Full Paper

A novel probiotic *Bacillus siamensis* B44v isolated from Thai pickled vegetables (*Phak-dong*) for potential use as a feed supplement in aquaculture

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The use of probiotic bacteria to control bacterial infection in farmed fish is of clear practical interest. The aims of this study were to isolate and select a probiotic *Bacillus* sp. and to evaluate the effects of its supplementation on the growth and disease resistance of hybrid catfish. *Bacillus siamensis* strain B44v, selectively isolated from Thai pickled vegetables (*Phak-dong*), displayed a high potential as a probiotic in catfish culture. This bacterium produced a bacteriocin-like substance and exhibited a broad-spectrum antibacterial activity inhibiting both Gram-positive and Gram-negative bacteria, especially the fish pathogens *Aeromonas hydrophila* and *Streptococcus agalactiae*. The susceptibility to all 14 antibiotics tested implies its less possibility to be the antibiotic-resistant bacterium. *Bacillus siamensis* strain B44v possessed interesting adhesion properties, as shown by its high percentages of hydrophobicity (64.8%), auto-agglutination (73.8%), co-aggregation (67.2% with *A. hydrophila* FW52 and 63.5% with *S. agalactiae* F3S), and mucin binding (88.7%). The strain B44v survived simulated gastrointestinal conditions and produced protease and cellulase enzymes. Hybrid catfish (*C. macrocephalus* ¥ *C. gariepinus*) were employed in the feed-trial experiments. Fish fed diet containing strain B44v (10^7 CFU/g feed) displayed not only no mortality but also growth improvement. At the end of the feed trial, fish were challenged by an intraperitoneal injection of *Aeromonas hydrophila* FW52. The *Bacillus siamensis* strain B44v fed fish survived (75.0%; *p* < 0.05) better than the controls (36.7%; *p* < 0.05) after a two week challenge. These collective results present for the first time the potential of *Bacillus siamensis* strain B44v for use as a bacterial probiotic in aquaculture.

Key Words: aquaculture; *Bacillus siamensis*; catfish; fermented food; probiotic; Thai pickled vegetables

Introduction

Thailand is one of the top fish producing nations in the world in which over 3.9 million tons of fish products were reported in the year 2007. The gross domestic product (GDP) of the fishery sector in 2008 was 104.2 billion baht, which accounted for 1.2% of the national GDP. The fish have been not only for domestic consumption but also for export, with Japan, U.S.A., and EU being the main markets. Since 1984, the propagated hybrid catfish (Clarias gariepinus ¥ Clarias macrocephalus) has been produced commercially and has increasingly gained popularity among farmers in Thailand. For decades, the hybrid catfish has been the second most important species for freshwater aquaculture in Thailand, next only to the Nile tilapia (FAO, 2012). Its meat is one of the most important protein sources because of its low cost, high availability, and high nutritional value. The aquaculture of hybrid catfish in Thailand has been expanding, and the current annual production is approximately 140,763 tons per year (De-
part of Fisheries, 2012). To meet the increasing demand for hybrid catfish, production has been carried out by means of fish farming. Hybrid catfish reared in intensive aquaculture are often exposed to stressful conditions resulting in poor growth, low immunity, and vulnerability to infectious diseases.

Bacterial infections are the most important causes of disease problems in farmed fish. Among bacteria identified as pathogens in hybrid catfish farming, *Aeromonas hydrophila* has been considered the most economically important pathogen, because its infection retards growth and causes an unmarketable appearance of infected fish (Na-Nakorn et al., 1994). To solve this problem, farmers frequently use antibiotics to treat bacterial diseases (Cabello, 2006). Extensive application of antibiotics leads to the development and spread of antibiotic-resistant bacteria and the presence of antibiotic residues in aquaculture products and the environment (Smith, 2008). Recently probiotics have been introduced as alternative methods to control diseases in aquaculture. Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal balance (FAO/WHO, 2006; Fuller, 1989).

