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In vitro Assessment of the Probiotic Characteristics of Three *Bacillus* Species from the Gut of Nile Tilapia, *Oreochromis niloticus*

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Abstract

Probiotics used in aquaculture are mostly from non-fish sources, as a result ineffective in eliciting the desired effects in aquatic animals. In this study, three *Bacillus* species were isolated from the digestive tract of freshwater fish *Oreochromis niloticus* and characterised based on their morphological, biochemical and evolutionary relationships. Their probiotic potentials were evaluated based on their ability to tolerate low pH, bile salt concentration, high temperatures, adhesion ability (auto-aggregation and hydrophobicity), haemolytic activity and antimicrobial activity including biosafety assay. Three *Bacillus* strains identified as *Bacillus velezensis* TPS3N (MK130897), *Bacillus subtilis* TPS4 (MK130899) and *Bacillus amyloliquefaciens* TPS17 (MK130898) were designated as TPS3N, TPS4 and TPS17, respectively. TPS3N and TPS17 were α-haemolytic, while TPS4 was γ-haemolytic. The three isolates had higher viability ability after exposure to higher temperatures (80 °C, 90 °C and 100 °C) and were resistant to low pH (1) and bile salt concentration (0.5%) as well as high cell surface hydrophobicity and auto-aggregation. The three isolates were compatible with one another and thus can be used in consortia. These strains were susceptible to gentamicin, cephalexin, ampicillin, ceftriaxone, kanamycin, amikacin, penicillin, cefoperazone, chloramphenicol, erythromycin, tetracycline, doxycycline, ciprofloxacin, clindamycin (except TPS4) and furazolidone (except TPS17). The antimicrobial assessment showed that among the three isolates, TPS3N and TPS17 exhibited good antimicrobial activity against the three fish pathogens (*Streptococcus agalactiae*, *Aeromonas hydrophila*, *Vibrio harveyi*), while TPS4 was effective against *Streptococcus agalactiae* only. The results of this work suggest that *Bacillus* strains TPS3N, TPS4 and TPS17 could be considered as potential probiotics in tilapia aquaculture.

Keywords *Bacillus velezensis* · *Bacillus subtilis* · *Bacillus amyloliquefaciens* · Probiotics · Nile tilapia

This article to be considered for publication has not been published previously and is not under consideration for publication elsewhere.

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Introduction

Tilapia is one of the most cultured freshwater aquaculture species second to carps with regard to total global aquaculture production [1, 2]. It is an important source of protein for people in most developed and developing countries with China having the highest production percentage [3, 4]. Tilapia aquaculture has increased dramatically over the years due to increasing demand and the wide adaptation of intensive culture practices. However, increased production of tilapia has led to the proliferation of diseases [5]. The use of probiotics to combat diseases is well elucidated in aquaculture [6]. Research into the use of probiotics came to the fore due to the adverse effects of antibiotics such as a change in the microbiota of aquaculture systems and resistance of the microorganism to the antibiotics in fighting diseases in aquaculture [7, 8]. Probiotics are considered effective and environmentally friendly substitutes to antibiotics [9] as have been shown over

the years not only to boost fish immunity and response to stress but also to improve growth and enhance the rearing water quality [10]. Probiotics when ingested in the right dose can stimulate the growth of other useful microorganisms and a reduction in pathogens thus improving the intestinal microbial balance of the host and lowering the risk of gastrointestinal diseases [11].

The digestive tract of fish provides a conducive environment for bacteria growth and survival, aiding the bacteria community to exhibit a variety of enzymatic potentials which in turn aids the fish's digestion [12, 13]. The enzymes synthesised by this microbial community include amylolytic, proteolytic, lipolytic and cellulolytic enzymes which are involved in the digestion of carbohydrates, proteins, lipids and cellulose, respectively [14, 15]. To be able to produce the enzymes mentioned above, the bacteria must be able to survive the gastrointestinal conditions and thus should be resistant to low pH and tolerant to gastric juice in order to transit through the stomach and the intestines [16, 17]. To be used as feed additives, probiotic bacteria are required to withstand high temperature since the feed production process requires high temperatures [18]. Haemolytic bacteria are considered unsafe for use as probiotics due to the virulent factor of haemolysin which causes anaemia and oedema in the host. Above all, probiotic bacteria should be able to synthesise proteins or bacteriocins that inhibit the growth of pathogenic bacteria [19]. Therefore, to promote production and to reduce disease symptoms in aquaculture for sustainable development, screening and selection of probiotics are crucial [19].

Among the numerous probiotics, *Bacillus* species have proven to be very useful due to their special qualities such as the production of non-pathogenic and non-toxic compounds, improvement of water quality and a sporulation capacity which gives them an advantage in terms of survival (heat-tolerance and longer shelf-life) in diverse environments compared to other probiotics [7, 11, 20, 21]. These qualities have translated into their ability to improve growth, and enhance immunity and response to stress, improving the rearing water quality and above all enhancing the resistance of fish to diseases [5, 10, 22].

Bacillus species as probiotics used in aquaculture have many sources including soil, water, decaying matter, commercial sources, and the gastrointestinal tract of fish and other vertebrates [23–25]. However, as stated by Kavitha et al. [7] and Ghosh et al. [26], most of the probiotics used in aquaculture are commercial probiotics which are often relatively ineffective because they are isolated from non-fish sources hence ineffective in the colonisation process in the fish gut [19]. It was also established that those isolated from the intestinal tract of fish are more effective on their host in comparison to others [22, 27, 28]; thus, it is advantageous to isolate probiotics from fish to be used in aquaculture. Taking all of the above into consideration, in the present study, we have isolated, identified and characterised three probiotic *Bacillus* species based on their probiotic traits including antagonism to selected fish pathogens, resistance to low pH and high temperature, non-haemolytic nature and bile tolerance from the intestines of Nile tilapia *Oreochromis niloticus*. We hope that the information provided in this study could be further used to test their efficacy in tilapia culture.

