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ORIGINAL ARTICLE

Aquaculture

Efficacy of *Bacillus* spp. isolated from Nile tilapia *Oreochromis niloticus* Linn. on its growth and immunity, and control of pathogenic bacteria

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Abstract

Probiotics can act as biological control agents against bacterial infection in aquatic animals, and also increase growth and stimulate immunity. In this study, two potential probiotics, *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2, isolated from Nile tilapia *Oreochromis niloticus* Linn. intestine, were investigated for their biological functions. Species of isolated bacteria were identified by a conventional microbiological assay, biochemical assay and 16S ribosomal RNA polymerase chain reaction analysis. Both *Bacillus* isolates could survive at a pH ranging from 2 to 9 for 6 h and for 2 h in bile salts. The candidate probiotics were tested for their inhibition activity against the pathogenic bacteria *Streptococcus agalactiae* and *Aeromonas hydrophila*, and their specific protease activity. The probiotics had no significant effect ($P > 0.05$) on the average weight, average daily growth, specific growth rate or feed conversion ratio of tilapia fry after an 8-week feeding trial. Furthermore, supplementation with probiotics did not increase the survival rate of tilapia challenged with *S. agalactiae*. Several immune parameters including lysozyme, phagocytic activity and respiratory burst activity of juvenile fish treated with probiotics were significantly higher than those of the control ($P < 0.05$), but levels of the superoxide anion and alternative complement activity did not significantly differ ($P > 0.05$). Stress tolerance to brackish water at 25 p.p.t. NaCl did not improve significantly in fish treated with probiotics ($P > 0.05$). These two probiotic *Bacillus* offer benefits in terms of disease control and stimulation of the immune response of cultured tilapia.

Keywords *Bacilli* · Probiotics · Disease resistance · Stress tolerance · Immunity

Introduction

Nile tilapia *Oreochromis niloticus* Linn. is the most common freshwater aquaculture fish species in Thailand with an annual domestic production of about 200,000 metric tons. Intensive culture to increase the productivity of these fish can cause stress and weaken their health, leading to disease outbreaks and ultimately economic loss. Many infectious agents have been identified in tilapia culture worldwide, including parasites, bacteria and viruses. The most common pathogenic bacteria in tilapia are *Streptococcus agalactiae* and *Aeromonas hydrophila*. Farmers treat bacterial infections with antibiotics, but these can be of high risk to consumers due to residual drug levels and the development of antibiotic resistant strains or strain mutation. To overcome these problems, and to protect fish from bacterial diseases, the safe application of beneficial bacteria that may induce an immune response against pathogenic bacteria has been reported for many fish and shrimp species in recent decades.

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The benefits of probiotics on growth, the intestinal microbial population and immunity of fish in aquaculture, and in particular in tilapia farming, have been extensively studied and reviewed in recent years (Najeeb et al. 2015; Truong-Giang et al. 2017; Goutam and Arun Kumar. 2017; Narayanan et al. 2018; Emmanuel et al. 2018).

Probiotics can stimulate innate immunity in fish and activate the expression of various cytokines (Nayak 2010). However, the mechanisms by which probiotics stimulate the immune system are not clearly understood. Amongst the many types of probiotics used in aquaculture, *Bacillus* spp. are amongst the most common, and are applied as single species or in combination with other probiotic species. *Bacillus* spp. possess many advantages such as high inhibition activity of pathogenic bacteria, immune enhancement, exoenzyme production and the ability to form spores.

In this study, we investigated the effects of dietary supplementation with *Bacillus* spp. KUAQ1 and KUAQ2, isolated from Nile tilapia intestine, which were characterized as potential probiotics by their inhibition of pathogenic bacteria, their protease activity, pH and bile tolerance. Experimental tilapia were tested for their growth performance, innate immunity, disease resistance and stress tolerance in response to probiotic application.

Materials and methods

Bacterial isolation and characterization

Bacillus spp. were isolated from the intestine of Nile tilapia from cage culture in Thailand using heat shock and cold shock activation. Briefly, approximately 1 cm of Nile tilapia intestine was excised and briefly washed with sterile saline solution. Samples were put into sterile glass test tubes, minced with sterile scissors, and ground with a sterile pestle until homogeneous. The homogenate was heated at 80 °C for 20 min and quickly chilled on ice for 1–2 min. Bacteria were cultured on trypticase soy agar (TSA) and kept at 30 °C overnight. Colonies were then subcultured on TSA to obtain single colonies and bacterial stocks in TSA containing 20% glycerol, then were kept at − 80 °C for further analysis.

For bacterial characterization and species' identification, isolated bacteria were cultured in trypticase soy broth (TSB) and genomic DNA was extracted by a conventional DNA extraction method (Kannika et al. 2017). Purified DNA was subjected to polymerase chain reaction (PCR) analysis for amplification of the 16S ribosomal RNA (rRNA) gene using primers 20F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1500R (5′-GTTACCTTGTTACGACTT-3′) (Kannika et al. 2017). The PCR master mix contained 1 × Dream *Taq* buffer, 0.25 mM dNTP, 0.5 µM forward primer and reverse primer, and 0.2 U Dream *Taq* (Thermo Scientific, USA). The reaction was performed at 94 °C for 3 min followed by 25 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. The amplified PCR product was then purified and ligated to pGEMT Easy Vector (Promega, USA) before nucleotide sequencing (Macrogen, Korea). The nucleotide sequence was searched for bacterial identity with data from the National Center for Biotechnology Information (NCBI) database using the BLASTN program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Eight isolates were selected according to probiotic characteristics and further identified by phylogenetic tree analysis.

Phylogenetic tree analysis

16S rRNA of eight selected *Bacillus* isolates was subjected to multiple sequence alignment with ClustalW and phylogenetic tree analysis using Mega X (Kumar et al. 2018). Maximum likelihood analysis was performed with 1000 bootstrap replication; 16S rRNA accession numbers of the other *Bacillus* spp. are listed in Table 1.

Bacterial inhibition assay

Bacterial inhibition activity of isolated *Bacillus* spp. was determined with the pathogenic bacteria *S. agalactiae* serotype III (Kannika et al. 2017) and *A. hydrophila* strain KUAQ1. Both bacteria were isolated from Nile tilapia and kept at − 80 °C in the Laboratory of Aquatic Animal Health Management, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. A cross streak assay was performed by streaking pathogenic bacteria (1×10^8 CFU/ml) on TSA as the first line. The second line was streaked with *Bacillus* spp. (the same amount as for the pathogenic bacteria) perpendicular with the first line (cross

Table 1 List of 16S ribosomal RNA (*rRNA*) genes from *Bacillus* spp. used for phylogenetic tree analysis

Organisms	Gene	Accession no.
Bacillus cereus ATCC 14579	16S rRNA	NR_074540.1
Bacillus anthracis strain ATCC 14578	16S rRNA	NR_041248.1
Bacillus amyloliquefaciens strain NBRC 15535	16S rRNA	NR_041455.1
Bacillus licheniformis strain ATCC 14580	16S rRNA	NR_074923.1
Bacillus subtilis strain 168	16S rRNA	NR_102783.2
Bacillus altitudinis strain J54	16S rRNA	MN148770.1
Bacillus xiamenensis strain 1E0018	16S rRNA	MK353500.1
Bacillus vallismortis strain 12a	16S rRNA	MF595074.1
Bacillus pumilus strain IESE:ST2	16S rRNA	KU962124.1
Bacillus stratosphericus strain A7	16S rRNA	KX262677.1
Bacillus aerius strain 24K	16S rRNA	AJ831843.1
Bacillus thuringiensis M61	16S rRNA	LN890147

streak). Plates were then incubated at 30 °C for 72 h and the inhibition area observed.

