Identification and characterization of potential probiotic *Bacillus* spp. for application in striped catfish (*Pangasianodon hypophthalmus* [Sauvage, 1878])

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ABSTRACT

One hundred and twenty isolates of *Bacillus* sp. were collected from the intestines of striped catfish (*Pangasianodon hypophthalmus* [Sauvage 1878]) reared in farms in three provinces in the southern part of Vietnam: An Giang, Can Tho and Dong Thap. Isolates were identified by multiplex and single PCR using specific primers designed on gene 16S rRNA for six species of *Bacillus* (*B. subtilis, B. amyloliquefaciens, B. cereus, B. megaterium, B. licheniformis and B. pumilus*). The *in vitro* analysis included inhibition activity against the pathogenic bacterium *Edwardsiella ictaluri*, protease activity, stomach acidity and bile tolerance test, and antibiotic susceptibility. Results indicated that the highest number of isolate was *B. megaterium* and none was identified as *B. licheniformis*. The strain *B. amyloliquefaciens* 54A showed high protease activity and *B. pumilus* 47B showed high inhibition activity against *E. ictaluri*. These two probiotic candidates were also resistant to stomach acid pH and low bile salt conditions after 3 h and 24 h of exposure. *Bacillus* sp. candidates displayed varying sensitivities to the tested antibiotics with the exception of spectinomycin.

Keywords: Isolation, characterization, probiotics, *Bacillus*, striped catfish, Vietnam

INTRODUCTION

The striped catfish (*Pangasianodon hypophthalmus* [Sauvage 1878]) is a well-known fish exported all around the world, with Vietnam the largest producer (FAO, 2010). It is a very important freshwater fish for Vietnamese aquaculture. Unfortunately, the increase in intensive farming in the Mekong Delta, South Vietnam, and high density fish stocking have induced disease outbreaks (Phan *et al*., 2009). Enteric septicemia of catfish caused by the gram negative bacterium, *Edwardsiella ictaluri*, is the most serious disease that infects this species, and it has severely affected the Vietnamese aquaculture industry (Crumlish *et al*,. 2002). *E. ictaluri* infection can cause 50-90% mortality, and it is prevalent in over 90% of the striped catfish farms in the Mekong Delta (Phan *et al*., 2009). With the rapid development of aquaculture, effective control of disease outbreaks is a big challenge for the industry. Many methods have been applied to prevent infection including the application of immunostimulants through diet, genetic selection for disease resistance, and monitoring water quality (Hawke *et al*., 1998; Dunham *et al*., 2002). Vaccines have been introduced but not commonly used and the result may not be consistent.

The use of antibiotics may be an effective treatment in serious cases of high fish mortality. However, there are some limitations due to the high cost of the drugs. Bacterial resistance and drug residue in the fish product can also affect human health

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(Baker, 2006). Therefore, a biological approach using beneficial microorganisms as probiotics has become an effective tool for the control of many diseases in aquaculture (Jose *et al*., 2006). The use of probiotics in aquaculture has increased due to their ability to increase productivity through disease prevention (Aditya *et al*., 2008). *Bacillus* sp. is an important candidate for the production of probiotics. It can sporulate and produce secondary products such as antibacterial substances and enzymes that are beneficial in the intestinal environment of humans and animals (Carlos *et al*., 2010). Enzymes produced by *Bacillus* sp. are capable of digesting carbohydrates, lipids, and protein (Hong *et al*., 2005). Many studies demonstrated *Bacillus* spp. efficacy in different fish species such as Indian carp, rainbow trout, grouper, and yellow croaker (Nayak *et al*., 2007; Newai-Fyzul *et al*., 2007; Sun *et al*., 2010; Ai *et al*., 2011). Consequently, we identified *Bacillus* spp. from the intestines of striped catfish and characterized potential *Bacillus* spp.

MATERIALS AND METHODS

Isolation of the genus *Bacillus*

Live and healthy striped catfish were collected from farms in An Giang, Dong Thap and Can Tho provinces in the South of Vietnam. The gut contents were collected by homogenizing 1 cm of mid gut in 1 ml of 0.85% (w/v) NaCl solution. The suspension was 10 fold diluted with sterile saline, then heated on a hot plate at 80°C for 15 min and cooled on ice for 5 min to eliminate vegetative cells. Aliquots (0.1 ml) of dilution were then spread onto tryptone soya agar (TSA) (Himedia, India) and incubated at 37°C for 24 h. Single colonies were selected and purified by re-streaking onto TSA for identification test.

Identification of selected isolates by PCR amplification

Gram positive, long rod and spore strains were selected after incubation for 72 h for screening *Bacillus* spp. by 16S rRNA gene amplification using the primer pair, forward (5'-AGAGTTTGATCCTG GCTCAG-3') and reverse (5'-AAGGAGGTGATC CAGCCGCA-3') (Gomaa and Momtaz 2007). DNA samples were extracted from bacterial cells suspension by using phenol/chloroform method (Russell and Sambrook, 2001). Amplification was carried out by using 20 µl of PCR mixture containing 1 µl of DNA template (100 ng•µl^{-1}), 2 µl of Dream *Taq* buffer (10× Dream *Taq* buffer plus 20 mmol•l^{-1} $MgCl_2$; Thermo Fisher Scientific, MA, USA), 2 µl of 10 mmol•l^{-1} dNTP (Thermo Fisher Scientific, MA, USA), 1µl of 10 µmol•l^{-1} each primer (Biodesign, Pathumthani, Thailand) and 0.2 µl of Dream *Taq* polymerase (5 U•µl^{-1}) (Thermo Fisher Scientific, MA, USA). Polymerase reactions (PCR) were performed as following: an initial denaturing step of 5 min at 95°C followed by 25 cycles of 95°C for 1 min, 67°C for 1 min and 72°C for 2 min, and then a final extension at 72°C for 10 min. The PCR was carried out in Takara PCR thermal cycler (Otsu, Shiga, Japan).

Bacillus spp. were used commonly in aquaculture including *B. subtilis* (Liu *et al*., 2010; Ai *et al*., 2011; Telli *et al*., 2014), *B. cereus* (Navin Chandran *et al*., 2013), *B. amyloliquefaciens* (Das *et al*., 2013), *B. licheniformes* (Avella *et al*., 2010), *B. megaterium* (Ating *et al*., 2013), and *B. pumilus* (El-Sersy *et al*., 2006; Leyton *et al*., 2012). They were identified by specific primers designed on 16S rRNA genes. Multiple sequence alignment program Clustal Omega online (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to find unique nucleotides of primers on each gene. The primer designing tool, Oligo CalC program (http://biotools.nubic.northwestern.edu/OligoCalc.html) was also applied to find the optimal primers. All primers used for *Bacillus* identification are listed in Table 1.

