A Study on the Effect of *Bacillus* spp. to Control the Pathogenic Bacteria in Aquaculture

Sanghamitra Nayak*, Chalor Limsuwan, Niti Chuchird and Supranee Pungpang

ABSTRACT

The present study was aimed to select potential probionts from five different strains of *Bacillus* spp. The test pathogens included *Vibrio* spp. of *V.vulnificus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. mimicus* and *V. cholera* (non 01), which were isolated from the white feces disease infected pacific white shrimp (*Litopenaeus vannamei*), and *V. harveyi* AQVH (Aquaculture *V.harveyi*) which was isolated from the diseased post larvae of pacific white shrimp. *V.vulnificus* and *V. fluvialis* included two strains of yellow and green respectively. *Aeromonas hydrophila* AQAH (Aquaculture *A.hydrophila*) was isolated from diseased Nile Tilapia (*Oreochromis niloticus*). The probiont strains were *Bacillus subtilis* B1, *B. pumilus* B2, *B. subtilis* B3, *B. subtilis* B4, and *B. subtilis* B5. The test of antagonism was carried out by cross streak and agar well diffusion assay (AWDA). Antagonistic activities were observed in B1, B2 and B5 against *A. hydrophila* by B1 and against all the *Vibrio* species by the other two strains. B1 and B5 were found to antagonize by inhibition to the pathogens while B2 colonized on the pathogens in cross streak method. In AWDA, these three probionts produced an antimicrobial substance evidenced by the presence of a clear zone after respective hours of incubation with the pathogens. B2 and B5 were found to retain antimicrobial activity up to seven days while B1 up to five days. There was no antagonism found in B3 and B4 against the selected pathogens. Based on the results of cross streak and AWDA, the probionts B1, B2 and B5 were subjected to co-culture experiments where all the *Bacillus* species were cultured individually with the target pathogen for 120 hours and compared with the monoculture of each strain to determine the potentiality of competitive exclusion of the test probionts for the target pathogens. It was observed that B1 was able to reduce the growth of *A. hydrophila* by about 61.81%, while B2 and B5 inhibited the growth of *Vibrio* spp. by more than 90 and 85%, respectively, at the end of 120 hours of co-culture. These results suggested that B1, B2 and B5 could be recommended to be used as effective probiotic for aquaculture practices.

Keywords: *Bacillus*, Probiotic, Aquaculture pathogen, competitive exclusion

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INTRODUCTION

Aquaculture is a fast growing and rapidly expanding multi-billion-dollar industry. It plays a major role in the economy of many Asian countries. Hence large-scale production facilities are being introduced to meet target for being profitable and a high economic turnover. This has led to stressful conditions in aquatic animal production, which include the threat of diseases and the deterioration of environment, which contribute to huge economic losses when they occur. Prevention and control of diseases have led during recent decades to a substantial increase in the use of antibiotics. However, the efficiency of antibiotics as a preventive measure has been questioned, given extensive documentation of the evolution of antibiotic resistance among pathogenic bacteria. The massive use of antibiotics encourages natural emergence of antibiotic resistant bacteria, which can transfer their resistant genes to other bacteria that have never been exposed to antibiotics (Karunasagar *et al*., 1994; Austin *et al*., 1995; Moriarty, 1997). This led to the suggestions of suitable alternatives to disease prevention methods, which could be the use of non-pathogenic bacteria such as probiotic and bio-control agents (Austin *et al*., 1995; Moriarty, 1997; Gatesoupe, 1999; Mishra *et al*., 2001). One of the commonly studied probiotics include the spore forming *Bacillus* spp. *Bacillus* spp. have been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Cherif *et al*., 2001; Cladera-Olivera *et al*., 2004; Duc *et al*., 2004; Barbosa *et al*., 2005). *Bacillus* spp. hold added interest in probiotics as they can be kept in the spore form and therefore stored indefinitely on the shelf (Hong *et al*., 2005). *In vitro* production of inhibitory compounds toward known pathogens for the considered species has often been used in the selection of putative probiotics (Verschuere *et al*., 2000).

Hence, the present study is going to analyze the potentiality of some *Bacillus* species. The strains were obtained from Novozymes Biologicals, Inc., for experimental purposes only on the pathogenic bacteria isolated from white feces infected pacific white shrimps (*Litopenaeus vannamei*) and diseased Nile Tilapia (*Oreochromis niloticus*) by focusing on the competitive and inhibitive potentiality against selected pathogenic bacteria in aquaculture.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Probionts

Five strains of *Bacillus* spp. including four different strains of *B.subtilis* and one strain of *B.pumilus* were taken (*B. subtilis* B1, *B. pumilus* B2, *B. subtilis* B3, *B. subtilis* B4 and *B. subtilis* B5) (Table 1).

Table 1. Results of cross streak and AWDA with *Bacillus* spp. against pathogenic bacteria

Indicator Strains	B1		B2		B3		B4		B5	
	Cross Streak	AWD	Cross Streak	AWD	Cross Streak	AWD	Cross Streak	AWD	Cross Streak	AWD
V. parahaemolyticus	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. alginolyticus	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. harveyii	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. vulnificus (G)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. vulnificus (Y)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. fluvialis (Y)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. fluvialis (G)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. cholarae (non 01)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. mimicus	--	--	Col	CZ	--	--	--	--	Inh	CZ
A. hydrophila.	Inh	CZ	--	--	--	--	--	--	--	--

CZ : Clear Zone Col: Colonization Inh: Inhibition

AWD : Agar well diffusion plate assay.