Materials and Methods

Sample Collection

Healthy (i.e. without any symptoms of infection (i.e., haemorrhage, ascites, lethargic, and detachment of scales) [5] samples of Nile tilapia, *O. niloticus*, of average weight 150 ± 5 g were obtained from a local fish farm (Zhanjiang, Guangdong Province, China) and transported alive to the laboratory in polythene bags containing oxygenated water for immediate use.

Isolation of Gut Bacteria

Fish were anesthetised by exposure to an overdose of ethyl 3-aminobenzoate methanesulfonate, tricaine methanesulfonate (Sigma-Aldrich, 150 mg L−1MS-222) and then killed by a blow to the head. Fish were cleaned externally with cotton dipped into 75% ethanol to remove or kill any external bacteria on their bodies. The fish guts were then dissected using sterile scissors, and the intestines removed and stripped carefully to remove all digesta content and washed three times using a physiological saline solution (PBS). The weight of the intestines was then taken, and equal proportions of PBS by volume added. The content was then homogenised using 15 ml Borosilicate glass tissue homogeniser (Shanghai Lenggu Instrument Company, Shanghai, China) under sterile conditions in ice to keep it cold. Afterwards, 0.5 ml of the gut homogenate was diluted with 4.5 ml PBS. This mixture was serially diluted using PBS, and 0.1 ml of the aliquot was spread on Luria-Bertani (LB) agar plates. The plates were incubated for 24 h at 30 °C. Single colonies were randomly selected and inoculated into LB media for mass culture under the same culture conditions. Repeated streaking of the isolates was done to obtain very pure colonies. Potential probiotic strains were characterised based on their basic morphology and identified by 16S rDNA gene sequence analysis using universal bacterial primers 27F (AGAGTTTGATCCTG GCTCAG) and 1492R (GGTTACCTTGTTACGACTT) through polymerase chain reaction (PCR) [29]. The PCR reaction system contained 1 μL of each primer, 1 μL template of each isolate, 12.5 μL of 10 × Ex*taq* buffer and 9.5 μL of double distilled water. For negative control, double distilled water was used as template, and for positive control, *Vibrio*

harveyi (previously isolated in our laboratory) [30] was used as template. The PCR amplification was initiated with denaturation at 96 °C for 5 min followed by 33 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min 30 s; the amplification was completed by holding the reaction mixture at 72 °C for 10 min. The PCR products were analysed by agarose gel (1% *w*/*v*) electrophoresis and later sequenced by Sangon Biotech Co., Ltd. (Guangzhou, China). The sequence homology was compared with 16S rDNA gene sequences available in the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) program from the National Center for Biotechnology Information (NCBI). Similarity analysis was carried out to help identify the types of probiotic strains. Also, a phylogenetic tree was constructed using Mega 7 software to establish evolutionary relationships. Identified probiotic strain sequences were then submitted to NCBI and accession numbers obtained.

Biochemical Characterisation

Biochemical characterisation (Table 2) of the selected probiotic strains was assayed using commercial kits (Huankai Microbial, Guangzhou, China) following the manufacturer's protocol and confirmed using *Bacillus cereus* (HBIG07-1) identification bar (Qingdao Hope Bio-Technology co., Ltd., Qingdao, China).

Growth of Bacteria in Luria-Bertani Broth

A single colony of probiotic bacteria from an LB agar plate was selected and inoculated into 5.0 ml of LB broth and incubated at 37 °C overnight. The next morning, 1 ml of the culture was inoculated into 100 ml sterile LB broth in 500-ml Erlenmeyer flasks and incubated at 37 °C with shaking (150 rpm) while monitoring their growth by measuring the absorbance at 600 nm at 2 h interval for 24 h [31].

Biosafety Assay

In order to assess the possible harmful effects of the probiotic bacteria isolates in Nile tilapia, 0.1 ml (10^8 CFU/ ml), each of the bacteria, was intraperitoneally injected into groups of three (each consisting of 10 Nile tilapia fish with an average body weight of 100 g) fish. A control group (10 fish) was injected with the same volume of sterile PBS (pH 7.2). The culture condition of fish was similar as previously described [32]. Fish were monitored daily to detect any clinical signs, and mortality rate was recorded for 10 days.

Antibiotic Susceptibility

Antibiotic susceptibility of the three selected isolates was evaluated against some antibiotics (listed in Table 3) using commercial antibiotics discs purchased from Hangzhou Microbial Reagent Co., Ltd., Hangzhou, China. Antibiotic discs were carefully placed on to Mueller-Hinton agar plates previously spread with the probiotic bacteria and incubated for 24 h at 37 °C. Susceptibility was observed by measuring (mm) the zone of inhibition as previously described by Patel et al. [33].

Resistance to Bile Salts

Resistance to bile was determined according to modified methods described by Argyri et al. [34]. Briefly, bacterial cells from an overnight culture were harvested (9000 g, 5 min, 4 °C), washed twice with PBS buffer (pH 7.2) and re-suspended in PBS solution (pH 7.4), containing 0.5% (*w*/*v*) bile salts (BBI Life Sciences, Shanghai, China). Resistance was evaluated in triplicates by counting viable colonies after incubation at 37 °C for 1 h, 2 h, 3 h and 4 h.

Resistance to High Temperature

The ability of the bacteria isolates was assessed according to Guo et al. [18] with slight modification to determine their resistivity to different temperatures since the processing of fish feed at times requires high temperatures. Overnight culture of isolates was washed twice with PBS (pH 7.4) and afterwards exposed to 80 °C, 90 °C and 100 °C temperature for 2 min, 5 min and 10 min, respectively, after which equal volume of sterile LB broth was added to the heat-treated isolates to determine their ability to grow after heat treatment. Growth was monitored by measuring absorbance at 600 nm after 12 h of incubation at 37 °C with continuous shaking (150 rpm).