Several microbial strains have been evaluated as probiotics in aquaculture including lactic acid bacteria (Pérez-Sánchez et al., 2011), *Bacillus* (Han et al., 2015), actinobacteria (Das et al., 2010) and yeasts (Tovar-Ramirez et al., 2010). Thai fermented foods have been reported to be a potential source of probiotic bacteria, such as *Lactobacillus* spp. from fermented pork, fermented fish, fermented tea leaves and pickled garlic (Klayraung et al., 2008) and *Bacillus* sp. from fermented soybean (*Thua nao*) (Inatsu et al., 2006). Lactic acid bacteria, especially in the genus *Lactobacillus*, have been well-studied probiotics for a long time. Recently, the gram positive spore forming *Bacillus* spp. have received increasing interest for use as probiotics in the fish farming industry (Martínez Cruz et al., 2012). Spore-forming *Bacillus* spp. have several advantages over other non-spore formers being used as probiotics. Their endospores are resistant to harsh environmental conditions and suitable for the formulation of stable probiotics, and they tolerate the acidic and alkaline conditions in gastrointestinal tracts. Basically the important criteria for screening probiotics include non-pathogenicity, antagonistic activity against pathogens, antibiotic susceptibility, tolerance to gastrointestinal conditions, digestive enzyme production, and intestinal mucosa adhesion (Fjellheim et al., 2010; Fontana et al., 2013). Therefore, in this study, we have sought to isolate *Bacillus* spp. from Thai indigenous fermented foods and to evaluate their probiotic properties for potential use in aquaculture.

**Materials and Methods**

**Isolation of Bacillus spp.** Various fermented foods were collected from local markets in northeastern Thailand. The spore-forming bacteria were isolated using heat treatment as described by Guo et al. (2006). Food samples were enriched in a Mixed Nutrient Broth (MNB: peptone, 5.0 g/l; beef extract, 3.0 g/l; MgSO₄·7H₂O, 0.5 g/l) and incubated at 35°C for 24 h prior to heat treatment at 80°C for 15 min, followed by serial dilution and plated on a Mixed Nutrient Agar (MNA) and incubated at 35°C for 24 h. Colonies with different morphologies were determined for Gram stain, endospore formation and catalase production prior to being stored in 20% (v/v) glycerol at −80°C for further experiments.

**Identification of probiotic Bacillus.** Taxonomic studies of *Bacillus* spp. were determined as described in Bergey’s Manual of Systematic Bacteriology (Logan and De Vos, 2009). The API 50 CHB (BioMerieux) was used to determine carbohydrate fermentation. Molecular characterization was performed according to Tongpim et al. (2014). The 16S rRNA gene was amplified through the polymerase chain reaction (PCR) using universal primers 8F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTACGACTT-3’). The PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen). Sequence homology was compared with 16S rRNA gene sequences available in the National Center for Biotechnology Information (NCBI) and the EzTaxon server (http://www.ezbiocloud.net/eztaxon). The sequences obtained were submitted to NCBI and accession numbers were obtained.

**Antimicrobial activity.** *Aeromonas hydrophila* FW52 and *Streptococcus agalactiae* F3S, the most common causative agents of fish infection in North-eastern Thailand previously isolated from diseased fish (Tongpim et al., 2009) were used as the indicator fish pathogens. The antimicrobial activity of *Bacillus* isolates was assessed against the indicator fish pathogens by a colony overlay assay as described by Barbosa et al. (2005). Examination of bacteriocin-like activity was performed by neutralizing culture filtrate to pH 7.0 and catalase treatment prior to antimicrobial evaluation by agar well diffusion assay (Elegado et al., 1997) against not only indicator fish pathogens but also several foodborne pathogens. The effect of enzymes on the bacterial culture supernatant was carried out following the methods described by Todorov et al. (2011) with slight modifications. Briefly, the bacterial culture supernatant was adjusted to pH 7.0 with 1N NaOH. Two-milliliter aliquots were incubated for 2 h in the presence of 1.0 mg/ml (final concentration) of proteinase K (Sigma), and subsequently tested for antibacterial activity against *A. hydrophila* FW52 and *S. agalactiae* F3S using an agar well diffusion method.