The extracted DNA of *Bacillus* spp. samples were amplified by multiplex PCR using specific primers of *B. cereus*, *B. amyloliquefaciens*, *B. licheniformis* and *B. megaterium*. A 20 µl of PCR reaction contained 1 µl of DNA templates (100 ng•µl^{-1}), 2 µl of *Taq* polymerase buffer (10 ×; Thermo Fisher Scientific, MA, USA), 1 µl of 10 mmol•l^{-1} dNTP, 0.4 µl of each primer (10 µmol•l^{-1}) (Biodesign, Pathumthani, Thailand), 1.2 µl of 25 mmol•l^{-1} $MgCl_2$ (Thermo Fisher Scientific, MA, USA) and 0.2 µl of *Taq*DNA polymerase (5U•µl^{-1}) (Thermo Fisher Scientific, MA, USA). The amplification was

Table 1. Primers used in this study

Target *Bacillus*	Primer name	Sequence (5'to 3')	Amplicon size (bp)	Reference
B. subtilis	Bsu1-F	GCTTACGGGTTATCCCGC	500	Kwon *et al*. (2009)
	Bsu1-R	CCGACCCCATTTCAGACATATC		Kwon *et al*. (2009)
B. cereus	20F	GAGTTTGATCCTGGCTCAG	219	Kawasaki *et al*. (1993)
	Bce-R	GAACCATGCGGTTCAAAATGTT		Present study
B. licheniformis	20F	GAGTTTGATCCTGGCTCAG	193	Kawasaki *et al*. (1993)
	Bl-R	GTAGCTAAAACCCACCTTTTATAA		Present study
B. pumilus	20F	GAGTTTGATCCTGGCTCAG	472	Kawasaki *et al*. (1993)
	Bpu-R	CAAGCAGTTACTCTTGCACTT		Present study
B. megaterium	20F	GAGTTTGATCCTGGCTCAG	500	Kawasaki *et al*. (1993)
	Bme-R	AAGGTATGAGCAGTTACTCTC		Present study
B. amyloliquefaciens	Ba1-F	GCGCAGTCCGTGCCTTAC	800	Peng *et al*. (2004)
	Ba1-R	TTACTGAGCTGCCGCCTGTAC		Peng *et al*. (2004)

followed conditions: denaturation at 94°C for 3 min followedby 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min.

To detect *B. pumilus*, 20 µl of PCR master mix contained 1µl of DNA templates (100 ng•µl^{-1}), 2 µl of *Taq* polymerase buffer (10×), 1 µl of dNTP mix (10 mmol•l^{-1}), 1 µl of each primer (10 µmol•l^{-1}), 0.8 µl of $MgCl_2$ (25 mmol•l^{-1}) and 0.2 µl of *Taq* DNA polymerase (5 U•µl^{-1}). A single PCR was performed for samples to identify *B. pumilus* under condition: 3 min of initial denaturation at 94°C followed by 25 cycles of denaturation at 94°C for 30 s, an initial annealing at 60°C for 30 s, and 72°C for 30 s, with an extension step at 72°C for 10 min.

Another single PCR amplification was also carried out using specific primer of *B. subtilis*. Each PCR reaction contained similar volume as mentioned above, except 1.2 µl of $MgCl_2$ (25 mmol•l^{-1}) was added into the reaction tubes. The PCR amplification was conducted with a denaturation step of 3 min at 94°C, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and final extension at 72°C for 10 min.

PCR products were separated by 1% of agarose gel electrophoresis, and DNA in the gel was stained with ethidium bromide (1µg•ml^{-1}), then visualized by exposure to UV light and photographed by a digital capture system (Gel doc, Bio-Rad, Richmond, CA, USA). The sizes of the DNA fragments were estimated using a 100 bp DNA ladder (Thermo Fisher Scientific, MA, USA). To confirm the identification of *Bacillus* spp. by specific primer, PCR products were transferred into plasmid and sent to Macrogen Korea for sequencing and comparison with the GenBank database. Plasmid preparation followed the method of Birnboim and Doly (1979).

Inhibition activity against pathogenic bacteria, *Edwardsiella ictaluri*

The inhibition against the growth of *E. ictaluri* was assessed by cross streak plate assay method (Velho-Pereira and Kamat, 2011). A single streaking line of *E. ictaluri* strain was streaked (3-4 mm width) on the surface of tryptone soya agar (TSA; Himedia, India) and then the samples of *Bacillus* spp. were crossed perpendicular to the original line. All of the plates were incubated at 28°C for 96 h to observe the inhibition and colonization.

Primary screening protease production of *Bacillus* spp.

Bacillus spp. isolates were screened for protease activity using skim milk agar plates (Liu *et al*., 2009) with some modification. The protease production was tested by dropping 10 µl of supernatant

filtrates of *Bacillus* spp. onto 5 mm diameter filter paper and placed on the surface of skim milk agar for colonization. The clear zone of protease activity on skim milk media was estimated after 24 h of incubation.

Characterization of protease specific activity

Screened isolates producing a halo diameter of ≥ 3 mm were selected from primary screening to analyze specific protease activity following the method of Beg *et al*. (2002) with some modification. Bacteria candidates were cultured at 37°C in trypticase soya broth. After incubation for 24 h, 700 µl of cell suspension was mixed with 700 µl of 50 mmol•1^{-1} Tris-HCl buffer and centrifuged at 15,000 ×g for 10 min at 4°C. The supernatant was collected and used as crude enzyme. The enzyme activity was determined in triplicate by incubating 125 µl enzyme solution with 250 µl of a 1% (v/v) azocasein in 100 mmol•1^{-1} Tris-buffer (pH 9.0) for 15 min at 37°C. The reaction was stopped by adding 1.2 ml of 10% (v/v) trichloroacetic acid (TCA), left for 15 min at room temperature and then, centrifuged at 8,000 ×g for 15 min at 4°C. Next, 0.6 ml of supernatant was added to 0.7 ml of 1 mol•1^{-1} NaOH. The absorbance of the mixer was read at 440 nm by spectrophotometer. The protein concentrations were determined according to the Bradford method (Bradford 1976) using bovine serum albumin as a standard. The protease activity was defined as amount of unit of protease mg^{-1} protein.

Antibiotic susceptibility analysis

The *Bacillus* spp. isolates were tested for antibiotic susceptibility using a standard disc diffusion method following the procedures of Clinical and Laboratory Standard Institute (2012). Eleven antibiotics used in Vietnam aquaculture were selected including amoxicillin (AML10), ampicillin (AMP10), erythromycin (E15), florfenicol (FFC30), neomycin (N30), spectinomycin (SH25), oxytetracycline (OT30), sulfamethoxazole (RL25), trimethoprim (W5), tetracycline (TE30) and ciprofloxacin (CIP5). Selected isolates were sub-cultured to a trypticase soy agar (TSA) and then suspended in trypticase soy broth (TSB). Bacterial suspension was adjusted to a concentration of approximately 1-2 × 10^8 CFU•ml^{-1} matched to a 0.5 McFarland turbidity standard. A sterile cotton swab was dipped into the suspension and used to seed inoculums onto the entire surface of a Mueller Hinton agar (MHA) plate. Antibiotic discs (Oxoid, Basingstoke, England) were placed gently on the surface of MHA plates, and the inhibition zone was calculated in mm of diameter after incubation at 37°C for 24 h. Interpretation of zone size was based on the standard zones in the table of Clinical and Laboratory Standard Institute (2007).

Acidic and bile salt tolerance

The aim of the analysis was to evaluate the viability of selected *Bacillus* cells under pH stress in stomach juice and bile salts in the intestine. Two selected potential strains for in vivo experiment were chosen as representative for exposure to acidic pH (2, 3) and bile salts (0.5, 1 and 2%) for 1, 3 and 24 h. Using 1 mol•1^{-1} HCl and 1 mol•1^{-1} NaOH, the sterilized nutrient broth was adjusted to pH 2, and 3 for the challenge and pH 7 for the control. The pH was measured by a Mettler Toledo pH meter (Mettler Toledo Group, Switzerland). Bile salt (Himedia, India) was added to the nutrient broth at concentration 0.5, 1 and 2% and 0% for the control. The challenge test was conducted on *Bacillus* vegetative cells and spore forms. *Bacillus* spp. was cultured in nutrient broth at 37°C for 24 h to collect the vegetative cell, and cultured on sporulation media (Ran *et al*., 2012) for 6 days to harvest the spore. *Bacillus* sp. suspension was adjusted to 10^8 CFU•ml^{-1} by spectrophotometer and then added to the challenge media at the ratio 1:100. The growth of viable cells was determined by observing the colonization on streaking single lines on the nutrient agar plates.