Compatibility of the Three Isolates

Compatibility study was done according to methods described by Rajyalakshmi et al. [35]. In brief, the three probiotic isolates were vertically streaked on LB agar plate 5 mm apart followed by perpendicular streak 10 mm apart from each other. The plates were incubated for 24 h at 37 °C, and compatibility was determined by observing the zone of inhibition among the isolates.

Antimicrobial Activity

Three pathogenic bacterial strains were previously isolated from diseased fish from Langye fish farms in the Gaozhu City of the Guangdong Province and identified as *Streptococcus agalactiae*, *Aeromonas hydrophila* and *Vibrio*

harveyi for use in our laboratory. These pathogens were tested against the *Bacillus* strains isolated from the fish used in the present study using the cross streak method and agar well diffusion method [36].

Auto-aggregation

The auto-aggregation of the selected *Bacillus* strains was analysed in accordance with the modified method by Lee et al. [37]. Bacterial cells were harvested by centrifugation at 9400*g* for 3 min, washed with PBS twice, re-suspended in the supernatant and then vortexed for 30 s. The absorbance was measured at 600 nm using a spectrophotometer (Shanghai Inesa Analytical Instrument Company, shanghai, China) at 0 h, 1 h, 2 h, 3 h and 24 h.

$$\text{Auto-aggregation}\ (\%) = (1 - A_t/A_0) \times 100$$

A_0 = Absorbance at 0 h at 600 nm.
A_t = Absorbance at 1, 2, 3, 24 h at 600 nm.

Cell Hydrophobicity

The cell hydrophobicity of the selected *Bacillus* strains was analysed according to Lee et al. [37]. Briefly, a 24 h culture was centrifuged at 9400*g* for 3 min, and the cells were washed and re-suspended with 2 ml of phosphate buffer saline (PBS, pH 7.4) twice. To determine the percentage hydrophobicity, its absorbance was measured at 600 nm and recorded as A_0. An equal volume of the cell suspension was mixed with each solvent, namely chloroform (an acidic solvent), xylene (a non-polar solvent) or ethyl acetate (a basic solvent), and vortexed for 5 min. The mixture was allowed to separate into two phases for 30 min. The absorbance was measured at 600 nm and recorded as A_1 and hydrophobicity (%) calculated as:

$$\text{Hydrophobicity}\ (\%) = (1 - A_1/A_0) \times 100$$

A_0 = Absorbance before mixing with solvent at 600 nm.
A_1 = Absorbance after mixing with solvent at 600 nm.

Haemolytic Activity

The selected probiotic *Bacillus* species were subjected to a haemolytic assay by streaking them onto agar plates supplemented with 7% sheep blood. The plates were incubated at 37 °C for 48 h, and the haemolytic zones were observed. The isolates were subsequently classified as α, β or γ-haemolysis. Isolates with a green zone around the colony were recorded as α-haemolysis, while those with a clear zone were denoted as β-haemolysis and those that did not produce any zone around the colony was referred to as no haemolysis [37, 38].

Determination of Optimal Growth and pH

Optimal growth and pH was assessed according to Kavitha et al. [7]. In brief, fresh overnight cultures of bacteria isolates were inoculated into LB broth with variable pH (1–10). The pH was adjusted with acetic acid (99%) and 5 N NaOH. The inoculated broths were incubated at 37 °C for 24 h and growth monitored using a spectrophotometer (Shanghai Inesa Analytical Instrument Company, shanghai, China) at 600 nm (OD) against the uninoculated broth.

Detection of Biofilm Formation (Congo Red Agar Method)

Biofilm production was detected according to the methods described by Kavitha et al. [7]. Briefly, the isolates were streaked on Mueller Hinton agar supplemented with 0.8 g/l of Congo red dye and incubated at 37 °C for 48 h. The presence of black colonies with a dry crystalline consistency indicated biofilm production, and red colonies indicated non-biofilm-producing strains.

Statistical Analysis

All the experiments were performed in triplicates, and the results were subjected to one-way analysis of variance (ANOVA). The differences in mean values were identified by Tukey's HSD tests ($P < 0.05$). Data were expressed as a mean ± standard error (SE). Data were analysed by SPSS (IBM SPSS STATISTICS, 16.0 package, IBM Corporation, New York, USA) for Windows version 7.0 (SPSS, Chicago, USA).

Results

Identification of Gut Bacteria

Three potential probiotic bacteria, TPS3N, TPS4 and TPS17, were selected following morphological and biochemical characterisation (Tables 1 and 2). 16S rDNA PCR (Fig. 1) gene sequence analysis revealed that the three isolates were *Bacillus* species (refer to supplemental data for sequences). The three isolates, TPS3N, TPS4 and TPS17, showed close sequence homology (99%) with *Bacillus velezensis*, *Bacillus subtilis* and *Bacillus amyloliquefaciens*, respectively. Phylogenetic analysis (Fig. 2) confirmed that the isolates TPS3N, TPS4 and TPS17 were closest to *Bacillus velezensis*, *Bacillus subtilis* and *Bacillus amyloliquefaciens*, respectively. The 16S rDNA gene sequences were submitted to GenBank (NCBI) and accession numbers obtained; thus, the isolates

Table 1 Morphological characterisation of the isolates

Morphological characterisation							
Isolate	Form	Surface	Texture	Size	Colour	Elevation	Margin
TPS3N	Circular	Dry	Rough	Medium	Creamy white	Flat	Entire
TPS4	Irregular	Dry	Rough	Medium	White	Crateriform	Entire
TPS17	Irregular	Mucoid	Rough	Large	White	Umbonate	Undulate

TPS3N, TPS4 and TPS 17 were designated as *Bacillus velezensis* TPS3N (MK130897), *Bacillus subtilis* TPS4 (MK130899) and *Bacillus amyloliquefaciens* TPS17 (MK130898), respectively.