**Survival in simulated gastrointestinal conditions.** Tolerance to simulated gastric and intestinal fluids was performed according to the procedures described by Huang and Adams (2004) with some modifications. The *Bacillus* cultures were grown at 35°C overnight in MNB. Bacterial cells were harvested by centrifugation at 5000 rpm, 4°C for 20 min (Himac CR20B2, Hitachi, Japan) prior to inoculation into simulated gastric fluid [3 mg/ml of pepsin (porcine stomach mucosa; Sigma) adjusted to pH 2.5] to obtain the initial cell concentration ~1.0 × 10⁶ CFU/ml (OD₅₇₀ = 0.1) and incubated at 35°C in a shaker incubator. Bacterial cell counts were performed on MNA plates at 0, 0.5, 1, 2, and 3 hours of incubation. Similarly, the overnight grown cultures were incubated in simulated in-
testinal fluid [1 mg/ml of pancreatin (porcine pancreas; Sigma) and 0.3% bile salts (Oxoid)] adjusted to pH 8. Aliquots were drawn at intervals of 0, 1, 2, 3, and 6 hours for dilution plate count on MNA plates. The percentage survival at each time interval was calculated by a comparison with the viable cell number at 0 h.

**Cellular auto-aggregation and co-aggregation assay.**

Auto-aggregation was performed according to the methods described by Nithya and Halami (2013) with slight modifications. *Bacillus* cultures grown overnight were harvested by centrifugation, washed twice, and resuspended in a phosphate buffered saline (PBS, pH 7.2) followed by a turbidity measurement at 600 nm (OD600). The bacterial suspension was then kept undisturbed at room temperature for 5 h followed by measuring the OD600 of the upper suspension fluid (OD1). The percentage of auto-aggregation (%AAg) was calculated according to the following formula:

\[
\text{AAg} (%) = 1 - \left( \frac{\text{OD}1}{\text{OD}0} \right) \times 100.
\]

Co-aggregation of probiotic *Bacillus* with bacterial fish pathogens, *A. hydrophila* FW52 and *S. agalactiae* F3S, were assayed according to the methods described by Collado et al. (2008). Cell suspensions of *Bacillus* sp. strain B44v, *A. hydrophila* FW52 and *S. agalactiae* F3S were prepared at concentrations of \(1.5 \times 10^7\), \(2.3 \times 10^9\) and \(3.0 \times 10^9\) CFU/ml, respectively. Two milliliters of strain B44v were mixed with 2 ml of individual fish pathogens, resulting in the population ratios of strain B44v: strain FW52 and strain B44v: strain F3S as 1:1.5 and 1:2, respectively.

**Hydrophobicity assays.** Cell surface hydrophobicity was assessed by measuring the bacterial cell adhesion to hydrocarbon according to previously described methods (Hori et al., 2008) with slight modifications. *Bacillus* cultures grown overnight were harvested by centrifugation, washed twice, and resuspended in PBS (pH 7.2) to obtain an OD600 of 0.5 (initial OD). Then, 3 ml of bacterial suspension was mixed with 0.6 ml of xylene by a vortex mixer for 1 min allowing a separation of the organic and aqueous phases for 30 min at room temperature. Then, the final OD of aqueous phase was measured. The percentage of cell surface hydrophobicity was calculated according to the following formula:

\[
\% \text{ hydrophobicity} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100,
\]

where \(A_0\) is the initial OD and \(A_1\) is the final OD.

**Mucin binding assay.** Adhesion to porcine gastric mucin type III (Sigma, USA) of the *Bacillus* was tested following the procedures described by Tallon et al. (2007). *Lactobacillus plantarum* 299v, a known adhesive strain, was used as a positive control. Assays were performed in four replicates and data were expressed as % adhesion according to the following formula:

\[
\% \text{ Adhesion} = \left( \frac{\log \text{ CFU of adhered bacteria}}{\log \text{ CFU of initial bacteria}} \right) \times 100.
\]

**Antibiotic susceptibility assay.** The susceptibility of the probiotic isolates was determined according to the method described by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) using antibiotic discs (Oxoid, England). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as the control bacterial strains.

**Enzyme production.** The probiotic *Bacillus* was evaluated for enzyme production on an agar media. Cellulase, amylase and protease were determined according to the methods described by Mohapatra et al. (2003). Tween medium was used to observe lipase production (Boekema et al., 2007).