RESULTS

Identification of *Bacillus* spp.

Nine types of colony morphology were classified by visual observation from 300 samples from the provinces of An Giang, Can Tho and Dong Thap. Most colonies were 3-4 mm in diameter and morphology included circular, entire margin, mucoid, convex elevation and irregular forms. Based on colony

morphology, we selected 120 representative isolates with 40 samples from each province. After 72 h of culture, the bacteria were Gram stained and all of them were Gram positive and spore formed. The pure isolates were identified as *Bacillus* family by universal primer. The results showed 100% identification as *Bacillus* spp. (Figure 1).

Based on species identification by specific primer, the selected isolates were classified as *B. subtilis*, *B. cereus, B. amyloliquefaciens, B. megaterium* and *B. pumilus*, respectively (Figures 2 to 4), with no finding of *B. licheniformis* in the collected samples. The percentages of *Bacillus* population gave *B. megaterium* (27.5% of the total), *B. amyloliquefaciens* (24.2%), *B. cereus* (21.7%), *B. subtilis* (16.7%), *B. pumilus* (3.3%) and unidentified *Bacillus* (6.6%) (Figure 5).

The distribution of the five different species of *Bacillus* in An Giang, Can Tho, and Dong Thap provinces are shown in Figure 6. The population of *B. subtilis, B. megaterium* and *B. pumilus* were 17.5, 30 and 5%, respectively, in An Giang and Can Tho. *B. megaterium* isolates were commonly found in An Giang and Can Tho, whereas *B. amyloliquefaciens* and *B. cereus* were widespread in Dong Thap province. In An Giang and Can Tho, low numbers of *B. pumilus* were found and 10% of isolates unidentified.

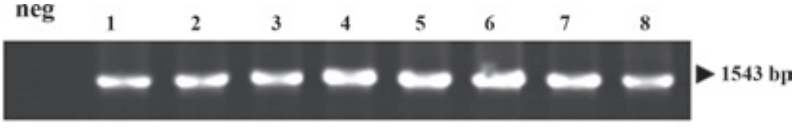

Figure 1. Detection of *Bacillus* spp. sampled in the gut of striped catfish by Polymerase Chain Reaction using universal primer designed on 16S rRNA gene (1,543bp) as indicator for *Bacillus* family. lane neg : negative control; lane 1 – 8: *Bacillus* isolates

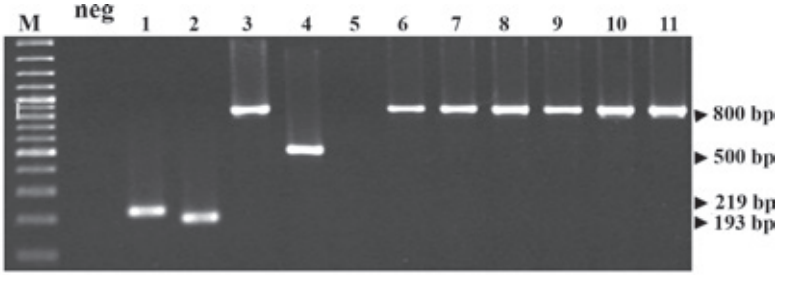

Figure 2. Multiplex PCR detection of 16S rRNA gene to identify 4 species: *B. cereus, B. amyloliquefacians, B. licheniformis, B. megaterium.* Lane M: 100 bp plus DNA ladder (Invitrogen), lane 1: *B. cereus* (positive control-amplicon size 219 bp); lane 2: *B. licheniformis* (positive control-amplicon size 193 bp); lane 3: *B. amyloliquefacians* (positive control- amplicon size 800 bp); lane 4: *B. megaterium* (positive control-amplicon size 500 bp); from lane 5 to 11: *Bacillus* isolates, lane (neg): negative control.

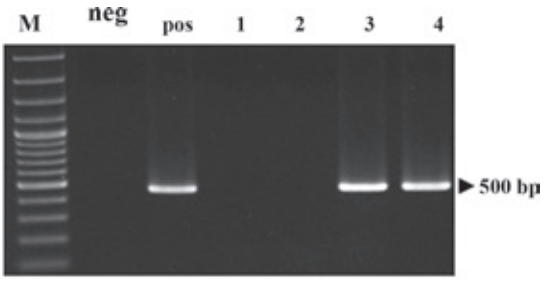

Figure 3. PCR detection of 16S rRNA gene by specific primer to identify *B. subtilis*. Lane M: 100 bp plus DNA ladder, lane neg: negative control; lane pos: positive control (amplicon size 500 bp); from lane 1 to 4: *Bacillus* isolates.

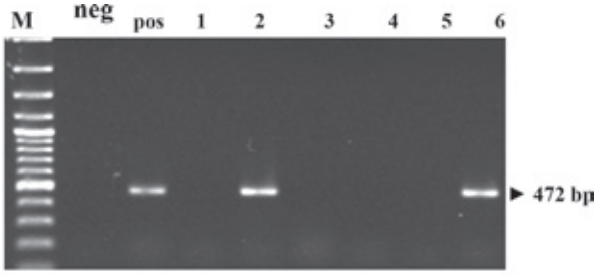

Figure 4. PCR detection of 16S rRNA gene by specific primer to identify *B. pumilus*. Lane M: 100 bp plus DNA ladder, lane neg: negative control; lane pos: positive control (amplicon size 472 bp); from lane 1 to 6: *Bacillus* isolates.

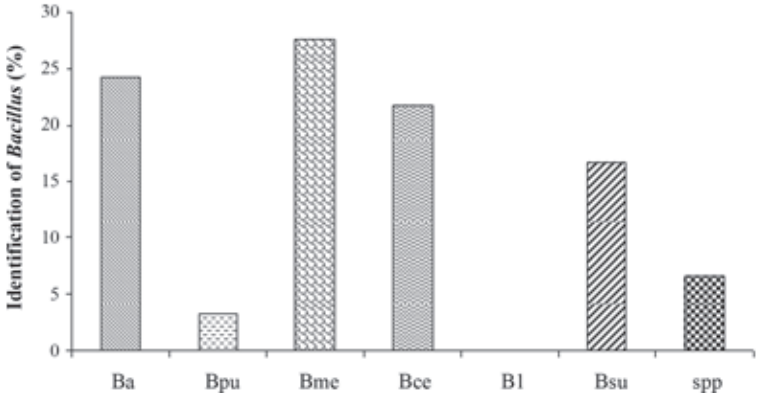

Figure 5. Percentage of *Bacillus* spp. classified by specific PCR identification from the total of 120 isolates. Ba: *B. amyloliquefaciens*, Bpu: *B. pumilus*, Bme: *B. megaterium*, Bce: *B. cereus*, Bl: *B. licheniformis*, Bsu: *B. subtilis*, spp: unidentified

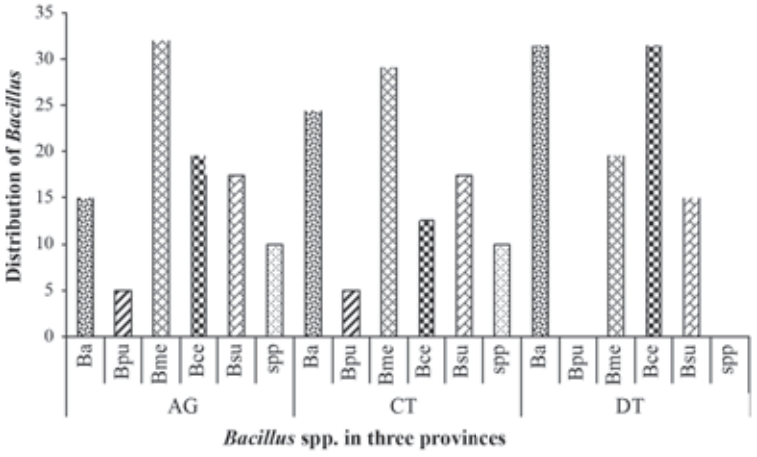

Figure 6. Distribution of *Bacillus* spp. in An Giang (AG), Can Tho (CT) and Dong Thap (DT) in every 40 samples in each province. Ba: *B. amyloliquefaciens*, Bpu: *B. pumilus*, Bme: *B. megaterium*, Bce: *B. cereus*, Bl: *B. licheniformis*, Bsu: *B. subtilis*, spp: unidentified.