Pathogenic Bacteria

Seven species of *Vibrio* spp. including *V. vulnificus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. mimicus*, and *V. cholera* (*non 01*) were isolated from the white feces disease infected pacific white shrimp), while *V. harveyi* AQVH was isolated from the diseased post larvae of pacific white shrimp. *V. vulnificus* and *V. fluvialis* included two strains of yellow and green respectively. *Aeromonas hydrophila* AQAH was isolated from diseased Nile Tilapia (*O.niloticus*) obtained from the Aquaculture Business Research Laboratory,Faculty of Fisheries, Kasetsart University.

Antagonism assay by Cross Streak Method

Bacillus spp. and *A. hydrophila* AQAH were cultured on Tryptic Soy Agar (TSA) and incubated at 30°C for 24 hours. *Vibrio* spp. were cultured on TSA supplemented with 1.5% NaCl (w/v). Antagonism tests were conducted on TSA (*A. hydrophila* AQAH) and TSA supplemented with 1.5% NaCl (w/v) (*Vibrio* spp. and *V. harveyi* AQVH) by cross streak method (Lemos *et al*., 1985). Pathogenic bacteria were 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 30°C for 120 hours. Inhibition activities and colonization effect were observed at 24, 48, 72, 96 and 120 hours.

Preparation of Cell-Free Neutralized Supernatant

To prepare the cell-free neutralized supernatant (CFNS), the overnight culture of antimicrobial producer strains of all the *Bacillus* spp. was inoculated (1%) into 10 ml TSB and incubated at 30°C for 10 days at 150 rpm. After every 24 hours, 5 ml of the culture was drawn from each flask and subjected to centrifugation at 10000 x g for 10 min at 4°C to pellet the cells. The supernatant was collected, pH was adjusted to 7.0 with 1mol/L of NaOH and filtered through a 0.22µm (Millipore Ireland B.V.) membrane filter and used for the antagonistic activity assay by means of agar well diffusion assay. The prepared CFNS was used immediately.

Antagonism assay by Agar Well Diffusion Plate Assay

The agar well diffusion plate assay (AWDA) method was carried out (Bauer *et al*. 1966 with modification) to determine antagonism characteristics. The indicator bacteria *Vibrio* spp. were cultured on TSB supplemented with 1.5% NaCl, while *A. hydrophila* was cultured on TSB. Suspension of each indicator bacteria strain (100 µl) containing 10^7 CFU ml^{-1} was spread on the plates made up of Tryptic Soy Agar and supplemented with 1.5% NaCl for *Vibrio* spp., and TSA for *A. hydrophila*. Wells of 5 mm in diameter were punched in the agar with a sterile tip and filled with 50 µl of CFNS. Plates were pre-incubated for 2 hours at 4°C to allow the diffusion of any inhibitory metabolites into surrounding agar, and then incubated at the optimum growth temperature (30°C) of the indicator microorganism. The plates were examined after 24 hours for the presence of a clear zone in the agar surrounding the well, which was considered a positive result. The measurements were recorded in triplicates. The diameter of the inhibition zone (clear zone) was measured in millimeters.

Co-culture of *Bacillus* spp. with pathogenic bacteria in Tryptic Soy Broth

Bacillus spp. Were tested for antagonistic activity against pathogenic bacteria in a co-culture experiment. *Bacillus* and pathogenic bacteria were separately pre-cultured in 10 ml TSB for 24 hours with shaking at 150 rpm. Tryptic Soy Broth was inoculated with 10^5 CFUml^{-1} pathogenic bacteria together with 10^5 CFU ml^{-1} of *Bacillus* spp. B1 was co-cultured with *A. hydrophila* AQAH in TSB. *Bacillus* spp. B2 and B5 were co-cultured with *V. harveyi* AQVH and all other strains of *Vibrio* spp. in TSB supplemented with 1.5% NaCl in the similar ratio of pathogen and probionts as in *A. hydrophila*. Each *Bacillus* and pathogenic bacteria had a control group (monoculture) to compare bacterial concentration. Flasks were incubated at room temperature. All combinations were tested in triplicates. Samples were collected after 0, 24, 48, 72, 96 and 120 hours for enumeration of the number of bacteria.

RESULTS

Antagonism assay by Cross Streak Method

After incubating for 48 hours and 96 hours, *Bacillus* B2 and *Bacillus* B5, respectively, showed colonization (where the *Bacillus* grew on the line of pathogenic bacteria) and inhibition effect (where there was no growth of pathogenic bacteria near the *Bacillus* line) against all the *Vibrio* spp. (Figure 1 and Table 1). However, these strains did not show any effect against *A. hydrophila* AQAH. *Bacillus* B1 showed inhibition effect against *A. hydrophila* AQAH after 72 hours of incubation (Figure 1 and Table 1). There was no effect by *Bacillus* spp. B3 and B4 against any of the target pathogenic strain (Table 1).

