymes for inhibition and/or digestion (Bruno and Montville, 1993)

Regarding to the growth of *Bacillus* spp., this experiment might confirmed that *Bacillus* spp. could grow in a wide range of environment, such as salinity up to 8% NaCl, pH 4-11 and temperature up to 50 °C, althought *B. sphaericus* could not grow at 50 °C but in the real culture, water temperature never raise so high as 50 °C.

Using *B. subtilis* mixed with *B. sphaericus* could increase weight of shrimp significantly difference ($P<0.05$) from control and other experiment groups, while others treatments had no significant different from the control ($P>0.05$).

About immunoenhancement ability of *Bacillus* spp., *Bacillus* had peptidoglycan in its cell wall which could increase immune of shrimp (Boonyaratpalin *et al*., 2000). In this research, *B. subtilis* had highest efficiency to improve immune parameters of black tiger shrimp consists of total hemocyte, phenol oxidase, superoxide anion and clearance ability. Others treatments containing *B. subtilis* in the mixtures also increased immune parameters significantly difference from the control as well. These results were similar to Rengpipat *et al*. (2000). But in our research we measured superoxide anion instead of phagocytic activity because superoxide anion produced in phagocytosis process (Bell and Smith, 1993).

Furthermore, after fed shrimp with *Bacillus* spp. for 4 weeks revealed that *Bacillus* spp. showed superiority in competition and colonization to *Vibrio* spp. in shrimp intestine.

CONCLUSIONS

In summary, it had been demonstrated that *B. pumilus*, *B. sphaericus* and *B. subtilis* had good properties to be used as probiotic for black tiger shrimp. *B. pumilus* showed colonization activity and produce inhibitory compounds while *B. sphaericus* and *B. subtilis* showed only inhibitory effects to *V. harveyi*. TEM studied found that cell morphology of *V. harveyi* colonized by *B. pumilus*, *B. sphaericus* and *B. subtilis* changed to smaller sizes compared with normal cell. Moreover, three species of *Bacillus* spp. could grow in a different environment, including salinity 0-8% NaCl, pH 4-11 and temperature 25-50 °C except *B. sphaericus* which could not grow at 50 °C. When fed shrimp with these *Bacillus* spp. showed immunoenhancement capability and also colonization and inhibition on *V. harveyi* in shrimp intestine. Therefore, these *Bacillus* spp. might be applied as good probiotic in shrimp aquaculture.

ACKNOWLEDGEMENTS

This research was supported by Kasetsart University Research and Development Institute (KURDI). We thank Professor Dr. Toshiaki Itami, Miyazaki University for immune techniques. Assoc. Prof. Kidchakarn Supamattaya, Prince of Songkla University, Thailand for chemical formula. Ms.Kanokphan Srimanopach, Department of Fisheries for VITEK 32 instrument, Ms. Patcharee Umrung, Central lab KU for TEM and Ms. Yuppade Paowpan, Central lab KU for SEM.

LITERATURE CITED

Bell, K. L. and V. J. Smith. 1993. *In vitro* superoxide production by hyaline cells of the shore crab *Carcinus maenas* **Dev. Comp. Immunol.** 17: 211-219.

Boeyé, A. and M. Herts. 1976. Numerical taxonomy of Bacillus isolates from North Sea sediments. **Int. J. Syst. Bacteriol.** 26:427-441.

Bonde, G. J. 1981. *Bacillus* from marine habitats : Allocation to phena established by numerical Techniques, pp. 181-215. *In* R. C. W. Berkeley and M. Goodfellow (eds.). **The Aerobic Endospore-Forming Bacteria : Classification and Identification**, Academic Press, London.

Boonyaratpalin, M., K. Supamattaya, J. Pongmaneerat, S. Boonyaratpalin and Y. Toride. 2000. The stimulatory effect of high and low molecule peptidoglycan (PG) on immune responses in black tiger shrimp, *Penaeus monodon* Fabricius. **Songklanakarin J. Sci. Technol**. 22 (Suppl.) : 689-696.

Bruno, M. E. C. and T. J. Montville. 1993. Common mechanistic action of bacteriocins from lactic acidbacteria. **Appl. Environ. Microbiol.** 59:3003– 3010.

Fuller R. 1992. **Probiotics.** The Scientific Basis. Chapman and Hall, London. 398 p.

Gullian, M., F. Thompson and J. Rodriguez. 2004. Selection of probiotic bacteria and study on their immunostimulatory effect in *Penaeus vannamei*. **Aquaculture** 233: 1-14.

Lowry, O.H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. **J. Biol. Chem.** 193: 265-275.

Martin, G. G., D. Poole, C. Poole, J. E. Hose, M. Arias, L. Reynolds, N. Mckrell and A. Whang. 1993. Clearance of bacteria injected into the hemolymph of the Penaeid shrimp, *Sicyonia ingentis*. **J. Inver. Pathol.** 62: 308-315.

Moriarty, D.J.W. 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. **Aquaculture** 164: 351-358.

Rengpipat, S., S. Rukpratanporn, S. Piyatirativarakul and P. Menasveta. 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). **Aquaculture** 191: 271-288.

Supamattaya, K., J. Pongmaneerat and T. Klowklieng. 2000. The effect of β–glucan (MacroGard®) on growth performance, immune response and disease resistance in black tiger shrimp, *Penaeus monodon* Fabricius. **Songklanakarin J. Sci. Technol.** 22 : 677-688.

Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete. 2000. Probiotic Bacteria as Biological Control Agents in Aquaculture. **Microbiol. Mol. Biol. R.** 64: 655–671.

Williams, S. T., and J. C. Vickers. 1986. The ecology of antibiotic production. **Microb. Ecol.** 12:43–52.