Inhibition activity of *Bacillus* spp.

Using the cross streak plate method, the appearance of a clear halo on the culture media indicated the presence of an inhibition activity. As summarized in Table 2, 36/120 *Bacillus* spp. isolates showed antagonism against *E. ictaluri* in this study. Six *Bacillus* spp. showed strong inhibition activity against *E. ictaluri* with inhibition zone over 10 mm including *B. amyloliquefaciens* (48C, 51G, 93B), *B. megaterium* (4A, 62D) and *B. pumilus* (47B).

Protease production of *Bacillus* spp. and their identification

Thirty-six strains of bacteria showing inhibition activity were selected to test their proteolytic activities by screening on skim milk agar. The results in Table 2 showed that 27 *Bacillus* spp. isolates produced small amounts of protease (clear zone ≤ 2 mm) or did not secrete protease.

Bacillus spp. candidates (1C, 31C, 47B, 54A, 78A and 93B) showing antibacterial zone ≥ 5 mm and proteolysis zone ≥ 3 mm were selected to analyze for specific protease activity. The specific protease activity indicated that the 54A strain yielded the highest protease of 55.64 U•mg^{-1}, followed by 93B at 45.04 U•mg^{-1} and 31C at 37.05 U•mg^{-1} (Table 3).

The results of specific PCR identification were confirmed by 16S rRNA gene sequencing which indicated that four *Bacillus* strains were *Bacillus amyloliquefaciens* including 31C (CP014100.1), 54A (CP013727.1), 78A (EU414203.1) with 99% ID and 93B (CP006845.1) with 100% ID. Strain 1C (CP009684.1) and 47B (CP012329.1) were identified as *B. subtilis* and *B. pumilus,* with 99% and 100% identities respectively (Table 3).

Antibiotic sensitivity of *Bacillus* spp.

Six *Bacillus* spp. strains (1C, 31C, 47B, 54A, 78A and 93B) exhibiting good inhibition of pathogenic bacteria and protease activity were further examined for antibiotic sensitivity by disc diffusion test. The diameter of the clear zone determined that all of these strains were sensitive to amoxicillin, ampicillin, erythromycin, florfenicol, neomycin, trimethoprim, tetracycline and ciprofloxacin at different levels. Particularly, all strains were highly inhibited by erythromycin, florfenicol, trimethoprim and ciprofloxacin with a wide range of inhibition zone (≥ 25 mm). However, some of the strains were resistant to oxytetracycline (strain 1C, 31C and 54A), sulfamethoxazole (strain 31C, and 93B) and especially, all of these strains showed resistance to spectinomycin (Table 4).

Table 2. Antimicrobial activity of 36 *Bacillus* spp. isolates against pathogenic bacteria and their protease screening

Species	Strain	Inhibition zone to *Edwardseilla ictaluri*	Protease clear zone (mm)
B. subtilis	1C	++ and colonize	5
B. megaterium	4A	+++ and colonize	2
B. subtilis	10A	+	5
unidentified	11A	++	0
B. subtilis	28B	+ and colonize	1
B. amyloliquefaciens	31C	++ and colonize	4
B. amyloliquefaciens	32B	+ and colonize	1
B. subtilis	32C	+ and colonize	1
B. amyloliquefaciens	36D	++ and colonize	2
B. amyloliquefaciens	39B	+	4
B. subtilis	41A	+	0
B. pumilus	47B	+++	3
B. amyloliquefaciens	48C	+++ and colonize	2
B. amyloliquefaciens	51G	+++	2
B. amyloliquefaciens	54A	++ and colonize	3
B. subtilis	55A	+ and colonize	2
B. subtilis	57C	+	<1
unidentified	58D	++	<1
B. megaterium	62D	+++	2
B. megaterium	63B	+	2
B. amyloliquefaciens	64A	+	2
B. amyloliquefaciens	64E	+	2
B. amyloliquefaciens	73C	++	<1
B. cereus	75B	++ and colonize	<1
B. amyloliquefaciens	76C	+	1
B. subtilis	77D	+	2
B. amyloliquefaciens	78A	++	3
B. subtilis	80A	+	<1
B. amyloliquefaciens	88A	+ and colonize	2
B. amyloliquefaciens	93B	+++	3
B. amyloliquefaciens	94B	++ and colonize	0
B. amyloliquefaciens	99B	+	3
B. amyloliquefaciens	100C	+ and colonize	1
B. amyloliquefaciens	101C	+ and colonize	2
B. amyloliquefaciens	103B	+ and colonize	3
B. amyloliquefaciens	115A	+ and colonize	1

(+) diameter of inhibition zone from 1-4 mm, (++) indicates a zone of inhibition from 5-9 mm, (+++) indicates a zone of inhibition over 10 mm, (-) indicates no inhibition zone.

Table 3. The protease activity of six *Bacillus* spp. and their identification

Strain	Protease activity (U^{-1} mg)	Identified on NSBI	ID %	Accession number
1C	29.57	*Bacillus subtilis*	99%	CP009684.1
31C	37.05	*Bacillus amyloliquefaciens*	99%	CP014100.1
47B	25.53	*Bacillus pumilus*	100%	CP012329.1
54A	55.64	*Bacillus amyloliquefaciens*	99%	CP013727.1
78A	29.11	*Bacillus amyloliquefaciens*	99%	EU414203.1
93B	45.04	*Bacillus amyloliquefaciens*	100%	CP006845.1

Table 4. Antibiotic susceptibility of six *Bacillus* spp. candidates to different antibiotics

Name of antibiotic	Diameter clear zone (mm) of 6 *Bacillus* spp. candidates					
	1C	31C	47B	54A	78A	93B
Amoxicillin (AML10)	20 (I)	20 (I)	23 (S)	24 (S)	22 (S)	24 (S)
Ampicillin (AMP 10)	23 (S)	20 (S)	26 (S)	25 (S)	23 (S)	22 (S)
Erythromycine (E15)	30 (S)	28 (S)	18 (S)	30 (S)	30 (S)	25 (S)
Florfenicol (FFC30)	28 (S)	25 (S)	25 (S)	30 (S)	31(S)	25 (S)
Neomycine (N30)	26 (S)	21 (S)	17 (S)	22 (S)	18 (S)	21 (S)
Spectinomycin (SH25)	10 (R)	13 (R)	8 (R)	12 (R)	10 (R)	13 (R)
Oxytetracycline (OT30)	12 (R)	8 (R)	20 (I)	14 (R)	15 (I)	26 (S)
Sulfamethoxazole (RL25)	20 (S)	8 (R)	21(S)	15 (I)	15 (I)	8 (R)
Trimethoprim (W5)	26 (S)	26 (S)	38 (S)	25 (S)	25 (S)	26 (S)
Tetracycline (TE30)	17 (S)	14 (I)	20 (S)	17 (S)	17 (S)	28 (S)
Ciprofloxacine (CIP5)	32 (S)	30 (S)	32 (S)	32 (S)	38 (S)	33 (S)

S - Sensitive; I - Intermediate; R – Resistant

Acidic and bile salt tolerance of *Bacillus* spp.