Biochemical and Morphological Characterisation

Tables 1 and 2 summarise the biochemical and morphological characteristics of the three isolates. The three isolates had similar biochemical characters and thus can use almost all the carbon sources examined except TPS3N which did not demonstrate an ability to use citrate as a carbon source. All isolates were negative for lactose fermentation, hippuric acid, gelatin liquefaction, methyl red and VP test. Only TPS4 was able to grow in lysozyme broth and was mannitol positive; thus, it is halophilic. However, they have different morphological characteristics (Fig. 3).

Growth Curve (in LB) Using OD

All the three isolates had their log phase beginning at approximately 2 h after incubation at 37 °C with continuous shaking (150 rpm). TPS3N attained its stationary phase earlier than TPS4 and TPS17 (Fig. 4).

Biosafety Assay

There were no pathological symptoms (i.e. oedema, haemorrhage, lesions, loss of scale and mucus) observed in both experimental and control fish after in vivo biosafety assay. Also, no mortalities were recorded. This confirmed that the isolates were non-pathogenic.

Antibiotic Susceptibility

The results of antibiotic susceptibility of the selected isolates are shown in Table 3. All the isolates were highly susceptible to most of the tested antibiotics except TPS3N and TPS17 which were resistant to ceftazidime. The isolate TPS4 and TPS17 were found to be moderately susceptible to ceftazidime and clindamycin and furazolidone, respectively. With regard to polymyxin, all the isolates were moderately susceptible.

Resistance to Bile Salt

The isolates were subjected to 0.5% bile salt resistance assay and survivability monitored by counting the number of colony forming units after 4 h of exposure and expressed as a percentage. The result revealed that more than 50% of the isolates

Table 2 Biochemical characterisation of the isolates

Biochemical characterisation							
Tests	TPS3N	TPS4	TPS17	Tests	TPS3N	TPS4	TPS17
Rhamnose	+	+	+	Haemolysis	α	γ	α
Sorbitol	+	+	+	Catalase	+	+	+
Inositol	+	+	+	VP	−	−	−
Adonitol	+	+	+	Methyl Red	−	−	−
Simon's citrate	−	+	+	Urease	+	+	+
Lactose fermentation	−	−	−	Gelatin liquefaction	−	−	−
Starch hydrolysis	+	+	+	Hippuric acid	−	−	−
Glucose	+	+	+	Mannitol	−	+	−
Arginine dihydrolase	+	+	+	Gram staining	+	+	+
Nitrate reduction	+	+	+	Biofilm production	−	−	−
Lysozyme broth	−	+	−	Spore formation	+	+	+

− negative, + positive

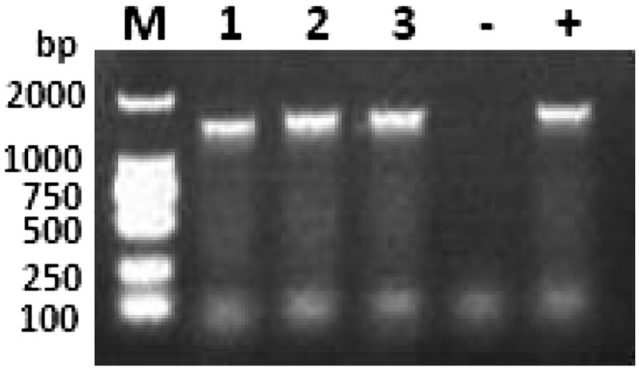

Fig. 1 16s rDNA amplification of the three isolates. Lane M: DNA marker DL2000. Lane 1: TPS3N. Lane 2: TPS4. Lane 3: TPS17. Lane −: negative control. Lane +: positive control

survived after 3 h of exposure. However, after 4 h of bile salt exposure, the percentage survivability of TPS3N dropped to 46.2%, while TPS4 and TPS17 remained above 50% (Fig. 5). Regarding TPS3N, significant ($P < 0.05$) reduction in percentage survival was observed at each hour after 2 h of exposure to bile salt. Significant reduction in percentage survival was observed in TPS4 after 1 h and 2 h (83.8% and 62.2%, respectively) after which the reduction remained insignificant to the fourth hour of exposure. In TPS17, a significant reduction in percentage survival was observed after 2 h and remained unchanged afterwards.

Resistance to High Temperature

After exposure to different temperatures (80 °C, 90 °C, 100 °C for 2 min, 5 min, 10 min, respectively), the three isolates gave promising results. High growth (OD) was observed in all the isolates exposed to the various temperatures in comparison to the control (isolates without exposure to higher temperatures). However, no significant ($P < 0.05$) differences were observed at the different times of exposure among the different temperatures (Fig. 6).

Compatibility of the Three Isolates

In the present study, when the isolates were characterised for their compatibility, no definite sign of suppression of the three bacteria isolates was observed on each other suggesting that they were compatible.

Antimicrobial Activity

In the present study, the three isolates were evaluated for their antimicrobial traits against three fish pathogens (viz. *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Vibrio harveyi*). It was noted that all the three selected isolates were found to inhibit at least one of the three tested pathogens (Table 4). TPS4 could not inhibit *Vibrio harveyi* and *Aeromonas hydrophila* both in the cross streak method and in the agar well diffusion method (Fig. 3).

Auto-aggregation

Auto-aggregation assay which is strongly correlated with cell adhesion to the digestive tract revealed that all the three isolates TPS3N, TPS4 and TPS17 had low cell adhesion ability (less than 30%) at the first 3 h. However, after 24 h, cell adhesion increased to 92.97%, 84.83% and 89.13%, respectively (Fig. 7).