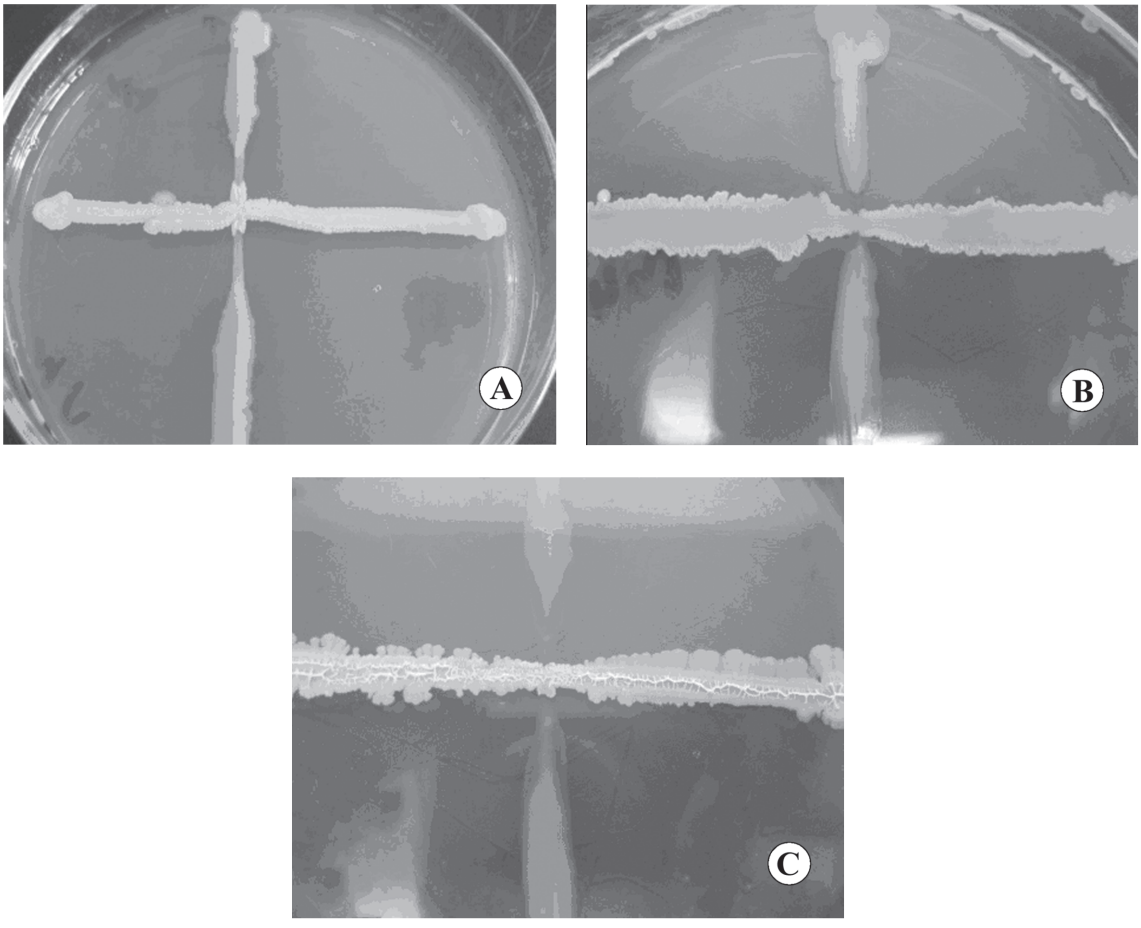

Figure 1. Effect of colonization (48 hrs) by B2 on *V. harveyi* AQVH (A); Effect of inhibition (96 hrs) by B5 on *V.harveyi* AQVH (B); and, Effect of inhibition (96 hrs) by B1 on *A. hydrophila* AQVH (C).

Antagonism assay by Agar Well Diffusion Plate Assay

The results showed that *Bacillus* B2 could produce a higher concentration of antibacterial substances than *Bacillus* B5 and B1. Among these, *Bacilli* B2 and B5 could produce antibacterial substances at the highest level on day 5, while B1could produce the maximum level at 72 hours (Figure 2) as measured by the size of the clear zones (Figure 3) . AWDA. However *Bacillus* B2 and *Bacillus* B5 showed the antagonism activity only against *Vibrio* spp., and this activity continued up to seven days, while B1 showed the antagonism only against *A. hydrophila* AQAH with the activity remaining up to five days. *Bacillus* spp. B3 and B4 showed no antagonism against any of the pathogenic strain (Table 1).