Specific protease activity analysis

To assess total secreted protease, *Bacillus* spp. in TSB (1×10^8 CFU/ml) were centrifuged to collect the culture media. Approximately 10 µl of cell-free medium was spotted onto sterile filter paper and placed onto the surface of TSA containing 5% skim milk. The clear zone surrounding the filter paper was measured after incubation at 30 °C for 24 h.

To determine the specific protease activity, selected *Bacillus* spp. from the skim milk test in TSB culture were mixed with 50 mM Tris-Cl, pH 8.0 and 200 mM NaCl. Culture medium was collected by centrifugation at 15,000 *g* for 30–60 min and the total protein content determined by Bradford analysis using bovine serum albumin standard. Then, 1 mg/ml of total protein was reacted with 5% azocasein (in 1 M NaOH) and incubated at 30 °C for 15 min. After incubation, 10% TCA was added to the mixture to stop the reaction and the mixture centrifuged at 8000 *g* for 15 min. The supernatant was mixed with 1 M NaOH (1:1 ratio) and the protease activity determined at 450 nm. The specific protease activity was calculated according to Eq. (1):

$$\text{Specific protease activity} = \frac{A_{450\,\text{nm}} \times \text{dilution factor} \times (1000/125) \times (60/15)}{\text{mg protein/ml}}. \quad (1)$$

Antibiotic sensitivity test

Antibiotic sensitivity tests of the selected probiotics were conducted with 14 antibiotics: novobiocin (5 µg), ampicillin (10 µg), erythromycin (15 µg), ciprofloxacin (5 µg), amoxicillin (25 µg), polymyxin B (300 units), neomycin (30 µg), trimethoprim (5 µg), florfenicol (30 µg), spectinomycin (25 µg), sulfadimethoxazone (25 µg), tetracycline (30 µg), oxytetracycline (30 µg) and enrofloxacin (5 µg) (Oxoid, UK) by using a disk-diffusion method with Mueller Hinton agar with bacteria at 10^6 colony-forming units (CFU)/ml. The cultures were incubated overnight at 30 °C and the results were interpreted in accordance with the recommendations of the Clinical and Laboratory Standards Institute (Formerly National Committee for Clinical Laboratory Standards 2004).

pH and bile salts tolerance tests

Bacillus spp. were grown in TSB and the cell number adjusted to obtain 10^8 CFU/ml. For the pH tolerance test, 0.1 ml of culture was adjusted to pH 2, 3, 4, 5, 6, 7, 8 and 9 by HCl or NaOH. For the bile salts tolerance test, 0.1 ml of bacterial culture was mixed with bile salts to obtain final concentrations of 0.5, 1, and 2%. Viability of bacteria was determined by streaking on TSA followed by culture for 1, 2, 4, and 6 h at 30 °C.

Experimental fish

Fry and juvenile Nile tilapia were used for different purposes. For growth performance, including average weight, average daily growth (ADG), specific growth rate (SGR) and feed conversion ratio (FCR) analysis, pathogenic bacterial challenge and stress test, Nile tilapia fry (initial average weight 2 g) from a commercial Good Agricultural Practices farm were used. For immune response, Nile tilapia juveniles (initial average weight 50 g) were used. Fish were acclimatized in 1-ton cement tanks with continuous aeration, and fed three times a day for 2 weeks before the experiment.

Diet preparation and feeding program

Selected *Bacillus* spp. were separately cultured in 50-ml tubes with 10 ml TSB, at 30 °C for 18–20 h. Then, the bacterial suspension was swabbed onto the sporulation medium (Difco sporulation medium) and incubated at 30 °C for 120 h. *Bacillus* spores on the surface of the agar were then transferred to 0.85% NaCl in 50-ml tubes and centrifuged twice at 13,000 *g* for 1 min. The bacterial suspension was heated to 85 °C for 15 min to induce complete spore formation. The concentration of spores was measured at an optical density of 600 nm (OD_{600}, where an OD_{600} of 1.1 is equivalent to 10^{12} CFU/ml. The *Bacillus* spore suspension was sprayed onto commercial feed to obtain 1×10^8, 3×10^8 and 5×10^8 CFU/g diets.

Four experimental diets including the control group (0.85% NaCl) and three probiotic supplement groups of 1×10^8 CFU/g diet, 3×10^8 CFU/g diet, and 5×10^8 CFU/g diet were fed to fish. Fifty tilapia fry were placed in 100-l glass tanks and fed twice daily with the normal diet and once daily with the probiotic supplement diet. The weights of 30% of fish per tank were determined every 2 weeks for 8 weeks to determine the growth parameters including ADG, SGR, total initial and final weights, and FCR. After 4 weeks of feeding, ten fish of each replicate were moved to a 30-l glass tank for the bacterial challenge test and stress test analysis. After 8 weeks of feeding, the total bacterial count in fish intestines was conducted using two fish from each replicate. All experiments were done in triplicate.

Immune parameter analysis

Twenty juvenile tilapia (average weight 50 g) were placed in a cement tank holding about 1 t of water and represented a replicate of each experimental group. The experimental feed and feeding scheme was the same as in the previous trial. After 4 weeks of feeding, fish serum was collected from the caudal vein of ten fish of each experimental diet and analyzed for lysozyme activity, superoxide dismutase (SOD) and hemagglutinin titers (alternative complement activity). White blood cells (WBC) were collected from the head kidney [adapted from Christybapita et al. (2007) and Ortuño et al. (2003)]; 5×10^6 cells/ml were used to measure phagocytosis activity and 6×10^5 cells/ml to measure respiratory burst activity.

Lysozyme activity assay

The lysozyme activity assay was modified from Parry et al. (1965). Briefly, 250 µl of *Micrococcus lysodeikticus* suspension [0.2 mg/ml in sodium phosphate buffer (pH 6.2)] was added to 10 µl of fish serum in a 96-well plate. The reaction was determined by measuring absorbance at 540 nm (A_{540}) for 0.5–6.5 min at room temperature. The activity of lysozyme (1 unit) in fish serum was calculated as the reduction in A_{540} of 0.001/min.