Cell Hydrophobicity

Adhesion of the selected isolates (TPS3N, TPS4, TPS17) to ethyl acetate, chloroform and xylene was tested to determine

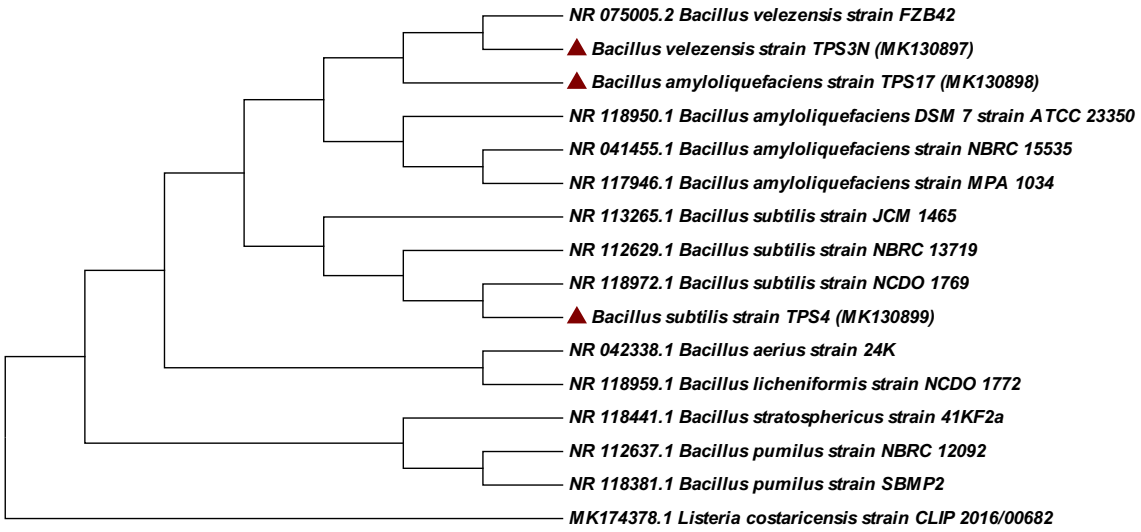

Fig. 2 Phylogenetic tree constructed by neighbour joining method showing the relatedness of TPS3N, TPS4 and TPS17 to other *Bacillus* species

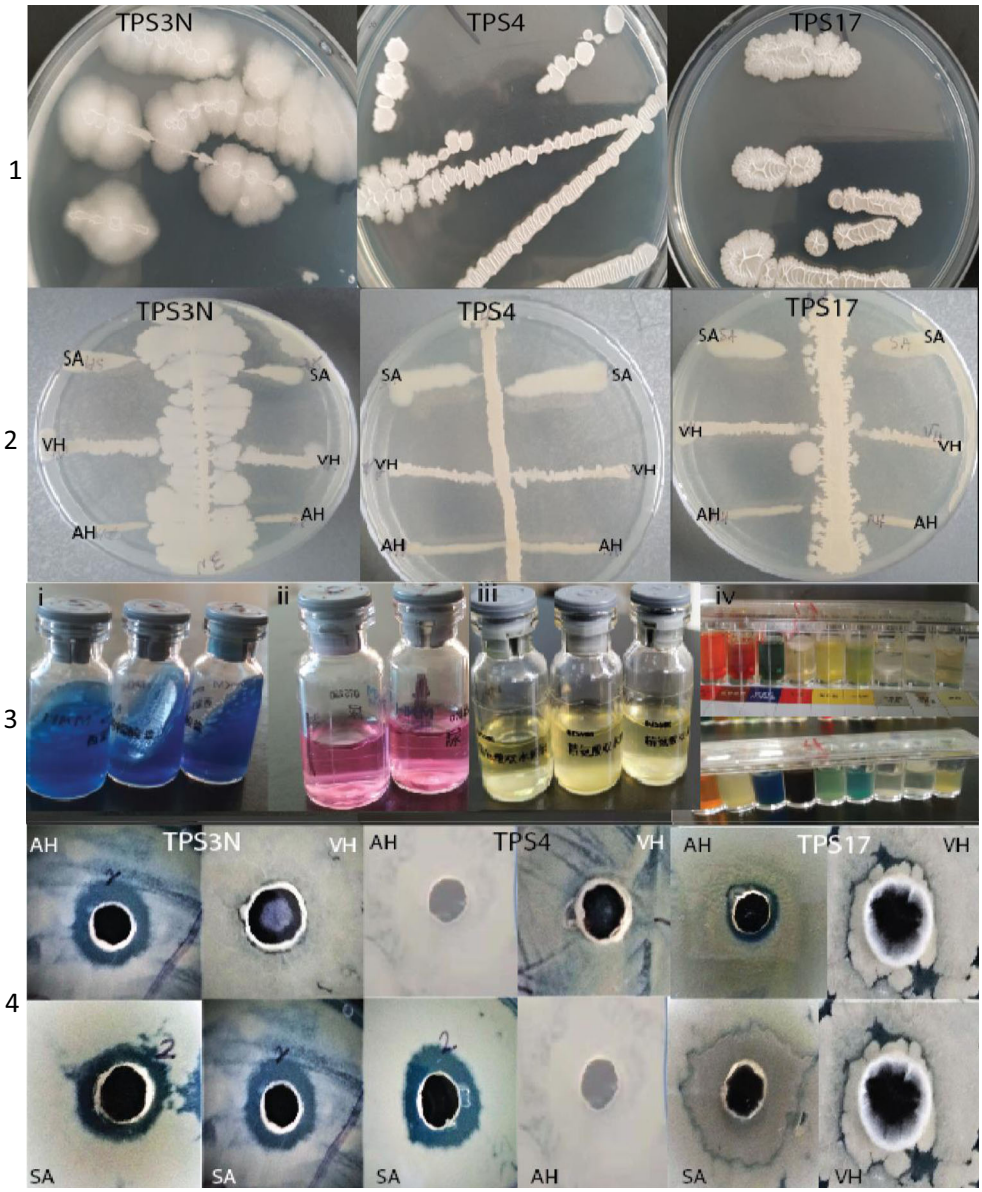

Fig. 3 Pictorial overview of the morphological, biochemical characteristics and antimicrobial activities of the three isolates. Lane 1: morphology of TPS3N, TPS4, TPS17. Lane 2: antimicrobial activities of TPS3N, TPS4, TPS17 (cross streak method). Lane 3: evidence of biochemical test of the isolates (3i: citrate reduction test, 3ii: urease test, 3iii: arginine dihydrolase test, 3iv: confirmatory test using *Bacillus cereus* identification bar). Lane 4: antimicrobial activities of TPS3N, TPS4, TPS17 (agar well diffusion method). SA: *Streptococcus agalactiae*, AH: *Aeromonas hydrophila*, VH: *Vibrio harveyi*

the adhesion capability of the bacterial to cell surfaces, and the results are shown in Fig. 8. Cell surface hydrophobicity of the isolates TPS3N and TPS17 to ethyl acetate was significantly lower ($P < 0.05$) than that of chloroform and xylene.