To make the mixed probiotic feed in the *in vivo* experiment, two strains of *Bacillus* spp. were selected to test for low pH and bile salt tolerance. The strain 54A (*B. amyloliquefaciens*), showing the highest protease activity and 47B (*B. pumilus*) with high inhibition zone to *E. ictaluri* were selected for this analysis.

The effect of low pH on the survival of probiotic 54A (*B. amyloliquefaciens*) and 47B (*B. pumilus*) is shown in Table 5. The spore and vegetative cells of 54A and 47B exhibited high tolerance in pH 2 and 3 after 1 h of incubation, with full colonization on the streak line. However, the vegetative cell of 47B grew weakly after 3 h and could not survive after 24 h of incubation. The strain 54A showed better tolerance in pH 2 and 3 than 47B, with good colonization after 3 h. The spore and vegetative cells of strain 54A and 47B were not tolerant for 24 h.

In the results of bile salt tolerance, the vegetative cells of 47B colonized well for 1 h at 0.5% and weakly after 3 h, then they acclimated and grew well after 24 h. However, the strain could not survive after 24 h at 1% and 2% of bile salt. The vegetative cells of 54A also grew weakly after 3 h and could not survive at 2% after 24 h. Compared to the vegetative cell, the spore of 47B also showed lower colonization after 3 h and tolerance after 24 h at 0.5% and 1% of

bile salt, but it could not grow after 24 h at 2%. The spore of 54A showed better tolerance in bile salt than 47B. Although it showed weak colonization after 3 h at 1% and 2% of bile salt, it acclimated well with better colonization after 24 h (Table 6).

DISCUSSION

The striped catfish (*P. hypophthalmus*) is one of the most important freshwater fish species in Vietnam, and exported to 80 countries around the world (FAO, 2012) with production of 1.2 million tons in 2012 (FAO Globefish, 2012). Applying new methods to maintain high yields of striped catfish and reduce damage to the environment would be very beneficial. Therefore, the use of probiotics can be considered as an effective alternative solution for disease prevention in the aquaculture industry. In aquaculture, the use of *Bacillus* sp. as a probiotic has developed rapidly and been applied in many species of aquatic animals (Hong *et al*., 2005). To our knowledge, little research has been conducted regarding *Bacillus* sp. probiotic application in catfish and there is no report about probiotics isolated from striped catfish intestines. Ran *et al*. (2012) analyzed antagonism of *Bacillus* spp. isolated from channel catfish intestine against *E. ictaluri* and *A. hydrophila*. In this study, *Bacillus* spp. from striped catfish intestines was identified and investigated with the aim of application as a feed supplement for the host.

Some molecular methods have been used for *Bacillus* spp. identification including pulsed field gel electrophoresis (PFGE), sequencing of 16S rRNA, gyrA, gyrB and rpoB genes, repetitive sequence-based PCR (REP-PCR) and random amplification polymorphic PCR (RAPD-PCR) (Liu *et al*., 1997; Yamada *et al*., 1999; Herman and Heyndrickx, 2000; Kwon *et al*., 2009). In this study, identification of *Bacillus* species has been proven successful by

Table 5. Survival of *Bacillus* spp. in low pH after period of incubation time

Strain		Survival appearance								
		pH 2			pH 3			pH 7		
		1h	3h	24h	1h	3h	24h	1h	3h	24h
47B	Vegetative cell	++	+	-	++	+	-	++	+	-
	Spore	++	++	-	++	++	-	++	++	-
54A	Vegetative cell	++	++	-	++	++	-	++	++	-
	Spore	++	++	-	++	++	-	++	++	-

(++), many colonies on the streak line; (+), a few colonies on the streak line; (-), no growth

Table 6. Survival of *Bacillus* spp. in bile salt after definite incubation time

Strain		Survival appearance											
		0.5%			1%			2%			0%		
		1h	3h	24h	1h	3h	24h	1h	3h	24h	1h	3h	24h
47B	Vegetative cell	++	+	++	++	+	-	++	+	-	++	++	++
	Spore	++	+	++	++	+	++	++	+	-	++	++	++
54A	Vegetative cell	++	+	++	++	+	++	++	+	-	++	++	++
	Spore	++	++	++	++	+	++	++	+	++	++	++	++

Legend: (++): many colonies on the streak line; (+): a few colonies on the streak line; (−): no growth

designing specific primers on 16S rRNA genes and running multiplex PCR. Compared to conventional microbiological methods, this PCR technique can save time and laboratory materials. Hence, this will be beneficial for the identification of large numbers of strains, and differentiation of closely related *Bacillus* species. Research by De Clerck and De Vos (2004) and Wang *et al*. (2007) using a combination of *rpoB* and *gyrB* gene sequencing can also improve identification and classification of closed *Bacillus* groups.

The *Bacillus* spp. population isolated in An Giang, Can Tho and Dong Thap provinces was detected by PCR method. Kim *et al*. (2010) identified and investigated *Bacillus* spp. diversity in *doenjang* using SDS-Page profiles of whole cell protein and the 16S rRNA gene sequencing method. *B. subtilis* was the most prevalent in the samples, and *B. licheniformis* and *B. amyloliquefaciens* were uncommonly recognized in 197 isolates. Derzelle (2015) analyzed molecular diversity of *B. anthracis* in the Netherlands using whole genome SNP discovery. Distribution of *B. thuringiensis* population in Yunnan and Hainan Provinces in China was investigated by identifying 31 holotype cry genes (Su *et al*., 2007). Distribution and diversity also depend on a variety of factors in the fish gut environment including variation of temperature, salinity, digestive physiology and feeding strategy (Ghanbari *et al*., 2015). In this study, *B. megaterium* was the most commonly found, and the next was *B. amyloliquefaciens* in a total of 120 isolates. *B. megaterium* is widespread in soil and it has also been found in cow feces, Emperor Moth caterpillars, and Greater Wax Moth frass (Vos *et al*., 1984). It can grow well in the temperature range 30-37^{o}C and has been found in Antarctica in a geothermal lake at temperatures up to 63^{o}C (Vos *et al*., 1984). There are many benefits from *B. megaterium* which has been an important microorganism in industry in recent years such as producing penicillin, amylase for use in the baking industry and glucose dehydrogenase for glucose examination (Vary *et al*., 2007). *B. megaterium* has also been applied in feed supplementation for the growth of shrimp *Litopenaeus vannamei* cultured in East Java of Indonesia (Ating *et al*., 2013). *B. amyloliquefaciens* is also beneficial in aquaculture to control Edwardseillosis in Catla (*Catla catla*) (Das *et al*., 2013), and applied for remediation for aquaculture pond water (Xie *et al*., 2013). It was very interesting to find high numbers of *B. megaterium* and *B. amyloliquefaciens* in this study.

The differences in the distribution of *Bacillus* species among the samples tested may be associated with the optimal conditions of the environment in various places (Darwin, 1859). Based on the results of variation in *Bacillus* spp. distribution in the three provinces, *B. subtilis, B. megaterium and B. pumilus* showed similar populations in An Giang and Can Tho, suggesting that the regional similarity of these two locations might lead to similar diversity of species. *B. megaterium* was the main species of *Bacillus* population in An Giang and Can Tho; in contrast, *B. amyloliquefaciens* and *B. cereus* were the most prevalent in Dong Thap province. Biofactors such as competition for food also affect species distribution and the more the environment changes, the more the species changes to adapt to its habitat (Darwin, 1859). A small number of *Bacillus* spp. were not identified in An Giang and Can Tho and they should be further studied for the possibility of discovering new species.