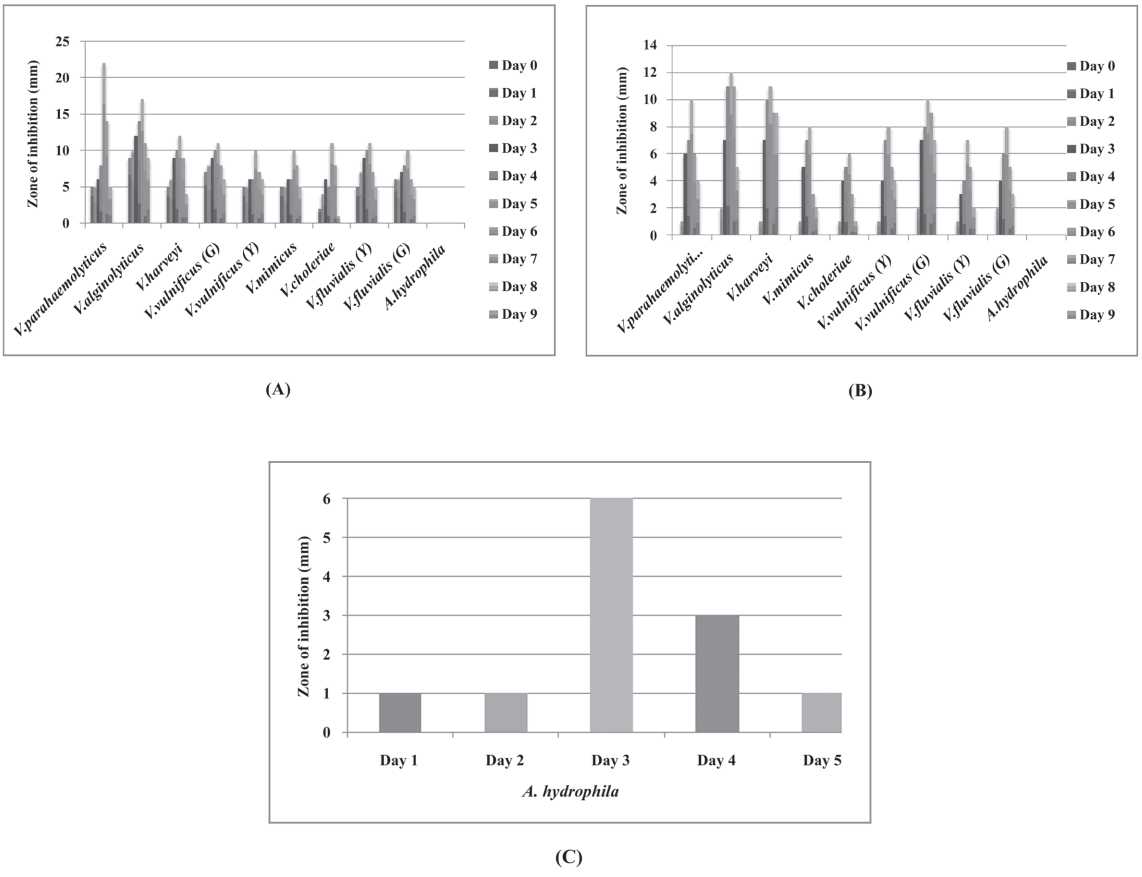

Figure 2. Zone of inhibition (mm), (A)–by B2; (B)–by B5; against *Vibrio* spp. (*V. parahaemolyticus, V. alginolyticus, V. harveyi, V. mimicus, V. choleriae* (non 01), *V. vulnificus* (Y and G), *V. fluvialis* (Y and G). and (C) - by B1; against *A. hydrophila* AQAH as revealed by the measurement (mm) of clear zone by AWDA. The measurements are the mean value of three independent experiments