Phagocytic activity assay

The phagocytic activity assay was performed by modifying the protocol of Ai et al. (2006). Briefly, 5×10^6 cells/ml WBCs were dropped onto a cover slip and allowed to adhere for 1.5 h. The cells were washed with sterile PBS thrice before adding 2×10^6 latex beads and were then incubated at room temperature for 2 h. The cover slip was then washed thrice with PBS to remove excess beads and the WBC stained with Diff-Quick stain. WBC with engulfed beads were counted for 300 WBCs on each cover slip and the percent phagocytic rate calculated according to Eq. (2).

$$\text{Phagocytosis (\%)} = \frac{\text{Active phagocytic cells} \times 100}{300}. \quad (2)$$

Respiratory burst activity (superoxide anion assay)

Superoxide anion (O_2^-) was used to determine respiratory burst activity through nitroblue tetrazolium (NBT) reduction reaction. Approximately 6×10^5 cells of WBC were added to a microtiter plate, mixed with NBT, and incubated at room temperature for 2 h. The supernatant was then decanted and the WBC fixed with 100% methanol for 5 min followed by washing with 75% methanol twice. Potassium hydroxide (2 M) and dimethyl sulfoxide were added to dried WBC on microtiter plates, mixed well and the reaction of the superoxide anion measured at A_{655}.

SOD activity assay

SOD activity was assessed by using the SOD Assay Kit (Sigma) according to the manufacturer's instructions. The reaction was measured at A_{450} after 20 min incubation at 37 °C. SOD activity was calculated as the percent inhibition rate.

Alternative complement activity

Alternative complement activity [ACH_{50}; serum dilution at which 50% hemolysis was induced (units/milliliters)] was determined using a modified method of Ortuno et al. (1998). Briefly, a twofold dilution of fish serum was prepared in a microtiter plate. Sheep red blood cells (2×10^6 cells) were added to the diluted serum to obtain final serum concentrations of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78% and 0.39%. The mixtures were incubated at room temperature for 1 h before centrifugation at 500 *g* for 5 min. Clear lysate was transferred to a new microtiter plate and measured at A_{540}. For complete (100%) hemolysis, sterile distilled water was used, whereas PBS was used for 0% hemolysis.

Challenge test with *S. agalactiae*

Ten fish were randomly collected from each replicate and intraperitoneally injected with 100 µl of *S. agalactiae* (10^8 CFU/ml). *S. agalactiae* (serotype III) were freshly prepared by inoculating a single colony of the bacterial strain into BHI broth and culturing at 32 °C for 18 h. *S. agalactiae* were collected by centrifugation at 3500 r.p.m. at 25 °C for 10 min, followed by washing and resuspension in 0.85% NaCl. The *S. agalactiae* suspension was adjusted to 10^8 CFU/ml with 0.85% NaCl before injection. Mortality was recorded for 2 weeks and the results presented as survival rates.

Salinity stress test

Ten fish per replicate were randomly collected from each experimental group and submerged in brackish water with salinity of 25 p.p.t. (NaCl). Mortality was recorded during 24 h.

Total intestinal bacterial counts

At the end of the 8-week feeding trial, tilapia intestines were collected from two fish per replicate, weighed and

washed with 0.85% NaCl then homogenized. The samples were treated with heat shock and cold shock as previously described and the total plate count determined. Total bacteria were counted after incubation at 30 °C for 18–24 h, expressed as CFU/grams intestine.

Statistical assay

All experimental data were analyzed by one-way ANOVA and Duncan's multiple range test using SPSS software (IBM SPSS Statistics 20) with a significance level of $P < 0.05$.

Results

Bacillus spp. isolation and characterization

The initial step of *Bacillus* spp. isolation from tilapia intestine was achieved by using a heat shock and cold shock method. A total of 55 bacterial isolates were collected and identified as Gram-positive bacteria by using a conventional Gram-staining method. However, only 52 isolates were identified as *Bacillus* spp. from 16S rRNA screening and DNA sequencing.

Bacterial inhibition assay

The functional analysis of the *Bacillus* spp. isolates determined their ability to inhibit and/or colonize pathogenic bacteria. Of 52 isolates, only 22 showed inhibition or colonization of *S. agalactiae* or *A. hydrophila* (Fig. 2a). Interestingly, most of the isolates showed colonization of *S. agalactiae* but not *A. hydrophila*. Eight isolates were further selected from the 22 isolates based on their degree of colonization and inhibition of the two pathogenic bacteria. Seven of these isolates colonized *S. agalactiae*; one isolate inhibited *S. agalactiae* and two isolates inhibited *A. hydrophila* (Table 2). Two isolates (PL3/2-1 and UD1/3-3) were selected for the feeding trial. The eight isolates were identified by BlastN, which demonstrated that all of them showed more than 90% identity to *Bacillus* spp. (Table 2). To verify the authenticity of the bacterial species, a phylogenetic tree analysis of all eight strains was conducted by comparing their rRNA with that of 12 other known *Bacillus* species. Interestingly, several isolated *Bacillus* in this study could not be exactly identified to species level even though they had high identity with known *Bacillus* in the database. The phylogenetic tree separated the *Bacillus* into three groups: group 1—*Bacillus vallismortis*, *Bacillus altitudinis*, *Bacillus aerius*, *Bacillus pumilus*, *Bacillus xiamenensis*, *Bacillus stratosphericus* and *Bacillus amyloliquefaciens*; group 2—*Bacillus licheniformis* and *Bacillus subtilis*; group 3—*Bacillus gaemokensis*, *Bacillus cereus* and *Bacillus thuringiensis* (Fig. 1). Among the isolated *Bacillus*, only CB1.4-3 was in the same clade as *Bacillus amyloliquefaciens*, whereas CC1.2-1 was in a sister clade with CB1.4-3 and *B. amyloliquefaciens*. Therefore, we assume that CB1.4-3 is *B. amyloliquefaciens*. UD1/3-3 and PL3/2-1 were found to share the same sister clade as *B. licheniformis* and *B. subtilis* but differed in divergence (Fig. 1). The two probiotics used for the feeding trial in Nile tilapia were *Bacillus* KUAQ1 (PL3/2-1) and KUAQ2 (UD1/3-3).

Specific protease activity analysis

Secreted exoenzymes are some of the beneficial products of probiotics that can increase nutrient utilization in the host digestive system. In this study, secreted protease was screened by using solid agar containing skim milk. Only 14 of the 52 isolates showed secreted protease activity (Fig. 2b). Only five *Bacillus* spp. with an expressed high protease activity were chosen to determine specific protease

Table 2 Probiotic properties of candidate *Bacillus* spp. isolated from the intestine of Nile tilapia

Isolate	Colonization and inhibition		Clear zone on skim milk agar	Specific protease activity	Species as identified by Blast N	% Identity	Accession no.
	Streptococcus agalactiae	*Aeromonas hydrophila*					
CB1.4-3	C+	I++	+++	34.176	*Bacillus amyloliquefaciens*	95.51	KJ934385.1
CC1.2-1	C++	–	++	33.536	*Bacillus subtilis*	100.00	AP019714.1
CM(2) 9/1-1	C+++	–	–	–	*Bacillus cereus*	97.76	LN890263.1
CM(3) 1/1-2	C+++	–	+	–	*Bacillus cereus*	90.82	KY974314.1
PL3/2-1	C+	–	+++	106.112	*Bacillus subtilis*	94.76	HG799984.1
UD1/3-3	I++	–	+	33.600	*Bacillus altitudinis*	100.00	MN148770.1
UD1/3-4	C+++	–	–	–	*Bacillus thuringiensis*	93.77	HG799964.1
UD1/9-2	C++	I++	+++	38.272	*Bacillus subtilis*	94.67	G799984.1