The selection of probiotic bacteria for aquaculture was based on the inhibition activity against the pathogen, and the increased resistance of the host to the pathogens (Moriarty, 1998). To evaluate and choose potential probiotics for control of the pathogen, *in vitro* antibacterial activity was selected as the primary criterion. Since *E. ictaluri* bacteria are the main cause of high mortality in striped catfish, identifying *Bacillus* strains that can be applied to inhibit the growth of this pathogen is essential. The genus Bacillus was the considered candidate because it was easy to find in the natural water environment and gastrointestinal tract of animals (Vos *et al*., 1984). However, in this study, there were not many candidates that showed strong inhibition to *E. ictaluri* with inhibition zone ≥ 10 mm and most were *B. amyloliquefaciens* and *B. megaterium*. Ran *et al*. (2012) found a *B. subtilis* group isolated from channel catfish intestine against *E. ictaluri* with antagonism zone of ≥10 mm and there were no *B. megaterium* and *B. amyloliquefaciens* in their selected isolates. Research by Newaj-Fyzul *et al*. (2007) showed that *B. subtilis* was antagonistic with *Aeromonas hydrophila*. Sugita *et al*. (1998)

demonstrated *Bacillus* strain NM12 isolated from dragonets (*Callionymus* sp.) against *Vibrio vulnificus.* Das *et al*. (2013) reported that the strain *B. amyloliquefaciens* FPTB16 inhibited *E. tarda* in catla (*Catla catla*). For the first criterion, we selected six *Bacillus* spp. including *B. amyloliquefaciens* (48C, 51G, 93B), *B. megaterium* (4A, 62D) and *B. pumilus* (47B) which showed strong inhibition activity to control the pathogen in striped catfish culture.

Another criterion that was also important for selecting the probiotics was producing an enzyme to encourage the growth performance of the host (Aditya *et al*., 2008). *Bacillus* strains have the advantage of a great variety of extracellular enzymes to support digestion (Moriarty, 1996). Some studies demonstrated that supplementation of probiotic *Bacillus* stimulated the production of extracellular enzymes and improved the growth of animals (Sun *et al*., 2010; Liu *et al*., 2012; Cha *et al*., 2013; Zokaeifar *et al*., 2014). Therefore, investigation of the production of extracellular enzyme from *Bacillus* species was important. Protein is considered as a major nutrient component in fish feed and required for fish growth. Protease produced by *Bacillus* can help protein digestion with better absorption in the gut to enhance fish growth. Previous research has demonstrated the effects of *Bacillus* spp. producing high protease on digestion and growth performance of aquatic animals. *Bacillus* subtilis E20 showed high proteolysis (16.6 U/mg) and enhanced the growth of white shrimp larvae, *Litopenaeus vannamei* at the level of 10^7 and 10^8 CFU/g diet (Liu *et al*., 2009), and grouper *Epinephelus coioides* at 10^8 CFU/g feed (Liu *et al*., 2012). Based on our results, the strains *B. amyloliquefaciens* 54A and 93B could be used as probiotics to enhance digestibility and increase the growth of striped catfish.

After animal feeding, probiotics are moved to the intestinal tract through different segments of the digestive system, and they are greatly influenced by stomach acid (pH 2-3) before transportation to the intestine (Hong *et al*., 2005). Therefore, the ability of resistance in stomach acid is an important characteristic of bacteria used as probiotic supplements in food. According to Liong and Shah (2005), acid resistance at pH 3 was the critical criterion in the tolerance test to select probiotic, and feed could be digested in the stomach for 90 min before moving to the intestine (Prasad *et al*., 1998; Haddadin *et al*., 2004). The results of this study indicated that vegetative cells and spore of *B. amyloliquefaciens* 54A and *B. pumilus* 47B are good tolerance candidates in low pH 2-3 for 3 h. Wang *et al*. (2010) reported that *Bacillus* strains including *B. licheniformes*, *B. amyloliquefaciens* and *B. subtilis* are able to survive in pH 2 for 3 h. The acid tolerance of *B. cereus* 141 in pH 2 and 4 for 1, 2 and 4 h was determined by Chen *et al*. (2009). However, not all *Bacillus* spp. are tolerant to gastric fluid; for example, *B. coagulans* strains are susceptible to acidic stomach juice (Hyronimus *et al*., 2000), or *B. cereus* isolates which commonly appeared in Biosubtyl products expressed very high susceptibility to gastric fluid (Duc *et al*., 2004).

The liver synthesizes bile salts and transfers them to the gall bladder for fatty acid digestion in the small intestine (John, 2011). Probiotic stability in bile acid is one criterion before it can be a beneficial product for animal or human health (Carlos *et al*., 2010). Gilliland *et al*. (1984) suggested that a level of 0.3-2% bile salts is the minimum inhibitory concentration (MIC) to select potential probiotic strains. In the previous studies, there are different results of bile tolerance on different strains and the explanation for these is still unknown (Charteris *et al*., 1998). For examples, *B. subtilis* cells were inhibited at bile salts 0.2% and not colonized at all in 1 h (Duc *et al*., 2003). Another study showed that 0.4, 0.2, and < 0.05% of bile salts were MIC for *B. subtilis*, *B. cereus* ĮP5832, and *B. clausii*, respectively (Spinosa *et al*., 2000). In our study, the spore forms of *B. pumilus* 47B and *B. amyloliquefaciens* 54A were resistant in bile salts at 2% for 3 h, therefore they could be used as probiotic supplement in the feed.

Antimicrobial substances have been widely used in Vietnamese aquaculture to treat bacterial infections for disease control (Jeney *et al*., 1998; Crumlish *et al*., 2002; Samira *et al*., 2007). Overuse of antibiotics in the aquaculture environment is the main cause of transferring antimicrobial resistant bacteria to terrestrial animals and humans (FAO/OIE/WHO, 2006). There are several related occurrences of

resistant gene transfer from probiotic microorganisms (SCAN, 1999; SCAN, 2003). Therefore, analysis of antibiotic susceptibility of the probiotic strains is very necessary to guarantee food safety for fish consumers. Our study found that six *Bacillus* candidates were susceptible to most antibiotics in the test, however, some were resistant to oxytetracycline and sulphamethoxazole and it is worth mentioning that all the isolates displayed resistance to spectinomycin. There are many reports about the antibiotic resistance of bacteria found in striped catfish farms (Crumlish *et al*., 2002; Samira *et al*., 2007; Dung *et al*., 2008). Further studies should investigate drug resistance gene transfer, and identify plasmid containing genes involved in antibiotic resistance on the *Bacillus* probiotic.

CONCLUSION

In conclusion, *B. megaterium* was the main *Bacillus* species in the intestines of striped catfish cultured in ponds. The population of *B. megaterium, B. subtilis and B. pumilus* in An Giang and Can Tho provinces was similar. Antibiotic resistance of *Bacillus* sp. was found in spectinomycin (SH25). The strains *B. amyloliquefaciens* 54A and *B. pumilus* 47B were selected as potential probiotics with properties of high pathogen inhibition, protease activity, and tolerance in gastric fluids, and bile salts. The selected isolates will be further evaluated through *in vivo* trials on growth performance, immune stimulation, pathogen resistance and improved stress tolerance of striped catfish.

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LITERATURE CITED

Aditya, K.W., K. Heinrich, M.J. Lategan and L. Gibson. 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. **Aquaculture** 274: 1-14.