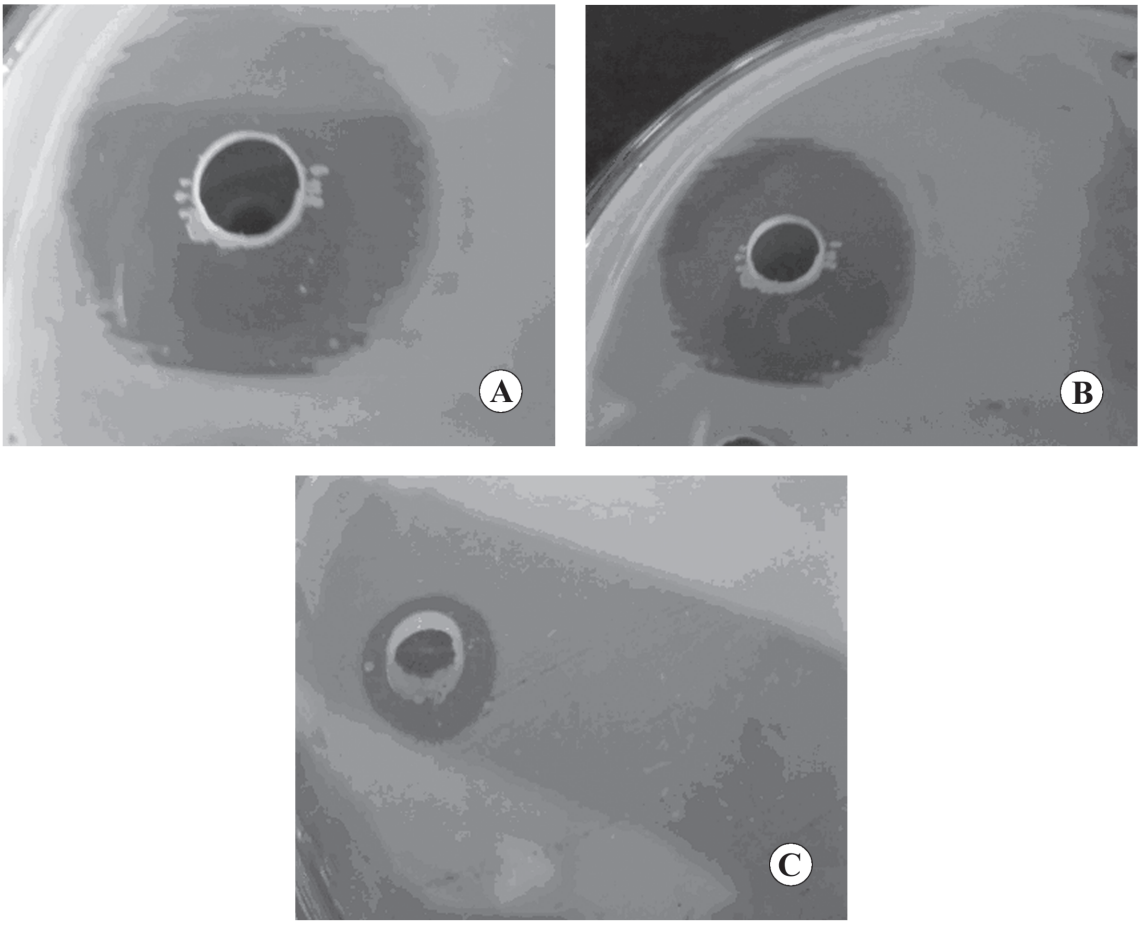

Figure 3. Inhibition zone, (A)-by B2 at 120 hrs; (B)–by B5 at 120 hrs; (C)-by B1 at 72 hrs against *V. parahaemolyticus, V. alginolyticus* and *A. hydrophila,* respectively

Co-culture of *Bacillus* spp. with pathogenic bacteria in Tryptic Soy Broth

Based on the result of cross streak and AWDA antagonism assays the *Bacillus* B2 and B5 were co-cultured with all the *Vibrio* spp. and B1 was cultured with *A. hydrophila* AQAH. The presence of *Bacillus* spp. (B2 and B5) led to a remarkable inhibition of growth of all strains of *Vibrio* spp. after 120 hours of incubation. Growth of *A. hydrophila* AQAH was reduced by 61.8% by B1 after 120 hours of co-culture (Figure 4). The growth reduction by B2 in the different strains of *Vibrio* spp. was more than 90%, and more than 85% at the end of 120 hours of co-culture experiment (Table 2), where as there was little difference observed in *Bacillus* spp. concentrations. The reduction of B1 was less than 5%, B2 was 4 to 7% and B5 was 4 to 8% at the end of 120 hours of the co-culture experiment compared to the control (monoculture) (Table 3).

Table 2. Reduction of growth of pathogenic bacteria (by percentage) in co-culture with *Bacillus* spp.

Bacteria species	**Percentage decrease of *A. hydrophila* with B1**					
	0 hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**
A.hydrophila	0.51±0.20	23.20±0.39	25.00±0.42	54.99±0.36	61.22±0.53	61.81±0.14
	Percentage decrease of *Vibrio* spp. with B2					
	0 hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**
V.alginolyticus	1.00±0.03 [a]	36.71±0.08 [a]	56.51±0.40 [a]	75.01±0.40 [a]	85.00±0.09 [a]	94.80±0.20 [a]
V.mimicus	0.76±0.12 [a]	34.94±0.10 [a]	55.72±0.40 [a]	73.70±0.60 [a]	84.00±0.72 [a]	92.43±0.18 [a]
V.vulnificus (Y)	0.60±0.08 [a]	33.33±0.20 [a]	54.39±0.35 [a]	73.39±0.14 [a]	84.76±0.31 [a]	91.61±0.15 [a]
V.vulnificus (G)	0.99±0.10 [a]	32.99±0.32 [a]	51.28±0.20 [a]	72.76±0.22 [a]	82.00±0.06 [a]	92.68±0.10 [a]
V.harveyi	0.67±0.19 [a]	34.23±0.11 [a]	54.82±0.27 [a]	74.57±0.10 [a]	84.11±0.26 [a]	91.65±0.28 [a]
V.luvialis (Y)	0.61±0.10 [a]	31.43±0.40 [a]	56.00±0.11 [a]	72.97±0.31 [a]	85.48±0.30 [a]	91.85±0.09 [a]
V.fluvialis (G)	0.93±0.41 [a]	30.91±0.43 [a]	54.17±0.33 [a]	69.71±0.09 [a]	81.13±0.13 [a]	91.21±0.20 [a]
V.choleriae (non 01)	0.83±0.20 [a]	32.71±0.46 [a]	53.00±0.13 [a]	71.60±0.13 [a]	81.95±1.30 [a]	88.93±0.37 [a]
V.parahaemolyticus	0.88±0.10 [a]	32.71±0.46 [a]	55.72±0.40 [a]	73.91±0.39 [a]	83.89±0.11 [a]	94.16±0.15 [a]
	Percentage decrease of *Vibrio* spp. with B5					
	0 hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**
V.alginolyticus	0.49±0.01 [a]	17.47±0.51 [a]	43.48±0.63 [a]	63.89±0.89 [a]	74.16±0.19 [a]	88.40±0.20 [a]
V.mimicus	0.51±0.20 [a]	18.07±0.90 [a]	44.29±1.38 [a]	64.62±0.31 [a]	75.60±0.21 [a]	89.64±0.69 [a]
V.vulnificus (Y)	0.48±0.08 [a]	17.39±0.39 [a]	42.86±0.20 [a]	63.06±0.69 [a]	75.23±0.75 [a]	89.68±0.89 [a]
V.vulnificus (G)	0.49±0.10 [a]	13.64±0.04 [a]	41.79±0.56 [a]	62.00±0.49	80.67±0.88 [a]	84.83±0.61 [a]
V.harveyi	0.40±0.09 [a]	18.03±0.35 [a]	44.83±0.37 [a]	62.71±0.19	83.33±0.21 [a]	89.98±0.11 [a]
V.fluvialis (Y)	0.48±0.21 [a]	17.94±0.90 [a]	44.00±0.65 [a]	64.86±0.52	82.73±0.27 [a]	88.19±0.29 [a]
V.fluvialis (G)	0.44±0.23 [a]	17.14±0.35 [a]	45.45±0.49 [a]	63.64±0.21	75.00±0.44 [a]	83.23±0.25 [a]
V.choleriae (non 01)	0.56±0.11 [a]	17.97±0.90 [a]	53.13±0.36 [a]	62.96±0.13	82.90±0.41 [a]	86.36±0.51 [a]
V.parahaemolyticus	0.49±0.23 [a]	18.64±0.45 [a]	46.00±0.38 [a]	63.77±1.20	84.44±0.55 [a]	88.19±0.90 [a]