Species identification of 16S rRNA of isolated bacteria was done by Blast N

C Colonization, *I* inhibition

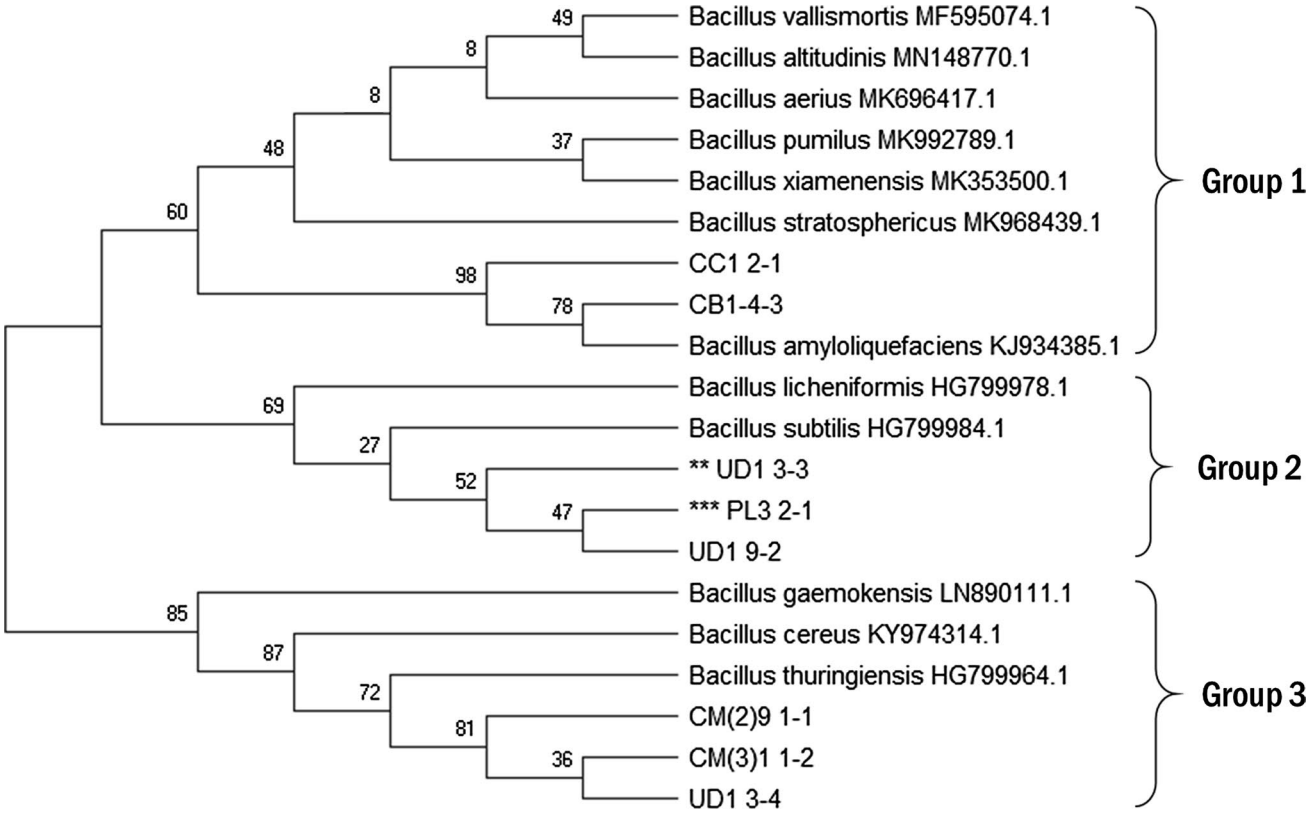

Fig. 1 Phylogenetic tree analysis of eight *Bacillus* spp. isolated from tilapia intestine using maximum likelihood with 1000 bootstraps. Asterisks indicate the *Bacillus* spp. used for the feeding trial [** KUAQ1 (PL3/2-1), *** KUAQ2 (UD1/3-3)]

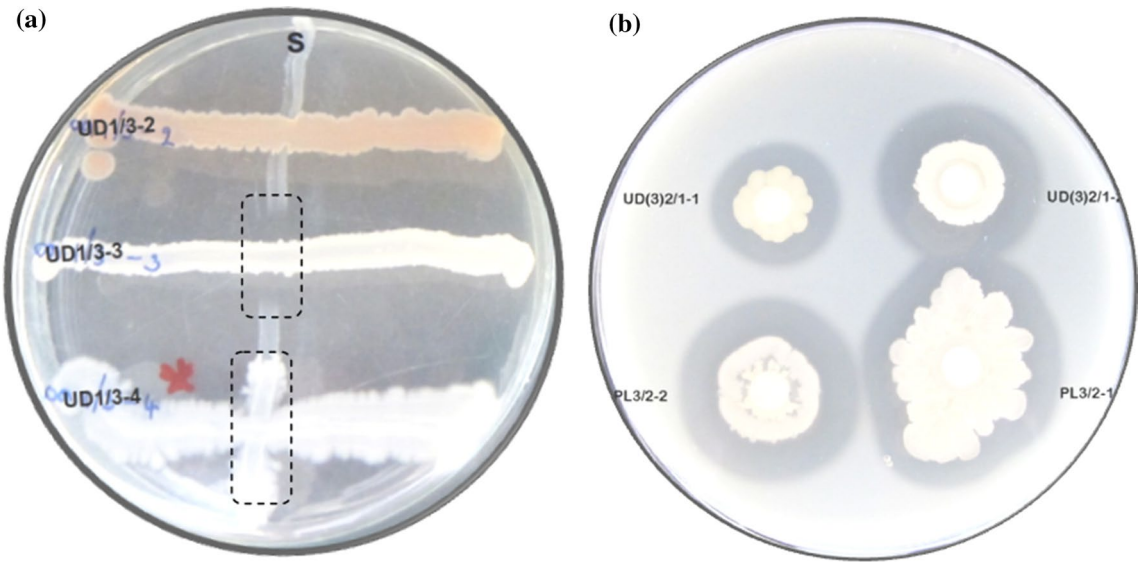

Fig. 2 Biological properties of probiotic candidates. **a** Interaction between *Streptococcus agalactiae* (*S*) and *Bacillus* spp. **b** Preliminary protease activity on skim milk agar

activity. *Bacillus* sp. KUAQ1 (isolate PL3/2-1) showed the highest specific protease activity (106.112 U/mg protein) for the degradation of azocasein; the remaining isolates showed similar activity ranging from 33.536 to 38.272 U/mg protein (Table 2).