Ai, Q.H., H.G. Xu, K.S. Mai, W. Xu and J. Wang. 2011. Effects of dietary supplementation of *Bacillus* subtilis and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*. **Aquaculture** 317: 155-161.

Ating, Y., A.G. Dodit, A. Maftuch and H. Martinah. 2013. Response of indigenous *Bacillus megaterium* supplementation on the growth of *Litopenaeus vannamei* (Boone), a new target species for shrimp culture in East Java of Indonesia. **J. Basic Appl. Sci. Res.** 3: 747-754.

Avella, M.A., G. Gioacchini, O. Decamp, P. Makridis and O.C. Bracciatelli. 2010. Application of multi-species of *Bacillus* in sea bream larviculture. **Aquaculture** 305: 12-19.

Baker, R. 2006. Health management with reduced antibiotic use-the U.S. experience. **Anim. Biotechnol.** 17: 195-202.

Beg, Q.K., R.K. Saxena and R. Gupta. 2002. De-repressionand subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. **Process Biochem.** 37: 1103-1109.

Birnboim, H.C. and J. Dolly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. **Nucleic Acids Res.** 7: 1513-1523.

Bradford, M.M. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** 72: 248–254.

Carlos, R.S., P.D.S.V. Luciana, R.S. Michele, B.P.M. Adriane, T.Y. Caroline, D.D.L. Juliano, P. Ashok and T.S. Vanete. 2010. The Potential of Probiotics: A Review. **Food Technol. Biotech.** 48: 413-434.

Cha, J.H., S. Rahimnejad, S.Y. Yang, K.W. Kim and K.J. Lee. 2013. Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. **Aquaculture** 402-403: 50-57.

Charteris, W., P. Kelly and L. Morelli. 1998. Ingredient selection criteria probiotic microorganisms in functional dairy foods. **Int. J. Dairy Technol.** 51: 123-136.

Chen, J.L., M.L. Chianga and J. Collins. 2009. Survival of the acid-adapted *Bacillus cereus* in acidic environments. **Int. J. Food Microbiol.** 128: 424-428.

Clinical and Laboratory Standard Institute. 2007. **Performance standards for antimicrobial susceptibility tests; seventeenth informational supplement.** Wayne, Pennsylvenia: Clinical and Laboratory Standard Institute

Clinical and Laboratory Standard Institute. 2012. **Performance standards for antimicrobial disk susceptibility tests; approved standard.** Wayne, Pennsylvenia: Clinical and Laboratory Standards Institute.

Crumlish, M., T.T. Dung, J.F. Turnbull, N.T.N. Ngoc and H.W. Ferguson. 2002. Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. **J. Fish Dis.** 25: 733-736.

Darwin, C.H.A. 1859. **The Origin of Species.** London, John Murrat, Albemale street.

Das, A., K. Nakhro, S. Chowdhury and D. Kamilya. 2013. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). **Fish Shellfish Immunol.** 35: 1547-1553.

De Clerck, E. and P. De Vos. 2004. Genotypic diversity among *Bacillus licheniformis* strain from various sources. **FEMS Microbiol. Lett.** 231: 91-98.

Derzelle, S., G. Girault, H.J. Roest and M. Koene. 2015. Molecular diversity of *Bacillus anthracis* in the Netherlands: Investigating the relationship to the worldwide population using whole-genome SNP discovery. **Infect. Genet. Evol.** 32: 370-376.

Duc, L.H., H.A. Hong and S.M. Cutting. 2003. Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. **Vaccine** 21: 4215-4224.

Duc, L.H., H.A. Hong, T.M. Barbosa, A.O. Henriques, S.M. Cutting. 2004. Characterization of *Bacillus* probiotics available for human use. **Appl. Environ. Microbiol.** 70:2161-2171.

Dung, T.T., F. Haesebrouck, A.N. Tuan, Z. Jeney and N.A. Tuan. 2008. Antimicrobial susceptibility pattern of *Edwardsiella ictaluri* isolates from natural outbreaks of *Bacillary Necrosis* of *Pangasianodon hypophthalmus* in Vietnam. **Microb. Drug Resist.** 14: 311-316.

Dunham, R.A., G.W. Warr, A. Nichols, P.L. Duncan, B. Argue, D. Middleton and H. Kucuktas. 2002. Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. **Mar. Biotechnol.** 4: 338-344.

El-Sersy, N.A., F.A. Abdelrazek and S.M. Taha. 2006. Evaluation of various probiotic bacteria for the survival of *Penaeus japonicus* larvae. **Fresenius Environ. Bull.** 15: 1506-1511.

FAO. 2010. **State of world fisheries and aquaculture.** Rome: FAO.

FAO. 2012. **Cultured aquatic species information programme *Pangasius hypophthalmus* (Sauvage, 1878).** http://www.fao.org/fishery/culturedspecies/ Pangasius_hypophthalmus/en. Cited 25 Dec 2012.

FAO GLOBEFISH. 2012. **Market report-Pangansius June 2012.** http//www. globefish.org/ pangasius-june-2012.html. Cited 30 Dec 2012.

FAO/OIE/WHO. 2006. **Antimicrobial use in aquaculture and antimicrobial resistance.** Geneva, Switzerland: Department of food safety, zoonoses and foodborne disease and World Health Organization.

Ghanbari, M., W. Kneifel and K. J. Domig. 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. **Aquaculture** 448: 464-475.

Gilliland, S.E., T.E. Staley and L.J. Bush. 1984. Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. **J. Dairy Sci.** 67: 3045-3051.

Gomaa, O.M. and O.A. Momtaz. 2007. 16S rRNA characterization of a *Bacillus* isolate and its tolerance profile after subsequent subculturing. **Arab J. Biotechnol.** 10: 107-116.

Haddadin, M.S.Y., S.S. Awaisheh and R.K. Robinson. 2004. The production of yoghurt with probiotic bacteria isolated from infants in Jordan. **Pak. J. Nutr.** 3: 290-293.

Hawke, J.P., R.M. Durborow, R.L. Thune and A.C. Camus. 1998. **Enteric Septicemia of Catfish.** Southern Regional Aquaculture Center Publication No 477.

Herman, L. and M. Heyndrickx. 2000. The presence of intragenically located REP-like elements in *Bacillus sporothermodurans* is sufficient for REP-PCR typing. **Res. Microbiol.** 151: 255–261.

Hong, H.A., L.H. Duc and S.M. Cutting. 2005. The use bacterial spore formers as probiotics. **FEMS Microbiol.** Rev. 29: 813-835.

Hyronimus, B., C. Le Marrec, A.H. Sassi and A. Deschamps. 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. **Int. J. Food Microbiol.** 61: 193-197.

Jeney, G., T.T. Dung, D.T.H. Oanh, Z. Jeney and N. A. Tuan. 1998. Preliminary results of the baseline survey on fish health management in freshwater aquaculture of the Mekong Delta of Vietnam. **J. Appl. Ichthyol.** 14: 223.

John, E.H. 2011. Guyton and Hall **Textbook of Medical Physiology.** U.S.: Saunders Elsevier

Jose, L.B.Z., I.D. Blas, R.Z. Imanol, C. David, V. Daniel, L.M.Z. Jose. 2006. The role of probiotics in aquaculture. **Vet. Microbiol.** 114: 173-186.

Kawasaki, H., Y. Hoshino, A. Hirata and K. Yamasato. 1993. Is intracytoplasmic membrane structure a generic criterion? It does not coincide with phylogenetic interrelationships among phototrophic purple nonsulfur bacteria. **Arch. Microbiol.** 160: 358-362.