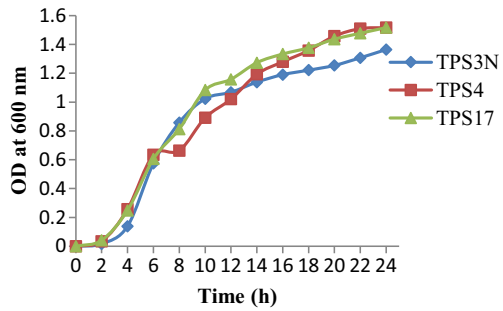

Fig. 4 Growth curve of the isolates measured at 600 nm

Haemolytic Activity

Regarding haemolytic activities, TPS3N and TPS17exhibited α-haemolysis, while TPS4 exhibited γ-haemolysis (Table 2).

Determination of Optimal Growth and pH

After exposure to different pH, TPS3N, TPS4 and TPS17 gave promising tolerance results. Though irregular, gradual increase in the growth of the isolates was observed within the pH range of 1.0–7.0 (TPS3N and TPS17) and 1.0–8.0 (TPS4). Decreased growth was observed as pH increased from 7.0 to 10.0 suggesting that the isolates could survive in extreme acidic as well as alkaline conditions. All the *Bacillus* strains displayed a significant difference ($P < 0.05$) in different pH at some points (Fig. 9).

Table 3 Antibiotics susceptibility of the isolates (mm)

Antibiotics	TPS3N	TPS4	TPS17	Antibiotics	TPS3N	TPS4	TPS17
Gentamicin (10 μg)	S	S	S	Cephalexin (30 μg)	S	S	S
Ampicillin (10 μg)	S	S	S	Ceftriaxone (30 μg)	S	S	S
Kanamycin (30 μg)	S	S	S	Amikacin (30 μg)	S	S	S
Penicillin (10 μg)	S	S	S	Clindamycin (2 μg)	S	I	S
Polymyxin (300 μg)	I	I	I	Cefoperazone (75 μg)	S	S	S
Chloramphenicol (30 μg)	S	S	S	Furazolidone (300 μg)	S	S	I
Erythromycin (15 μg)	S	S	S	Ceftazidime (300 μg)	R	I	R
Tetracycline (30 μg)	S	S	S	Doxycycline (30 μg)	S	S	S
Ciprofloxacin (5 μg)	S	S	S				

S susceptible, *R* resistant, *I* intermediate

Detection of Biofilm Formation (Congo Red Agar Method)

Congo red agar method was used to screen and ascertain the ability of the isolates to produce biofilm. None of the isolates formed black colonies indicating negative biofilm production.

Discussion

Attempts to curb the situation of fish diseases in aquaculture have led to the discovery and use of probiotics as a safer alternative to the widely used antibiotics due to their adverse effects [8, 39]. *Bacillus* species as probiotics have characteristics that make them outstanding among all other probiotics [11, 21]. Also, the positive effects of *Bacillus* species in tilapia aquaculture have been confirmed by many researchers [5, 10, 22], and those isolated from the gastrointestinal tract of a healthy fish are considered the best source for controlling many infectious diseases in fish [27].

In this current investigation, we isolated and assessed the probiotic potentials of three *Bacillus* species, viz. *Bacillus velezensis* TPS3N (MK130897), *Bacillus subtilis* TPS4 (MK130899) and *Bacillus amyloliquefaciens* TPS17 (MK130898) from the gut of Nile tilapia using in vitro methods. The isolated strain TPS3N, TPS4 and TPS17 were identified using morphological characteristics and biochemical tests and further confirmed by 16S rDNA gene sequence. The three isolates utilised a wide range of carbon sources namely rhamnose, sorbitol, inositol, adonitol, citrate, glucose, starch, and mannitol as well as the amino acid arginine which suggests that they could be helpful in the digestion of carbohydrates, and the hydrolysis of amino acids thus can be used as probiotics and for the production of value-added products in food industries as have been reported in other studies [7, 27, 37].

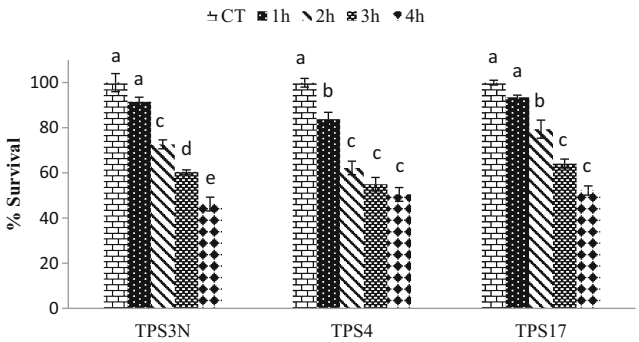

Fig. 5 Bile tolerance of the isolates after 4 h at 37 °C. Values are presented as mean ± SE. Significant differences are indicated by different letters ($P < 0.05$)

Unlike other probiotics, *Bacillus* species produce spores that are more heat tolerant [18, 40], resistant to low pH and a high percentage of bile concentration [41, 42], and have the abilities to germinate and survive in the gut of fish [41, 43, 44]. Gastric (low pH) and intestinal (high bile concentration) tolerance are the prerequisites for probiotics to survive and colonise the gut to produce beneficial traits [45, 46]. Also, heat treatment is an essential process during feed production to increase the palatability and kill pathogenic vegetative cells [18]. In this study, isolates TPS3N, TPS4 and TPS17 exhibited sporulation ability which translated into their ability to withstand pH as low as 1 (Fig. 9), 0.5% bile concentration (Fig. 5) and higher viability after heat treatment compared to the control (Fig. 6). It could, therefore, be deduced that higher temperatures (80 °C, 90 °C, 100 °C) activated the bacteria strains, hence the higher growth rates. Similar studies support the sporulation capacity of *Bacillus velezensis* [47, 48], *Bacillus subtilis* [18] and *Bacillus amyloliquefaciens* [28]. The higher viability of the strains after heat treatment strongly suggests that they could be used as feed additives and hence are good potential probiotics.