Antibiotic sensitivity test

Bacillus sp. KUAQ1 (isolate PL3/2-1) and *Bacillus* sp. KUAQ2 (isolate UD1/3-3) were selected for the antibiotic sensitivity test. Both isolates were susceptible to most of the tested antibiotics including ampicillin (10 µg), erythromycin (15 µg), ciprofloxacin (5 µg), amoxycillin (25 µg), polymyxin B (300 units), trimethoprim (5 µg), florfenicol (30 µg), sulfadimethoxazone (25 µg) and enrofloxacin (ENR 5 µg). There were only three antibiotics that both isolates showed intermediate resistance to: novobiocin (5 µg), neomycin (30 µg) and tetracycline (30 µg). Interestingly, *Bacillus* sp. KUAQ1 showed intermediate resistance to oxytetracycline (30 µg), whereas *Bacillus* sp. KUAQ2 showed susceptibility to this antibiotic.

pH and bile salts tolerance test

To determine the viability of *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2 under the conditions of the gastrointestinal tract, the viability of bacterial spores was tested in a range of pH and percentages of bile salts. Both *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2 spores could resist a wide range of pH (2–9) for 6 h (Fig. 3); however, *Bacillus* sp. KUAQ1 appeared to have a much higher tolerance to a higher pH than *Bacillus* sp. KUAQ2. Both *Bacillus* spp. could grow for a period of 2 h in bile salts (0.5, 1 and 2%); the viability of bacteria decreased after 4 h (Fig. 4).

Growth performance

To determine the effect of *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2 on growth performance, different concentrations of bacteria were added to commercial feed and fed once a day to fish for 2 months. At the end of the feeding trial, none of the growth performance parameters significantly differed ($P > 0.05$) between the probiotic supplement diet groups and the control group (Fig. 5). The FCR of the probiotic supplement groups improved when compared with the control but the differences were not significant ($P > 0.05$). The FCR of the control group and the 1×10^8, 3×10^8 and 5×10^8 CFU/g supplement diet groups were 1.31 ± 0.04, 1.27 ± 0.07, 1.26 ± 0.09 and 1.24 ± 0.01, respectively (Fig. 5).

Immune parameter analysis

After 4 weeks of feeding, the lysozyme activities of the control group (2433.3 ± 928.8) and the probiotic supplement diet groups were not significantly different ($P > 0.05$), although there was a significant difference between the 1×10^8 and 5×10^8 CFU/g diet groups. The lysozyme activities of the probiotic supplement diet groups were 2908.3 ± 1124.91 (1×10^8 CFU/g), 2416.6 ± 787.19 (3×10^8 CFU/g) and 1500.0 ± 868.33 unit ml^{-1} (5×10^8 CFU/g), respectively (Fig. 6).

Phagocytic activity of tilapia significantly differed between treatments ($P < 0.05$). The control group had a phagocytic activity of $16.50 \pm 8.00\%$, which did not significantly differ from those of the probiotic supplement diet groups of 1×10^8 CFU/g ($17.78 \pm 9.36\%$) and 3×10^8 CFU/g ($26.72 \pm 10.9\%$) ($P > 0.05$), but the phagocytic activity of tilapia fed with 5×10^8 CFU/g *Bacillus* spp. ($46.17 \pm 15.73\%$) was significantly higher than that of the other groups (Fig. 7).

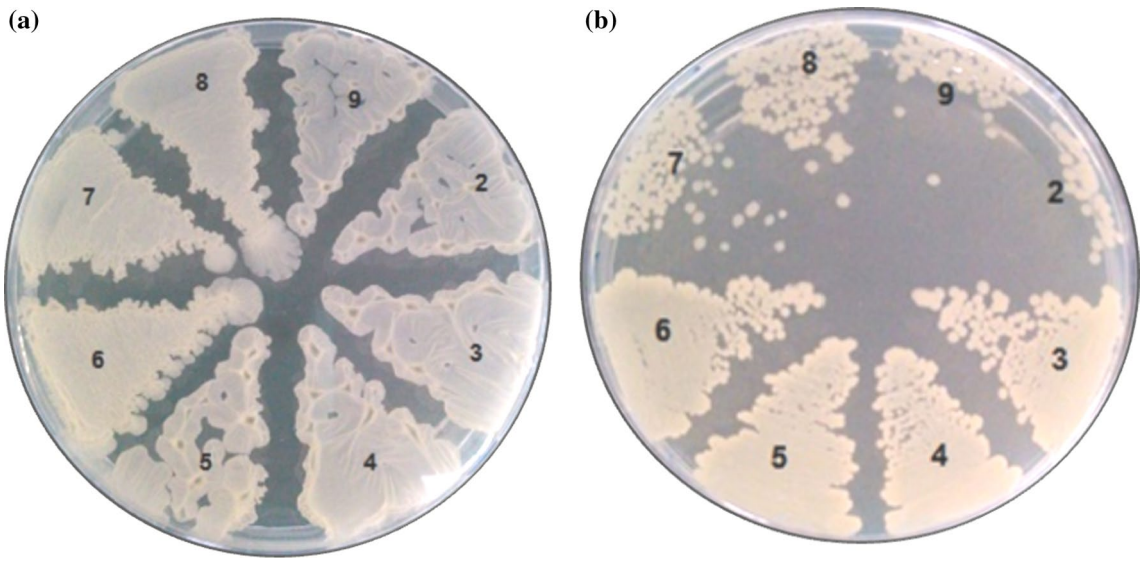

Fig. 3 pH tolerance test at pH 2–9 of **a** *Bacillus* sp. KUAQ1 and **b** *Bacillus* sp. KUAQ2 at 6 h

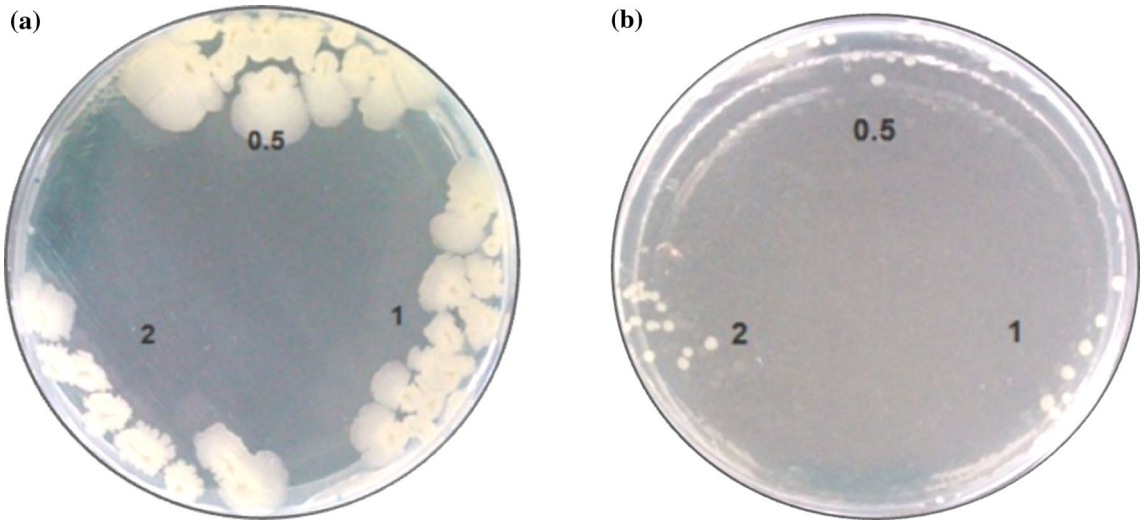

Fig. 4 Bile salts tolerance test at 0.5%, 1% and 2% of **a** *Bacillus* sp. KUAQ1 and **b** *Bacillus* sp. KUAQ2