**Feed trial and pathogen challenge test in hybrid catfish.** Hybrid catfish (*C. macrocephalus × C. gariepinus*) were fed a basal diet and allowed to acclimatize for 2 weeks. Fish weighing 10–11 g were randomly distributed into nine 60 l tanks (15 fish per tank) and divided into 3 groups. Two groups were fed a basal diet supplemented with each probiotic (10^7 CFU/g feed) strain B44v and strain B51f. The probiotic *Bacillus* added were mostly in spore form as they were cultivated in MNB for 24 h prior to mixing with fish feed followed by heating at 50°C for 1 h to reduce the moisture content. The third group was fed only a basal diet. Chlorine-free water was used throughout the course of the experiment, during which the water temperature ranged from 26.8–31.4°C. Low-pressure electric air pumps provided aeration via airstones and rearing was carried out under static aerated water conditions. Unconsumed feed and fish feces were siphoned out together with 70% water changing daily throughout the experiment period. Fish were fed twice daily (3% of body weight) for one month and growth performance was evaluated based on weight gain, feed conversion ratio (FCR), and specific growth rate (SGR). Afterwards, each fish was challenged by an intraperitoneal injection of 10^6 CFU *A. hydrophila* FW52 and kept under observation for another two weeks. Fish mortality was recorded and the percent survival was determined.

All experiments conducted with the fish were approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of the National Research Council of Thailand (record No. AEKKU 42/2013; reference No. 0514.1.12.2/58).

**Statistics.** A statistical analysis of the data was performed using SPSS 19.0. Student’s t-test was used to calculate statistical significance for the results of *in vitro* assays (auto-aggregation, co-aggregation and hydrophobicity). The one-way analysis of variance (ANOVA) and Duncan multiple range tests were used to evaluate the statistical significance of fish growth and survival results. Differences were considered significant when the p-value was <0.05.

**Results**

**Isolation, selection and identification of probiotic Bacillus spp.**

Using heat treatment, the 180 *Bacillus* isolates obtained were Gram-positive, catalase positive, motile and en-
related to a sequence similarity of 99.93%. Strain B51f was closely related to strain B44v and strain B51f as indicated by API identification test kits, shown in Table 3, identified the isolates as probiotic strains. The isolates could inhibit the growth of the test fish pathogens (1379 bp) and strain B51f (1403 bp) were determined. The nearly complete 16S rRNA gene sequences of strain B44v and strain B51f were deposited in GenBank under the accession numbers KC631806 and KC631807, respectively.

A novel probiotic Bacillus for catfish

**Table 1.** Antibacterial activity of Bacillus siamensis strain B44v and Bacillus sp. strain B51f against fish and foodborne pathogens.

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Source</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas hydrophila FW52</td>
<td>Diseased fish</td>
<td>18 ± 0.5 15 ± 0.7</td>
</tr>
<tr>
<td>Streptococcus agalactiae F3S</td>
<td>Diseased fish</td>
<td>20 ± 0.5 13 ± 0.8</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>Hospital</td>
<td>13 ± 0.5 0</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td>Oyster</td>
<td>11 ± 0.5 0</td>
</tr>
<tr>
<td>Staphylococcus aureus DMST 562</td>
<td>DMST</td>
<td>11 ± 1.5 0</td>
</tr>
<tr>
<td>methicillin-resistant S. aureus (MRSA)</td>
<td>Hospital</td>
<td>12 ± 0.5 0</td>
</tr>
<tr>
<td>Salmonella Enteritidis</td>
<td>Fermented fish</td>
<td>12 ± 1.5 0</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>River water</td>
<td>14 ± 0.5 0</td>
</tr>
<tr>
<td>Salmonella Typhimurium DMST 8023</td>
<td>DMST</td>
<td>14 ± 1.2 0</td>
</tr>
<tr>
<td>Shigella sp.</td>
<td>Salad</td>
<td>15 ± 1.2 0</td>
</tr>
<tr>
<td>Escherichia coli O157:H7</td>
<td>Cow dung</td>
<td>12 ± 1.1 0</td>
</tr>
<tr>
<td>E. coli DMST 4212</td>
<td>DMST</td>
<td>13 ± 0.2 0</td>
</tr>
<tr>
<td>Bacillus cereus DMST 5040</td>
<td>DMST</td>
<td>20 ± 1.1 0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa DMST 4739</td>
<td>DMST</td>
<td>12 ± 1.1 0</td>
</tr>
</tbody>
</table>

DMST: Department of Medical Sciences Type culture collection, Thailand. Values represent mean ± standard deviation (n = 3).