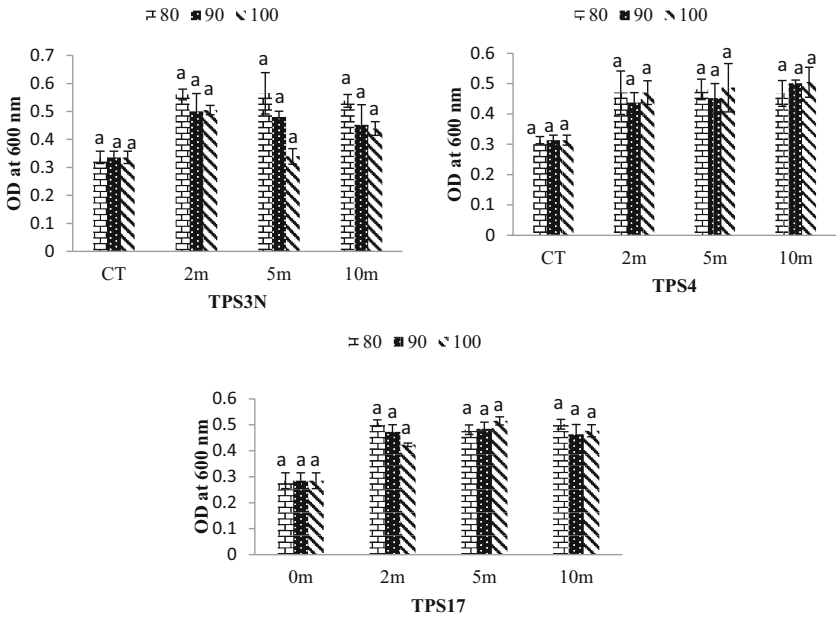

Fig. 6 Resistance of the isolates to high temperatures. Values are presented as mean ± SE. Significant differences are indicated by different letters ($P < 0.05$)

Colonisation and adhesion to epithelial cells and mucosal surfaces are important characteristics of good probiotics since it guarantees the ability to resist the fluctuation of the intestinal content and it also inhibits the pathogenic bacteria adhesion by occupying all the space of the intestine as well as inhibits inflammatory reactions [49–51]. An indirect method of determining the adhesion ability of probiotic bacteria is the determination of the auto-aggregation and the hydrophobicity of the bacteria [52, 53]. In this study, TPS17 showed much higher hydrophobicity (97.5% and 97.1%) with chloroform and xylene respectively indicating bacterial adhesion to hydrocarbons compared to TPS3N (92.8% and 95.8%) and TPS4 (85.1% and 96.6%). With regard to ethyl acetate, however, the highest hydrophobicity was observed in TPS4 (90.6%) as against TPS3N (78.1%) and TPS17 (74.9%). The hydrophobicity results in this study are relatively higher than that of *Bacillus* species in a study conducted by Lee et al. [37] and Manhar et al. [54] suggesting higher electron donation (chloroform) and acceptance (ethyl acetate) [55] of our strains hence higher adhesion to epithelial cells. Also, there is a strong correlation between auto-aggregation and cell adhesion to the digestive tract, which is one of the prerequisites for a good probiotic bacteria. TPS3N, TPS4 and TPS17 showed high auto-aggregation (93.0%, 84.8% and 89.1%, respectively) after 24 h which agreed with a similar study conducted by Liu et al. [56].

The safety prerequisites for the selection of a probiotic strain are the absence of haemolytic activity, and antibiotic resistance [34] as haemolysin is considered a virulent factor due to its ability to initiate infection by entering small lesions in the mucous membranes and skin of any host

Table 4 Antimicrobial activity of the isolates against selected fish pathogens

Pathogenic bacteria	Isolates		
	TPS3N	TPS4	TPS17
Streptococcus agalactiae	+ + + +	+ + + +	+ + +
Vibrio harveyi	+	−	+ +
Aeromonas hydrophila	+ + +	−	+ +

Values were calculated as inhibition zone diameter minus well diameter (mm)

+ zone of inhibition between 1 and 2 mm, + + zone of inhibition between 2 and 4 mm, + + + zone of inhibition between 4 and 6 mm, + + + + zone of inhibition above 6 mm, − no inhibition

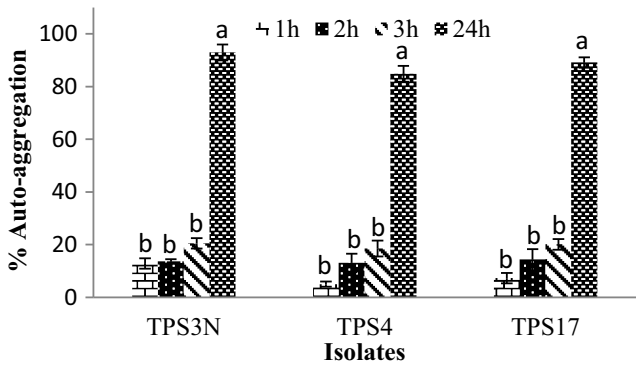

Fig. 7 Auto-aggregation of the isolates after 24 h. Values are presented as mean ± SE. Significant differences are indicated by different letters ($P < 0.05$)