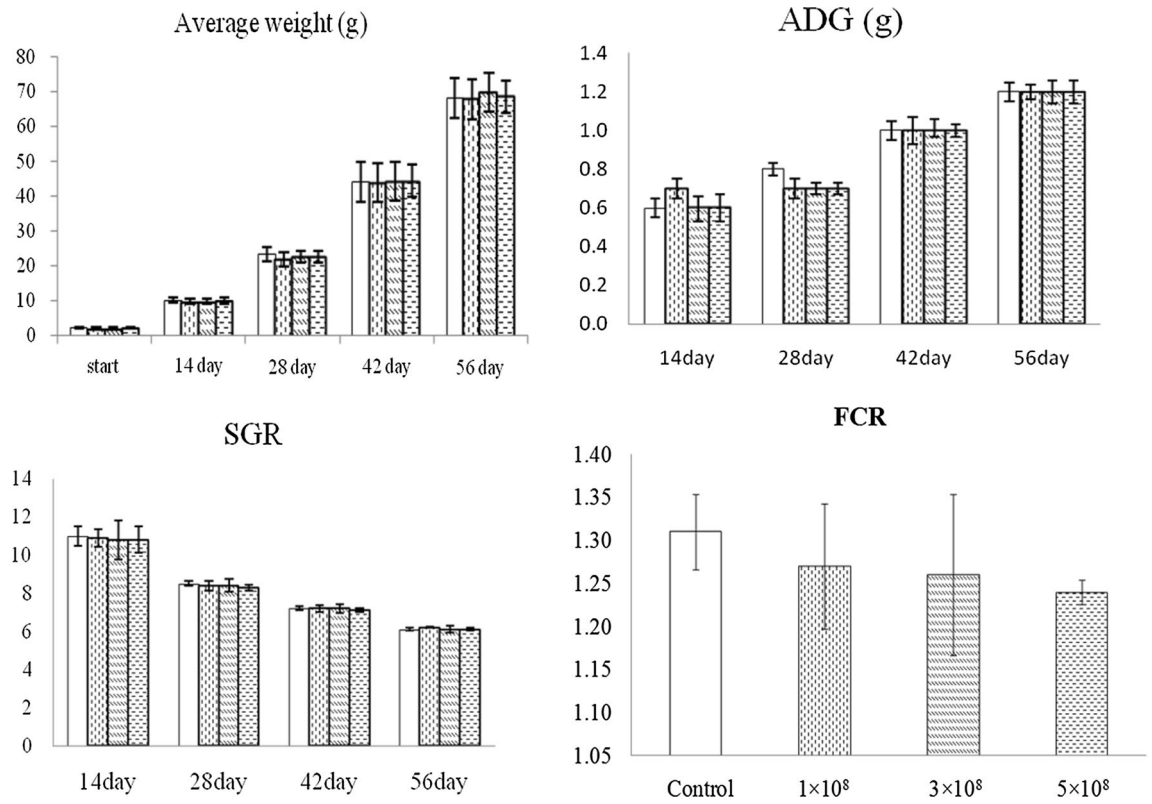

Fig. 5 Growth parameters: average weight, average daily growth (*ADG*), specific growth rate (*SGR*) and feed conversion ratio (*FCR*) ($n=50$). No significant differences were found for any parameter ($P>0.05$)

The respiratory burst activities (superoxide anion) of the control and probiotic supplement diet groups were 0.017 ± 0.009, 0.044 ± 0.017, 0.017 ± 0.007 and 0.033 ± 0.010 U ml^{-1}, respectively and significantly differed ($P<0.05$) (Fig. 8). However, neither the SOD nor the ACH_{50} significantly differed among tested groups ($P>0.05$) (Figs. 9, 10). The SOD of the control and probiotic supplement diet groups were 0.30 ± 0.26, 0.53 ± 0.29, 1.12 ± 0.78 and 0.37 ± 0.33 U ml^{-1}, respectively. ACH_{50} was 248.67 ± 67.36, 228.53 ± 64.80, 212.40 ± 23.43 and 243.32 ± 20.00 U ml^{-1}, respectively.

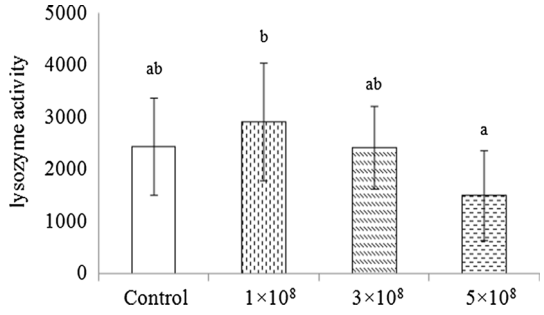

Fig. 6 Lysozyme activity of Nile tilapia fed with three dosages of probiotics for 4 weeks ($n=10$). Bars with different letters indicate significant difference ($P<0.05$)

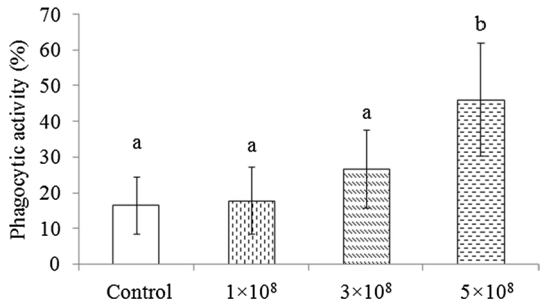

Fig. 7 Phagocytic activity of Nile tilapia fed with three dosages of probiotics for 4 weeks ($n=10$). Bars with different letters indicate significant differences ($P<0.05$)

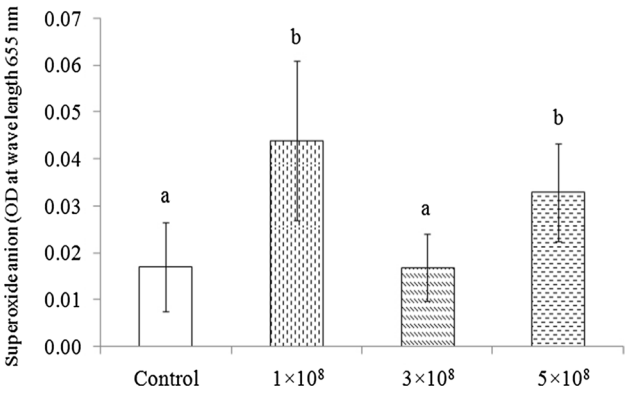

Fig. 8 Superoxide anion of Nile tilapia fed with three dosages of probiotics for 4 weeks ($n=10$). Bars with different letters indicate significant difference ($P<0.05$)

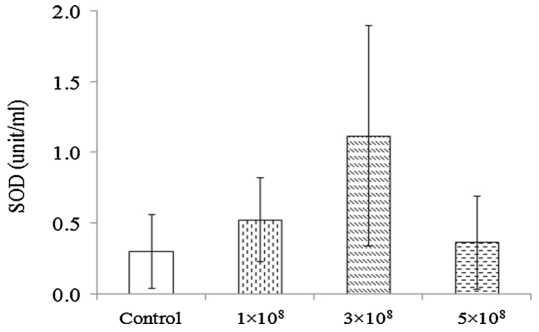

Fig. 9 Superoxide dismutase of Nile tilapia fed with three dosages of probiotics for 4 weeks ($n=10$). No significant differences between means were found ($P>0.05$)