**Table 2.** Effect of some enzymes on bacteriocin activity of B. siamensis strain B44v against the fish pathogens A. hydrophila FW52 and S. agalactiae F3S.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. hydrophila FW52</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>0</td>
</tr>
<tr>
<td>Lipase</td>
<td>16 ± 0.4</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>15 ± 0.7</td>
</tr>
<tr>
<td>α-Amylase</td>
<td>15 ± 0.2</td>
</tr>
</tbody>
</table>

Values represent mean ± standard deviation (n = 3).

Survival in gastrointestinal conditions, auto- and co-aggregation, surface hydrophobicity, and mucin binding

Bacillus siamensis strain B44v and Bacillus sp. strain B51f tolerated simulated gastrointestinal tract conditions. Strain B44v displayed a pronounced tolerance to gastric fluid (for 3 h) and intestinal fluid (for 6 h) with a 96.5 and 91.5 percentage survival, respectively; whereas strain B51f showed an 84.9 and 74.5 percentage survival, respectively (Table 4).

Auto- and co-aggregation, cell surface hydrophobicity, and the mucin binding ability of strains B44v and B51f are shown in Table 4. The auto-aggregation of strains B44v and B51f after 5 hours of incubation were 73.8% and 91.5 percentage survival, respectively; whereas strain B51f showed a higher cell surface hydrophobicity (64.8%) than strain B51f (42.9%). In addition, it displayed a higher mucin binding ability (88.7%) than strain B51f (78.6%), while L. plantarum 299v, a reference adherent strain, displayed 97.6% (Table 4).
Antibiotic susceptibility and enzyme production

*Bacillus siamensis* strain B44v was susceptible to all tested antibiotics including gentamycin (10 μg), vancomycin (30 μg), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), chloramphenicol (30 μg), penicillin G (10 U), teicoplanin (30 μg), cephalothin (30 μg), cefotaxime (30 μg), trimethoprim-sulfamethoxazole (25 μg), norfloxacin (10 μg), and ofloxacin (5 μg). However, *Bacillus* sp. strain B51f was susceptible to all tested antibiotics except clindamycin, chloramphenicol, penicillin G and cephalothin. Using plate assay, *B. siamensis* strain B44v produced protease and cellulase while *Bacillus* sp. strain B51f produced protease and amylase enzymes. Neither organism produced lipase.

Feed trial and pathogen challenge test in hybrid catfish

No mortality and no disease symptoms were observed in the hybrid catfish during 30 days of feeding diets with, and without, *Bacillus* supplementations. The effects of dietary supplements on the growth performances are shown in Table 5. After 30 days of feeding, a significantly better (p < 0.05) growth performance (SGR and weight gain) was observed in the fish groups fed diets containing strains B44v and B51f compared with the control group. Moreover, the FCR of the fish fed diets containing *Bacillus* was significantly lower (p < 0.05) than the FCR of the control fish, indicating the higher efficiency of diets containing probiotic *Bacillus* than the control diet.

After the fish were challenged with *A. hydrophila* FW52, and reared for another two weeks, it was clearly seen that both the probiotic *Bacillus* sp. enhanced protection against *A. hydrophila* FW52 infection (Fig. 2). The average survival of fish fed *B. siamensis* strain B44v and *Bacillus* sp. strain B51f was 75.0% and 63.9%, respectively, which was significantly higher (p < 0.05) than that of the control fish (36.7%).

Discussion

The use of probiotic bacteria as an alternative method for the prevention of bacterial disease, and growth en-
The genera material fish pathogens in aquaculture in Thailand belong to useful antagonistic property as the two most striking bac-
timents of probiotics, as ingested strains need to survive an

industrial level with stability and extended shelf life.