Mean values within the same column with same superscripts are not significantly different at P= 0.05

Table 3. Reduction of growth of *Bacillus* spp. (in percentage) in co-culture with pathogenic bacteria.

Bacteria species	**Percent decrease of B1 with** *A. hydrophila*					
	0 hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**
A.hydrophila	0.68±0.34	4.50±0.60	3.84±0.85	4.00±0.75	5.45±0.24	4.16±0.73
	Percentage decrease of B2 with *Vibrio* **spp.**					
	0 hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**
V.alginolyticus	0.21±0.04 [a]	4.00±1.01 [a]	7.27±0.40 [a]	8.41±0.20 [a]	8.18±0.55 [a]	7.33±0.56 [a]
V.mimicus	0.14±0.06 [a]	3.87±0.60 [a]	5.45±0.25 [a]	6.33±0.90 [a]	6.15±0.34 [a]	5.86±0.58 [a]
V.vulnificus (Y)	0.66±0.49 [a]	4.13±0.41 [a]	4.36±1.02 [a]	4.80±0.10 [a]	5.69±1.10 [a]	5.32±0.36 [a]
V.vulnificus (G)	0.79±0.63 [a]	1.33±0.33 [a]	3.64±0.76 [a]	6.00±0.96 [a]	6.00±0.70 [a]	6.00±0.22 [a]
V.harveyi	0.30±0.14 [a]	1.32±0.51 [a]	7.25±0.29 [a]	7.20±0.70 [a]	6.12±0.57 [a]	6.67±0.38 [a]
V.fluvialis (Y)	0.45±0.38 [a]	2.67±1.21 [a]	3.45±0.31 [a]	7.60±1.00 [a]	7.54±0.18 [a]	5.43±0.19 [a]
V.fluvialis (G)	0.67±0.22 [a]	2.00±0.50 [a]	3.64±0.97 [a]	7.96±0.37 [a]	7.69±0.54 [a]	6.65±0.20 [a]
V.choleriae (non 01)	0.56±0.29 [a]	2.56±0.51 [a]	6.73±0.13 [a]	6.40±0.40 [a]	5.54±1.02 [a]	4.00±0.69 [a]
V.parahaemolyticus	0.22±0.21 [a]	2.20±0.21 [a]	5.27±1.62 [a]	5.20±0.78 [a]	4.92±0.51 [a]	5.54±1.44 [a]
	Percentage decrease of B5 with *Vibrio* **spp.**					
	0 hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**
V.alginolyticus	0.13±0.08 [a]	7.69±1.02 [a]	9.68±0.11 [a]	9.76±0.71 [a]	9.09±0.70 [a]	8.62±0.09 [a]
V.mimicus	0.28±0.23 [a]	6.41±0.51 [a]	6.13±0.59 [a]	6.10±0.99 [a]	5.87±0.46 [a]	5.34±0.32 [a]
V.vulnificus (Y)	0.77±0.22 [a]	6.15±0.37 [a]	6.61±0.30 [a]	6.22±0.30 [a]	6.36±0.21 [a]	6.55±0.35 [a]
V.vulnificus (G)	0.79±0.60 [a]	2.30±1.03 [a]	6.45±0.32 [a]	6.22±0.80 [a]	6.36±0.36 [a]	6.72±0.53 [a]
V.harveyi	0.52±0.42 [a]	5.77±0.62 [a]	8.39±0.32 [a]	8.41±0.38 [a]	7.27±0.27	7.24±0.23 [a]
V.fluvialis (Y)	0.65±0.53 [a]	7.95±0.50 [a]	7.26.±0.59 [a]	6.95±0.51 [a]	6.82±0.59 [a]	6.55±0.55 [a]
V.fluvialis (G)	0.90±0.58 [a]	1.96±1.00 [a]	3.23±0.21 [a]	4.90±0.84 [a]	5.00±0.89 [a]	5.17±0.51 [a]
V.choleriae (non 01)	1.03±0.48 [a]	2.57±0.21 [a]	4.83±0.12 [a]	4.88±0.53 [a]	4.55±0.31 [a]	4.82±0.40 [a]
V.parahaemolyticus	0.38±0.23 [a]	3.51±0.42 [a]	6.28±0.50 [a]	6.34±0.34 [a]	6.36±0.96 [a]	6.21±0.21 [a]