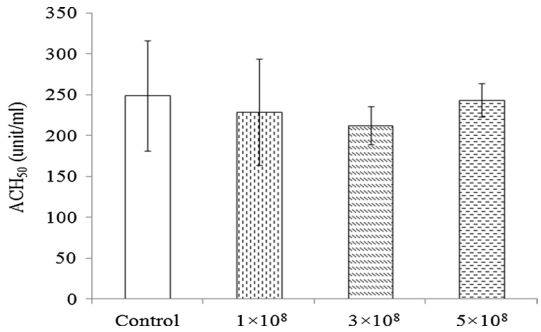

Fig. 10 Alternative complement activity (ACH_{50}) of Nile tilapia fed with three dosages of probiotics for 4 weeks ($n=10$). No significant differences between means were found ($P>0.05$)

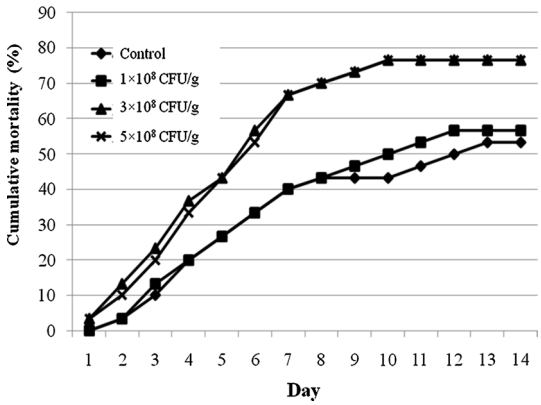

Fig. 11 Cumulative mortality of Nile tilapia during the challenge test with *S. agalactiae* ($n=30$) for 14 days. *CFU* Colony-forming units

Challenge test with *S. agalactiae*

The disease resistance of tilapia against experimental challenge with *S. agalactiae* (serotype III) was evaluated after fish were fed the probiotic supplement diets for 4 weeks. During the 14-day postchallenge period, mortality was observed on day 2 and steadily increased during the first 7 days (Fig. 11). Percentage survival rate of the control, 1×10^8, 3×10^8 and 5×10^8 CFU/g of probiotic supplement diet were $46.67\pm15.28\%$, $43.33\pm23.09\%$, $23.33\pm23.09\%$ and $23.33\pm15.28\%$, respectively, after 14 days. However, the average survival rate did not significantly differ between the groups ($P>0.05$) (Fig. 12).

Salinity stress test

Tilapia fed with different concentrations of *Bacillus* spp. spores were tested for their tolerance to salinity. During the 24-h stress period, mortality due to salinity stress was observed from 6 h onwards. The highest mortality rate was recorded at 24-h post stress induction. The mortality rates did not significantly differ between any of the tested groups ($P > 0.05$) (Fig. 13).

Total intestinal bacterial count

After the 8-week feeding trial, *Bacillus* spp. in tilapia fry intestines were isolated by heat shock and cold shock and a total count performed. The total *Bacillus* counts of all probiotic supplement diet groups were significantly higher than that of the control group ($P < 0.05$) (Fig. 14).

Discussion

Probiotics are currently used as an alternative biological method for the control of disease and also for the promotion of growth in farmed fish and shrimp worldwide. Several bacterial probiotics have been identified that have a positive effect on fish health, e.g., species of *Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Microbacterium*, and *Staphylococcus* (Nayak 2010; Balcazar et al. 2006; Lakshimi et al. 2013; Tuan et al. 2013; Lazado et al. 2015). In this study, we identified *Bacillus* spp. from the intestine of Nile tilapia and determined their biological potential for use as probiotics. Our isolation method allowed *Bacillus* spp. to sporulate by simple heat shock and cold shock activation (Pakingking et al. 2015; Molinari et al. 2003). This method should eliminate most contaminating bacteria in fish intestine, and only spores of *Bacillus* spp. could grow on the culture medium used. The identification of the isolated bacteria as Gram positive was confirmed by conventional Gram staining; the bacteria had a long rod shape and their spore morphology could be observed under a microscope.

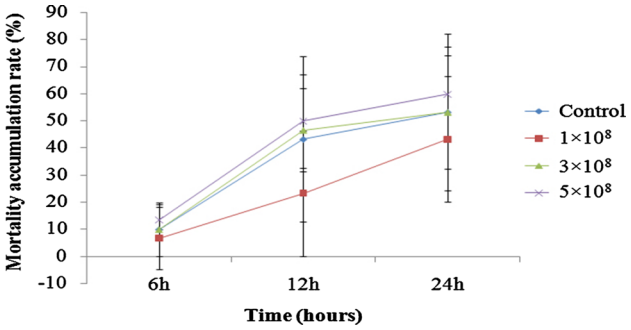

Fig. 13 Mortality rates (%) of Nile tilapia during the stress test with 25 p.p.t. NaCl

Molecular identification in our study indicated that the *Bacillus* isolates used for our feeding trial (*Bacillus* sp. KUAQ1 and KUAQ2) are closely related to *B. subtilis*. *Bacillus* spp. isolated from the intestines of a few species of freshwater fish for use as probiotics have been recently reported, e.g., from striped catfish (Ho et al. 2017), hybrid catfish (Khotchanalekha et al. 2018), Indian major carp (Kavitha et al. 2018), tilapia and African catfish (Kato et al. 2016) and Nile tilapia (Srisapoome and Areechon 2017). Although 52 different isolates of *Bacillus* spp. were isolated and identified from tilapia intestine in our preliminary study, only some isolates exhibited probiotic properties. We tested these isolates for several biological activities and related properties to confirm their potential as probiotics.

Secreted protease is an extracellular enzyme produced from probiotics beneficial to a host's digestive system. In this study, only some of the isolated *Bacillus* spp. showed secreted protease activity, of which *Bacillus* isolate PL3/2-1 showed the highest activity (106.112 U/mg protein). Secreted protease or extracellular enzymes are considered common characteristics of *Bacillus* spp., e.g., several enzymes are secreted by *B. amyloliquefaciens* such as protease (Cho et al. 2003), gelatinolytic enzymes (Sai-Ut et al.