Antimicrobial activity is considered one of the major mechanisms through which probiotics function and, consequently, is also one of the principle criteria for strain selection when screening potential probiotics (Dobson et al., 2012). This study reveals that Bacillus siamensis strain B51f, derived from indigenous fermented foods, displayed strongly antagonistic activity against the bacterial fish pathogens, Aeromonas hydrophila and Streptococcus agalactiae F3S (Co-F3S). Both strains of Bacillus effectively inhibited Gram-positive and Gram-negative bacteria, indicating their broad spectrum as a useful antagonistic property as the two most striking bacterial fish pathogens in aquaculture in Thailand belong to the genera Aeromonas and Streptococcus (Maisak et al., 2013). Besides fish pathogens, the bacteriocin-like substance from B. siamensis strain B44v inhibited several food-borne pathogens (Table 1) suggesting potential applications in human foods. The isolation process, involving heating at 80°C for 15 min, resulted in spore-forming bacteria. The spore-bearing Bacillus species are among the commercially available probiotic products in use today such as Enterogermina®, Bio-Kult®, Sustenex® and Flora-Balance® (Cutting, 2011). This is one of the advantages of Bacillus products as they can be produced at an industrial level with stability and extended shelf life.

Tolerance to acid and bile is one of the primary requirements of probiotics, as ingested strains need to survive an acid condition in the stomach and bile in the small intestine. In this study, B. siamensis strain B44v survived (>90%) the simulated gastrointestinal tract conditions better than the Bacillus sp. strain B51f. Another important criterion for selecting potential probiotic strains is the adhesion ability to the intestinal epithelium (Ouwehand et al., 1999). The determination of the auto-aggregation and hydrophobicity of bacteria is an indirect method of determining the adhesion ability of probiotics to intestinal mucosa (Collado et al., 2008). B. siamensis strain B44v had a higher auto-aggregation and hydrophobicity than the Bacillus sp. strain B51f. Similarly, a mucin binding assay showed the superiority of strain B44v to strain B51f. Strain B44v strongly adhered to porcine gastric mucin at 88.7%. Adhesion to intestinal mucosa is a prerequisite for the bacterial colonization of the host gut (Ouweland et al., 1999). In addition, both Bacillus strains were shown to co-aggregate with the fish pathogens A. hydrophila FW52 and S. agalactiae F3S at greater than 50% (see Fig. 1). Bacterial co-aggregation has a considerable significance in the host gut, as the co-aggregation ability of bacterial probiotics might interfere with the ability of pathogenic bacteria to infect the host and can prevent colonization of the pathogens (Spencer and Chesson, 1994).

Antibiotic susceptibility is another crucial requirement for bacterial probiotics. In this study, the B. siamensis strain B44v was susceptible to all tested antibiotics while the Bacillus sp. strain B51f was resistant to 4 out of 14 antibiotics (Table 1). Antimicrobial activity is considered one of the major mechanisms through which probiotics function and, consequently, is also one of the principle criteria for strain selection when screening potential probiotics (Dobson et al., 2012). This study reveals that Bacillus siamensis strain B51f, derived from indigenous fermented foods, displayed strongly antagonistic activity against the bacterial fish pathogens, Aeromonas hydrophila and Streptococcus agalactiae F3S (Co-F3S). Both strains of Bacillus effectively inhibited Gram-positive and Gram-negative bacteria, indicating their broad spectrum as a useful antagonistic property as the two most striking bacterial fish pathogens in aquaculture in Thailand belong to the genera Aeromonas and Streptococcus (Maisak et al., 2013). Besides fish pathogens, the bacteriocin-like substance from B. siamensis strain B44v inhibited several food-borne pathogens (Table 1) suggesting potential applications in human foods. The isolation process, involving heating at 80°C for 15 min, resulted in spore-forming bacteria. The spore-bearing Bacillus species are among the commercially available probiotic products in use today such as Enterogermina®, Bio-Kult®, Sustenex® and Flora-Balance® (Cutting, 2011). This is one of the advantages of Bacillus products as they can be produced at an industrial level with stability and extended shelf life.