Mean values within the same column with same superscripts are not significantly different at P= 0.05

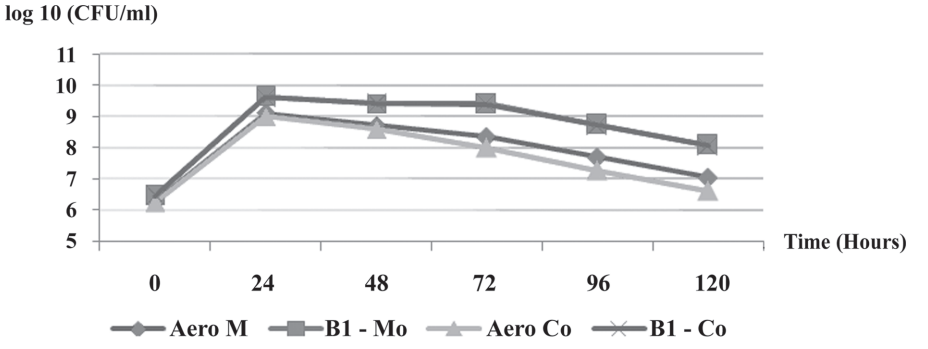

Figure 4. Growth of B1 and *A. hydrophila* in co-culture and monoculture

DISCUSSION

Several studies on probiotics have been published during the last decade. The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. The use of probiotics in human and animal nutrition is well documented and recently, efforts to apply probiotics in aquaculture have started (Gatesoupe, 1999; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000). An expert with the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) stated that probiotics are live microorganisms, which when consumed in adequate amount, confer a health benefit for the host (FAO /WHO, 2001). Probiotics proposed as biological control agents in aquaculture belong to the following groups: lactic acid bacteria (*Lactobacillus* and *Carnobacterium*), genus *Vibrio* (*V. alginolyticus*), genus *Bacillus*, and genus *Pseudomonas*. However other genera or species have also been mentioned such as *Aeromonas* and *Flavobacterium*.

In this study, we focused only on genus *Bacillus* spp. which showed antagonistic activity to pathogenic bacteria in aquaculture in many studies. In this study we found that *Bacillus* B2 colonized all the strains of *Vibrio* spp. from 48 hours as seen in the cross streak method, with the level of colonization increasing up to 96 hours. The same strain started producing an antimicrobial substance after 24 hours of incubation (as revealed by the result of AWDA) against all target *Vibrio* pathogens. The secretion of this antimicrobial substance continued up to seven days. The highest production of antimicrobial substances was observed on the 5th day by this strain of *Bacillus* against almost all target pathogenic strains. This result could explain the reduction of *Vibrio* strains in the co-culture experiment which strongly supports the evidence of 85-90% of reduction of *Vibrio* strains compared to the control (monoculture) at the end of 120 hours while co-cultured with B2. *Bacillus* B5 started showing inhibition against all the target pathogenic *Vibrio* strains from 72 hours and the effect kept increasing up to 96 hours as revealed by the result of cross

streak method. However production of antimicrobial substance for this strain was observed from 48 hours by AWDA. According to the result of AWDA the strain was able to produce the antimicrobial substance up to seven days against all the target pathogenic *Vibrio* strains and showed the highest level of activity on the 5th day of the experiment. This result also supported the growth reduction of all *Vibrio* strains at around 80-85% compared to control at the end of 120 hours while co-cultured with *Bacillus* B5. B1 showed the inhibitory effect only against *A. hydrophila* AQAH, with the zone of inhibition observed from 72 hours in cross streak method, and diameter of inhibition increasing until 96 hours. This result was different in the AWDA where we found that *Bacillus* B1 antagonized the target pathogen *A. Hydrophila* AQAH from 24 hours of incubation. However, the production of antimicrobial substance continue until the 5th day while the highest level was observed at 72 hours, which was similar to the result of cross streak method. In the co-culture experiment, B1 was found to reduce the growth of *A. hydrophila* AQAH to 61.22% at the end of 96 hours, which was not much different from the percent decrease of *A. hydrophila* AQAH at the end of 120 hours (61.81%). There duction of *Bacillus* bacteria was 4.16% for B1, 4 to 8 % for B2, and 4 to 9% for B5, at the end of 120 hours of the co-culture experiment compared to the monoculture (control). These can be attributed to lack of nutrients in the culture media at the end of 120 hours of incubation. *Bacillus* B3 and B4 did not produce any antagonism by cross streak and AWDA against any of the pathogenic bacteria, therefore these strains were not selected for the co-culture experiment.