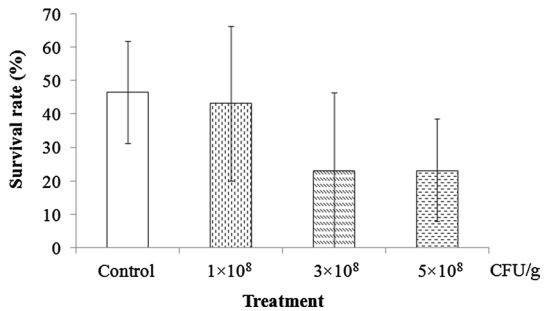

Fig. 12 Survival rate at the end of the challenge test with *S. agalactiae* ($n = 30$). No significant differences between means were found ($P > 0.05$)

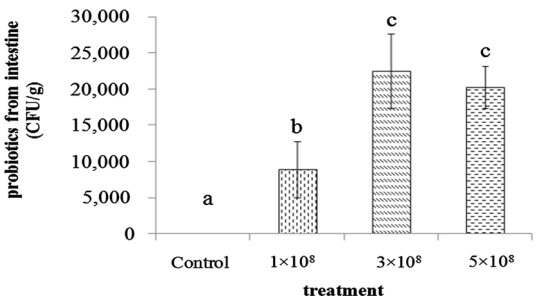

Fig. 14 Total counts of *Bacillus* spp. in intestines of tilapia fry at the end of the feeding trial ($n = 6$). Bars with different letters indicate significant differences ($P < 0.05$)

2013) and xylanase (Saputra et al. 2016). Moreover, protease secreted from *Bacillus clausii* provided an alternative means of controlling the pathogenesis of other bacteria. Serine protease from *B. clausii* O/C could neutralize the cytotoxic effects induced by the purified toxins of *Clostridium difficile* and *B. cereus* (Ripert et al. 2016), and *B. amyloliquefaciens* applied as a probiotic in Nile tilapia improved growth and enhanced fish immunity against *A. hydrophila* (Saputra et al. 2016). Interestingly, *Bacillus* sp. KUAQ2 (UD1/3-3) isolated in this study also exhibited inhibition activity against *A. hydrophila*. Therefore, the two isolates, *Bacillus* spp. as KUAQ1 (PL3/2-1) and KUAQ2 (UD1/3-3), were selected to determine their probiotic properties in a Nile tilapia feeding trial. Moreover, it is interesting that several novel *Bacillus* spp. were also isolated in this study; their biological functions will be determined in later studies.

The methods used for probiotic application in aquaculture may affect the response of the treated organisms. The oral administration of probiotics is the most suitable method for application to tilapia to enhance and directly activate their immunity and growth. Probiotic supplements in fish feed have been reported to enhance various parameters, especially growth (Reda and Selim 2015; Yan et al. 2015; Lukkana et al. 2015; Chaudhary and Qazi 2014; Krishnan 2014; Ahmed et al. 2014). The results of our study indicated that feeding probiotics to 2-g tilapia for 8 weeks did not affect their growth (i.e., in terms of average weight, ADG and SGR), or FCR, when compared with the control. This agrees with studies on the probiotic *Pediococcus acidilactici* in Nile tilapia (Standen et al. 2013), where no significant difference in growth or feed utilization was found. Similarly, *B. subtilis* and *L. acidophilus* used as probiotics did not promote the growth of adult tilapia (Ridha and Azad 2015), or that of other species, such as red tilapia (Wing-Keong et al. 2015) common carp (Huang et al. 2015), or marine fish (Hauville et al. 2016). This might have been due to the fact that laboratory conditions during these trials, such as temperature, dissolved oxygen etc. and nutrients from feed, were sufficient for the optimal growth of the fish (Pirarat et al. 2006).

However, supplementing feed with probiotics might enhance fish immunity and control disease caused by pathogenic bacteria. The pathogenic bacteria challenge in this study demonstrated that 1×10^8, 3×10^8 and 5×10^8 CFU/g probiotic supplement diets did not prevent *S. agalactiae* infection. In contrast, *B. pumilus* used as a probiotic in tilapia induced resistance against various bacterial diseases such as those caused by *S. agalactiae* (Srisapoome and Areechon 2017) and *Edwardsiella ictaluri* in striped catfish (*Pangasianodon hypophthalmus*) (Ho et al. 2017). It should also be noted that a combination of probiotics, e.g., *B. licheniformis* with *B. subtilis*, enhanced several parameters of growth and disease resistance in fish (Abarike et al. 2018). Therefore, future trials using a combination of two of more *Bacillus* species should be conducted to evaluate synergistic effects on the growth and disease resistance of Nile tilapia.

The effects of probiotics in stimulating host innate immunity was assessed to explain possible immune responses and to optimize the dose of probiotics in aquaculture feed. Lysozyme produced by WBC (leucocytes), especially neutrophils and macrophages, can hydrolyze the peptidoglycan layer in the cell walls of bacteria when fish have been infected by them (Schmekel et al. 2013). In this study, the group fed with the low dose of probiotics (1×10^8 CFU/g feed) showed the highest lysozyme level. The group receiving the highest dose of probiotics was likely to produce less lysozyme, which would explain why there was no significant difference in the lysozyme level between this group and the control. The role of lysozyme is well recognized in the non-specific immunity of animals and might be activated by foreign substances. However, in the common carp (*Cyprinus carpio*) (Kazuń et al. 2018), brown trout (*Salmo trutta*) (Balcázar et al. 2007) and rainbow trout (*Oncorhynchus mykiss*) (Panigrahi et al. 2005) probiotics did not influence the activation of lysozyme.

The hemagglutinin titer is used to analyze the alternative complement activity based on the ability to break down sheep red blood cells. The alternative complement is an element of non-specific immunity, which is mostly induced at the cell wall of microorganisms. The alternative complement did not significantly differ between the treatments, which contrasts with most other reports (Pirat et al. 2006; Liu et al. 2012; Giri et al. 2012; Wang et al. 2008; Safari and Atash 2013), although Ridha and Azad (2015) found a similar result to ours. Thus, adding probiotics to feed in this study had no impact on the alternative complement pathway.

The superoxide anion level an indicator of respiratory burst, a reaction that helps destroy microorganisms after phagocytosis through the creation of O_2^- which is converted to H_2O_2 by SOD (Gottfredsen et al. 2013). In our study, the superoxide anion volume of fish fed with probiotics was significantly higher than that of the control group, with the exception of the group fed a dose of 3×10^8 CFU/g feed. The SOD level did not significantly differ between any experimental group, i.e., when probiotics were applied at 3×10^8 CFU/g feed, SOD was not significantly higher than in the other treatments. It is possible that when SOD is secreted at a high level the superoxide anion is converted to H_2O_2, which would have resulted in a lower superoxide anion level than in the other treatment groups at the same stage.

After fish had been fed with probiotics for 4 weeks their phagocytic activity was higher than that of the control group, especially in the group fed 5×10^8 CFU/g feed ($P<0.05$). This process is a result of the increased action of phagocytic cells, including neutrophils, monocytes and macrophages, that engulf foreign cells. Peptidoglycan in the cell wall

of *Bacillus* can stimulate specific proteins called pattern recognition molecules, which can subsequently stimulate the immune response of the host animal (Lee et al. 2012; Richard et al. 2014). Moreover, Alexandre et al. (2014) also reported that some probiotics can also stimulate the secretion of cytokines from macrophages.

In conclusion, among the *Bacillus* isolates from Nile tilapia intestine examined in this study, *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2 showed the highest probiotic properties. They exhibited a positive effect in vitro including antimicrobial activity against *S. agalactiae*, and tolerance to a wide range of pH and to bile salts. However, neither of these probiotics showed a positive effect on the growth of Nile tilapia. Innate immunity tended to increase when the diet of Nile tilapia was supplemented with the probiotics. However, probiotic supplementation may not be economically viable according to the preliminary results of our study. Therefore, more studies need to carried out on the mechanisms of immune activation and fish excretion in response to probiotic supplementation in this species.

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