Kim, T.W., Y.H. Kim, S.E. Kim, J.H. Lee, C.S. Park and H.Y. Kim. 2010. Identification and distribution of *Bacillus* species in *doenjang* by whole-cell protein patterns and 16S rRNA gene sequence analysis. **J. Microbiol. Biotechnol.** 20: 1210-1214.

Kwon, G.H., H.A. Lee, J.Y. Park, J.S. Kim, J. Lim, C.S. Park, D.Y. Kwon, Y.S. Kim and J.H. Kim. 2009. Development of RAPD-PCR method for identification of *Bacillus* species isolated from Cheonhhukjang. **Int. J. Food Microbiol.** 129: 282-287.

Leyton,Y.E., R.A. Varas Psija and C.E. Riquelme. 2012. Probiotic activity of bacteria associated with egg capsules of *Concholepas* (common name 'Loco'). **Aquac. Res.** 43: 1089-1095.

Liong, M.T. and N.P. Shah. 2005. Acid and bile tolerance and cholesterol removal ability of *Lactobacilli* strains. **J Dairy Sci.** 88: 55-66.

Liu, C.H., C.H. Chiu, S.W. Wang and W. Cheng. 2012. Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. **Fish Shellfish Immunol.** 33: 699-706.

Liu, K.F., C.H. Chiu and Y.L. Shiu. 2010. Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. **Fish Shellfish Immunol.** 28: 837-844.

Liu, C., C. Chiu, P. Lin, W. Cheng and C.H. Liu. 2009. Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease producing probiotic, *Bacillus subtilis* E20 from natto. **J. Appl. Microbiol.** 107: 103-141.

Liu, P.Y.F., S.C. Ke and S.L. Chen. 1997. Use of pulsed-fields gel electrophoresis to investigate a pseudo-outbreak of *Bacillus cereus* in a pediatric unit. **J. Clin. Microbiol.** 35: 1533-1535.

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

Moriarty, D.J.W. 1996 Microbial biotechnology: a key ingredient for sustainable aquaculture. **Infofish Int.** 4: 29 - 33.

NavinChandran, M., P. Iyapparaj, S. Moovendhan, R. Ramasubburayan, S. Prakash, G. Immanuel and A. Palavesam. 2013. Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in *P. monodon*. **Fish Shellfish Immunol.** 40: 122-130.

Nayak, S.K., P. Swain and S.C. Mukheriee. 2007. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita*. **Fish Shellfish Immunol.** 23: 892-896.

Newai-Fyzul, A., A.A. Adesiyun, A. Mutani, A. Ramsubhag, J. Brunt. 2007. *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchusmykiss Walbaum*). **J. Appl. Microbiol.** 103:1699-1706.

Peng, Y., X.J. Yang , L. Xiao and Y.Z. Zhang. 2004. Cloning and expression of a fibrinolytic enzyme (subtilisin DFE) gene from *Bacillus amyloliquefaciens* DC-4 in *Bacillus subtilis*. **Res. Microbiol.** 155: 167-173.

Phan, L., T. Bui, T. Nguyen and B. Ingram. 2009. Current status of farming practices of striped catifish, *Pangasianodon hypophthalmus* in the Mekong Delta, Vietnam. **Aquaculture** 296: 227-236.

Prasad, J., H.S. Gill and J. Smart. 1998. Selection and characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. **Int. Dairy. J.** 8:993-1002.

Ran, C., C. Abel, A.W. Malach and P.K. Gopal. 2012. Identification of *Bacillus* strains for biological control of catfish pathogens. **Plos One** 7: 1-9.

Russell, D.W. and J. Sambrook. 2001. **Molecular cloning: a laboratory manual.** Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory.

Samira, S., H.N.N. Kha, T.L. Hung, J. Lazard and D. Montet. 2007. Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. **Food Control** 18: 1391-1396.

SCAN. 1999. **Opinion of the scientific steering committee on antimicrobial resistance. European commission, health and consumer protection directorate-general. (SCAN) Scientific Committee on Animal Nutrition.** http://europa. eu.int/comm/food/fs/sc/ssc/out50_en.pdf. Cited 13 June 2013.

SCAN. 2003. **Opinion of the scientific committee on animal nutrition, on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance.** European Commission, Health and Consumer Protection Directorate-General. (SCAN) Scientific Committee on Animal Nutrition. http://europa.eu.int/comm/food/fs/ sc/scan/out108_en.pdf. Cited 22 June 2013.

Su, X.D., C.L. Shu, J. Zhang, D.F. Huang, J.X. Tan and F.P. Song. 2007. Identification and distribution of *Bacillus thuringiensis* isolates from primeval forests in Yunnan and Hainan provinces and Northeast Region of China. **Agric. Sci. China** 6: 1343-1351.

Sugita, H., Y. Hirose, N. Matsuo and Y. Deguchi. 1998. Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. **Aquaculture** 165: 269-280.

Sun, Y.Z., H.L. Yang, R.L. Ma and W.Y. Lin. 2010. Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*. **Fish Shellfish Immunol.** 29: 803-809.

Spinosa MR., T. Braccini, E. Ricca, M. De Felice, L. Morelli, G. Pozzi and M.R. Oggioni. 2000. On the fate of ingested *Bacillus* spores. **Res. Microbiol.** 15: 361-368.

Telli, G.S., M.J. Ranzani-Paiva, C. Dias-Dde, F.R. Sussel, C.M. Ishikawa, L. Tachibana. 2014. Dietary administration of *Bacillus subtilis* on hematology and non-specific immunity of Nile tilapia *Orechromis niloticus* raised at different stocking densities. **Fish Shellfish Immunol.** 39: 305-311.

Vary, P., R. Biedendieck, T. Fuerch, F. Meinhardt, M. Rohde, W. Deckwe and D. Jahn. 2007. *Bacillus megaterium* from simple soil bacterium to industrial protein production host. **Appl. Microbiol. Biotechnol.** 76: 957-967.

Velho-Pereira, S. and N.M. Kamat. 2011. Antimicrobial screening of actinobacteria using a modified cross streak method. **Indian J. Pharm. Sci.** 73: 223-228.

Vos, P., G. Garrity, D. Jones, N.R. Krieg, W. Ludwig, F.A. Rainey, K.H. Schleifer and W.B. Whitman. 1984. **Bergey's *Manual of Systematic Bacteriology.*** Springer-Verlag, New York.

Wang, L.T., F.L. Lee, C.J. Tai, H. Kasai. 2007. Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. **Int. J. Syst. Evol. Micr.** 57: 1846-1850.

Wang, Y., H. Zhang, L. Zhang, W. Liu, Y. Zhang, X. Zhang and T. Sun. 2010. *In vitro* assessment of probiotic properties of *Bacillus* isolated from naturally fermented congee from Inner Mongolia of China. **World J. Microb. Biot.** 26: 1369-1377.

Xie, F., T. Zhu, F. Zhang, K. Zhou, Y. Zhao and Z. Li. 2013. Using *Bacillus amyloliquefaciens* for remediation of aquaculture water. **SpringerPlus** 2:119.

Yamada, S., E. Okashi, N. Agata and K. Ventkateswaran. 1999. Cloning and nucleotide sequence analysis of gyrB of *Bacillus cereus, B. thuringiensis, B. mycoides* and *B. anthracis* and their application to the detection of *B. cereus* in rice. **Appl. Environ. Microbiol.** 65: 1483-1490.

Zokaeifar, H., N. Babaei, C.R. Saad, M.S. Kamarudin, K. Sijam and J.L. Balcazar. 2014. Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp, *Litopenaeus vannamei.* **Fish Shellfish Immunol.** 36: 68-74.