In this co-culture experiment, pathogenic bacteria were used at high concentrations. Previous studies have shown that the number of luminous bacteria in coastal areas ranged from $0.7x10^1$ to $7x310^1$ CFU ml^{-1} (Sudthongkong, 1996), while in fresh water areas, total bacteria varied from $3.1x10^1$ to $1.0x10^3$ CFU ml^{-1} (de Sousa and Silva-Souza, 2001). In fish pond waters, total bacteria ranged from 1.8 $\pm$ 0.9 x10^2 to 6.0 $\pm$1.2 x 10^4 CFU ml^{-1} (Al-Harbi, 2003). In this present study it was observed that in spite of applying a high amount of pathogenic bacteria (10^5CFU ml^{-1}) the experimental strains of *Bacillus* spp. (except B3 and B4) exhibited potential antagonism against the target pathogenic strains

Moriarty (1998) and Rengpipat *et al.* (1998) reported that the possibility of increasing shrimp production in large bodies of water by adding the probiotic *Bacillus*. Similarly other studies have reported that probiotic could improve water quality (Homma and Shinohara, 2004; Manpal *et al.*, 2003).

In conclusion, the result of the present study gave evidence that *Bacillus* B2, B5 and B1 produced antimicrobial substance that could inhibit the growth of pathogenic *Vibrio* spp. and *A. hydrophila*, respectively. The activity of the strains remained up to five days for B1 and seven days for both B2 and B5. All these three strains were able to competitively exclude the pathogens during *in vitro* co-culture experiments. Thus these strains can be used as an effective probiotic in aquaculture. However, commercial scale *in vivo* experiments will be an area for future research along with the elucidation of the mechanism of antagonistic action between these probionts and pathogens.

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LITERATURE CITED

Al-Harbi, A.H. 2003. Faecal coliforms in pond water, sediments and hybrid tilapia *Oreochromis niloticus* x *Oreochromis aureus* in Saudi Arabia. **Aquaculture and Research**.34(7): 517.

Austin, B., L. Stuckey, P. Robertson, I. Effendi and D. Griffith.1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* & *Vibrio oradalli*. **Journal of Fish Diseases.**18: 93- 96.

FAO/WHO. 2001. Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina.

Barbosa, T.M., C.R Serra, R.M La Ragione, M.J Woodward and A.O Henriques.2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. **Applied and Environmental Microbiology.** 71(2), 968–978.

Bauer, A.W., W.M Kioby, J.C. Sherris and M. Turck 1966. Antibiotic susceptibility testing by a standard single disc method. **American Journal of Clinical Pathology** 45: 493–496.

Cherif, A., H.Ouzari, D. Daffonchio, H. Cherif, Ben Slama, K. Hassen, A. Jaoua and S. Boudabous, 2001. Thuricin 7: a novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7. **Letters in Applied Microbiology** 32: 243–247.

Cladera-Olivera, F., G.R Caron and A.Brandelli 2004. Bacteriocin-like substance production by *Bacillus licheniformis* strain P40. **Letters in Applied Microbiology** 38: 251–256.

de Sousa, J.A. and A.T. Silva-Souza. 2001. Bacterial community associated with fish and water from Congonhas River, Sertaneja, Paraná, Brazil. **Brazlian Archives of Biology and Technology.** 44:4.

Duc, L.H., H.A Hong, T.M Barbosa, A.O Henriques, S.M Cutting, 2004. Characterization of Bacillus probiotics available for human use. **Applied and Environmental Microbiology.**70(4): 2161–2171.

Gatesoupe, F.J. 1999. The use of probiotics in aquaculture. **Aquaculture** 180: 147–165.

Gomez-Gil, B.A.,Roque and J.F.Turnbull. 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. **Aquaculture** 191: 259–270.

Homma, H and T. Shinohara. 2004. Effects of probiotic Bacillus cereus toyoi on abdominal fat accumulation in the Japanese quail (*Coturnix japonica*). **Animal Science Journal.** 75: 37-41

Hong, H.A, L.HDuc and S.M Cutting 2005. The use of bacterial spore formers as probiotics. **FEMS Microbiology Reviews.** 29: 813–835.

Karunasagar, I.,RPai, G.R. Malathi and I, Karunasagar.1994. Mass mortality of *P.monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. **Aquaculture**128: 203-209.

Lemos, M.L., A.E. Toranzo and J.L. Barja. 1985. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. **Microbial Ecology.**11: 149-163.

Manpal, S.R and Paul Raj. 2003. Efficacy of gut probionts in enhancing growth in *Penaeus indicus* post larvae. In: **Aquaculture Medicine,** Singh, I.S.B., S.S. Pai, R. Philip, A. Mohandas, (Eds.)., Centre for Fish Disease Diagnosis and Management, CUSAT, Kochi, India, pp: 117-122.

Mishra, S., S. Mohanty, P. Pattnaik and S. Ayyappan.2001. Probiotics- Possible application in aquaculture. **Fishing Chimes.** 21: 31-37.

Moriarty, DJW. 1997. The role of microorganisms in aquaculture ponds. **Aquaculture** 151, 333- 349.

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

Rengipipat, S., W. Phianphak, W. Piyatiratitivorakul and P. Menasveta. 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. **Aquaculture** 167: 301- 313.

Sudthongkong, C. 1996. Identification and drug resistance of luminous bacteria in coastal area of Inner Gulf of Thailand. **The AAHRI Newsletter.** 5: 2.

Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete. 2000. Probiotic bacteria as biological control agents in aquaculture. **Microbiology and Molecular Biology Reviews.** 64:655 –67.