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<table>
<thead>
<tr>
<th>Diet</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Weight gain (g)</th>
<th>FCR</th>
<th>% SGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.17 ± 0.07a</td>
<td>34.34 ± 5.03a</td>
<td>24.17 ± 3.67a</td>
<td>1.55 ± 0.13a</td>
<td>1.78 ± 0.02a</td>
</tr>
<tr>
<td>Strain B44v</td>
<td>10.18 ± 0.05b</td>
<td>41.91 ± 5.03b</td>
<td>31.72 ± 5.01b</td>
<td>1.18 ± 0.17b</td>
<td>2.45 ± 0.08b</td>
</tr>
<tr>
<td>Strain B51f</td>
<td>10.15 ± 0.07c</td>
<td>40.52 ± 1.93c</td>
<td>29.37 ± 1.88c</td>
<td>1.31 ± 0.02c</td>
<td>2.33 ± 0.09c</td>
</tr>
</tbody>
</table>

Each value = mean ± SD of three replicates. Values = average performances of 45 fish per treatment. Mean values in the same column with different superscript letters vary significantly (p < 0.05). FCR and SGR represented feed conversion ratio and specific growth rate, respectively.

Fig. 1. Ability of the Bacillus siamensis strain B44v and the Bacillus sp. strain B51f in auto-aggregation and co-aggregation with Aeromonas hydrophila FW52 (Co-FW52) and Streptococcus agalactiae F3S (Co-F3S).

An asterisk denotes significant differences between the control and treated groups (p < 0.05).

Fig. 2. Percentage survival of probiotic-fed catfish and control fish after being challenged with A. hydrophila FW52 for 14 days. Different letters denote significant differences between the treated groups (p < 0.05).
antibiotics tested. The susceptibility of strain B44v to antibiotics ensures its inability to transfer antibiotic-resistant genes to recipient bacteria in the gut, thus preventing the development of antibiotic-resistant pathogens.

The potential probiotic *B. siamensis* strain B44v could produce cellulase and protease, whereas the *Bacillus* sp. strain B51f produced protease and amylase enzymes. Ability to produce some hydrolytic enzymes is beneficial to the host. Enzymes increase the digestion of macromolecules in animal feed and improve feed intake by reducing digesta viscosity and increasing nutrient absorption in host animals (Ray et al., 2012).

Feed trial using basal diets containing each of the probiotic strains (*B. siamensis* strain B44v and *Bacillus* sp. strain B51f) resulted in well grown fish with no disease symptoms. Interestingly, some probiotic *Bacillus* species have been reported as fish growth promoters (Han et al., 2015; Liu et al., 2012). The present study has demonstrated that a fish diet containing 10^7 CFU/g of either *B. siamensis* strain B44v or *Bacillus* sp. strain B51f was sufficient to significantly improve the growth performance of hybrid catfish, indicating its positive nutritional effect. The growth enhancement efficiency obtained in the present study was better than those reported by El-Haroun (2007) in an African catfish (*Clarias gariepinus*) fed diet containing the growth promoter Biogen® (a mixture of *B. subtilis*, alcin, and hydrolytic enzyme) in terms of SGR and FCR.

In this study, the *B. siamensis* strain B44v exhibited a superiority of probiotic properties compared with the *Bacillus* sp. strain B51f, including species identity, antibiotic susceptibility, and broader antimicrobial activity, and, therefore, it is considered as a promising bacterial probiotic for fish farming. This is the first report of *Bacillus siamensis* displaying probiotic properties in hybrid catfish.

**Conclusion**

Based on the criteria investigated in this study, the *B. siamensis* strain B44v displayed a high potential for use as a probiotic in hybrid catfish culture. It has a broad-spectrum antibacterial activity inhibiting both Gram-positive and Gram-negative bacteria, especially the fish pathogens *A. hydrophila* and *S. agalactiae*. The strain B44v survived well in simulated gastrointestinal conditions, it showed a tendency to adhere to intestinal mucosa, it produced protease and cellulase enzymes, and was susceptible to all antibiotics tested. Feed-trial and challenge tests performed in hybrid catfish revealed that this bacterium significantly stimulated the growth, and offered protection against bacterial infection, in hybrid catfish. It was clearly seen that the *B. siamensis* strain B44v displayed remarkable *in vitro* and *in vivo* probiotic properties, and thus can be considered as a probiotic for feed supplementation in aquaculture. However, a further investigation into the strain B44v’s effects on fish immunity would be useful to reaffirm its probiotic properties.

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