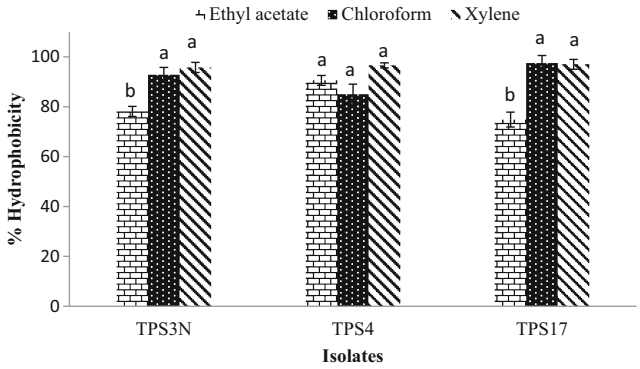

Fig. 8 Cell surface hydrophobicity of the isolates to various solvents. Values are presented as mean ± SE. Significant differences are indicated by different letters ($P < 0.05$)

[19, 27]. No or γ-haemolysis and α-haemolysis are considered to be safe, and β-haemolysis is considered harmful [57]. In this study, isolates TPS3N and TPS17 exhibited α-haemolysis, while TPS4 showed γ-haemolysis. A similar observation was made by Lee et al. [37] and Kavitha et al. [7]. Out of the 17 antibiotics tested, all the three isolates were highly susceptible to 15. Intermediate susceptibility was observed in polymyxin for all the isolates, also in clindamycin and ceftazidime for TPS4 and furazolidone for TPS17.

In a previous study by Saarela et al. [58], it was revealed that food produced using mono species probiotics had a sour and acidic taste. This has inspired us to investigate the compatibility of our strains in order to be used as multispecies probiotics, and the results showed that all the three isolates are compatible with one another which is in agreement with Rajyalakshmi et al. [35] who also observed compatibility among some *Bacillus* species.

Biofilms have great significance for public health, as biofilm-forming microorganisms exhibit dramatically reduced susceptibility to antimicrobial agents [59] despite the advantages associated with biofilm formation [60, 61]. Meanwhile, antibiotics-resistant strains are considered unsafe for use as probiotics. In this study, isolates TPS3N, TPS4 and TPS17 tested negative for biofilm formation test. Similarly, in an experiment conducted by Kavitha et al. [7], only one isolate out of three isolates formed biofilm.

Fish diseases caused by *Streptococcus* [62], *Aeromonas* [63] and *Vibrio* [64] species have been reported in aquaculture. Earlier reports indicated that several species of *Bacillus* have antimicrobial properties against several Gram-positive and Gram-negative pathogenic bacteria. In this study, aside from TPS4 which was effective against *Streptococcus agalactiae* only, TPS3N and TPS17 showed great antimicrobial effects against the three pathogenic bacteria, viz. *Streptococcus agalactiae*, *Aeromonas hydrophila* and *Vibrio harveyi* suggesting that the three *Bacillus* strains TPS3N, TPS4 and TPS17 could be used to fight fish disease in aquaculture.

Conclusion

The study showed that all three strains, TPS3N, TPS4 and TPS17 isolated from the gut of Nile tilapia, *O. niloticus*, possess characteristics such as high survivability after heat treatment, non-haemolytic nature, wide antimicrobial activities and safety confidence including antibiotic susceptibility. Overall, the features identified in these bacteria strains show that they might have great potential as probiotics for aquaculture use. However, in vivo assessment is required to ascertain their applications in aquaculture environment especially in tilapia culture.

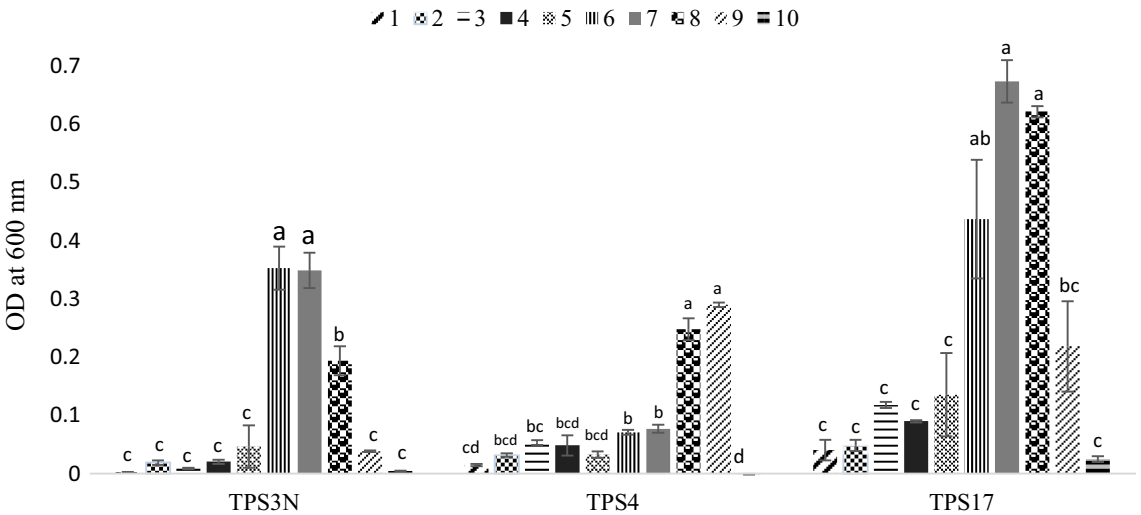

Fig. 9 Bacterial growth at different pH (1.0–10.0) levels. Values are presented as mean ± SE. Significant differences are indicated by different letters ($P < 0.05$)

Authors' Contributions Yishan Lu and Felix K.A. Kuebutornye conceived and designed the experiment. Felix K.A. Kuebutornye, Zhiwen Wang and Yuan Li carried out field experiments and laboratory analysis of data. Emmanuel D. Abarike drafted and proofread the manuscript and Michael Essien Sakyi analysed some data, edited and proofread the revised manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Animal Rights All fish were handled following the U.K animal act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

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