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Research article

Isolation and Selection of Probiotic Bacteria from Nile Tilapia *(Oreochromis niloticus)* as Probiotics for Promoting Fish Growth

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Abstract: *Bacillus* spp. and lactic acid bacteria (LAB) were isolated from samples of Nile tilapia body (gastrointestinal tract, mucus, and fish scales) and fishpond water in Songkhla province, Thailand. Fifty-one bacterial isolates were obtained, and only 44 Gram-positive isolates were tested for their probiotic properties. These isolates were selected based on the ability to inhibit serious pathogens in tilapia, namely *Streptococcus agalactiae* and *Aeromonas hydrophila*; only isolated bacteria that can inhibit both fish pathogens were selected. Hence, 6 selected bacteria were further tested for their nutrient digestion, adhesion, and tolerance to acids and bile salts. It was found that only 5 isolates passed those tests. There were three isolates of bacilli and two isolates of LAB. The five isolates were identified using the 16s rRNA gene method and API test kits, and only two isolates (*Bacillus subtilis* HW3B and *Lactiplantibacillus plantarum* DW5L) that could be safe for fish and humans were selected for further studies as probiotics for fish cultivation.

Keywords: Nile tilapia; Fish pathogens; Probiotics

1. Introduction

The aquaculture system and the food used for raising aquatic animals have developed greatly due to the increasing demand for aquatic animals. However, in raising aquatic animals, there is waste from the remaining food that the animals cannot eat, including excreta. The waste will accumulate in the water and the environment. For example, ammonia produced from the digestion of protein in food, primarily in fish hepatocytes, is excreted through the gills [1]. The excess ammonia causes poor water quality, leading to fish weakness and high sensitivity to infection by pathogens. Due to such problems, probiotics have a greater role in aquaculture. For the above reasons, microorganisms with probiotic properties have been selected to solve such problems. Especially the ability to promote the growth of aquatic animals and inhibit pathogens. Probiotics used in aquaculture are mainly in the lactic acid bacteria (LAB) and *Bacillus* spp. group [2].

The first evidence of the application of probiotics in aquatic animals was in 1986 when Kokasa used *Bacillus toyoi* spores in the diet of yellowtail fish (*Seriola quinqueradiata*) to promote growth [3]. Following that, many more studies

were aimed at selecting bacteria for use as probiotics, such as Worananthakij's study. Worananthakij [4] selected probiotics from the gastrointestinal tract of tilapia, which mainly included *Bacillus* spp. and LAB. Several studies also use *Bacillus* spp. and LAB to inhibit pathogenic bacteria in aquaculture. For example, bacteria that can inhibit and resist *Aeromonas hydrophila* include *Lactococcus lactis*, *Lactobacillus plantarum*, and *Lactobacillus fermentum* [5], as well as *Bacillus subtilis* [6] and *Bacillus pumilus* [7]. Additionally, *Bacillus subtilis* has been shown to inhibit *Streptococcus agalactiae* [8].

Currently, in aquaculture, probiotics serve multiple functions, including facilitating the decomposition of organic matter in aquaculture ponds, reducing concentrations of nitrogen and phosphorus by transforming them into non-toxic forms for aquatic animals, and promoting the growth of aquatic plants and algae through the conversion of organic matter into inorganic matter. Additionally, probiotics enhance the growth of aquatic animals and provide resistance against pathogens, stimulate the immune system in aquatic organisms, and aid digestion by producing beneficial nutrients such as amino acids, fatty acids, and vitamins [9].

Hence, in this study, we isolated LAB and *Bacillus* spp. from tilapia bodies and water in tilapia ponds to select them based on probiotic properties. The main requirement of probiotics focused on the ability to inhibit serious fish pathogens and digest waste in rearing water. Then, the selected bacterial strains were identified before being used as probiotics to search for safe strains. The outcome of this study may benefit fish farmers in the future.

2. Materials and Methods

2.1 Sample collection and isolation of potential probiotic bacterial strains

Ten Nile tilapia (*Oreochromis niloticus*) samples were collected from various locations in Songkhla province, including 3 samples from the Aquatic Science and Innovative Management Division (AQ), 2 samples from Klong Rian Market (RN), and 5 samples from Songkhla Inland Fishery Research and Development Center (KH). Additionally, 5 samples of fishpond water were collected from various locations (AQ, 3 samples, and KH, 2 samples) in Songkhla Province.

Bacterial strains were collected from Nile tilapia samples weighing 500-800 g by swapping the mucus around the fish's body with a sterile loop. The fish were euthanized by placing them in a sterile bag and submerging them in ice water before surgical procedures to collect fish scales. Then, a cloth was used to wipe the fish's body. Ethanol at 70% (v/v) was applied to clean the fish's abdomen before surgery to remove the gut. The gut was weighed and placed in 0.85% (w/v) normal saline solution (NSS) before being shaken in the stomacher at 230 rpm for 30 s. The liquid portion from this shaking was collected and used to isolate LAB and *Bacillus* spp.

2.2 Isolation of lactic acid bacteria (LAB)

One ml liquid portion was added into 9 ml NSS to dilute 10 times and with a ten-fold serial dilution to reach a 10^{-5} dilution. Subsequently, the pour plate technique was used to isolate LAB by adding one ml of every appropriate diluent into melted de Man, Rogosa, and Sharpe (MRS) agar, containing 0.04% (w/v) bromocresol purple and 0.02% (w/v) sodium azide. This agar plate was incubated at 30 °C for 1-2 days. Yellow colonies appeared after incubation, and these colonies were investigated for LAB characteristics by Gram staining and catalase test, following the procedure of Axelsson (1998)[10]. LAB appeared as Gram-positive and non-catalase-producing characteristics.

2.3 Isolation of *Bacillus* spp.

The diluents in Section 2.2 were used to isolate *Bacillus* spp. by a modified method from Phianpak *et al*. [11]. One ml of diluent was added into 9 ml of melted tryptic soy agar (TSA) medium in the test tubes, and the mixture was immersed in a water bath at 80 °C for 10 min. After heating, the mixture was thoroughly mixed and poured into sterile petri dishes to solidify. After 1-2 days of incubation, colonies were isolated and

examined for characteristics by Gram staining and microscopy. *Bacillus* spp. was identified as Gram-positive, rod-shaped, and endospores formation.

2.4 Investigation of probiotic properties

2.4.1 Antagonistic effect of potential probiotics against Aeromonas hydrophila and Streptococcus agalactiae

Isolated LAB and *Bacillus* spp. were tested for their ability to inhibit serious pathogens in Nile tilapia, specifically *A. hydrophila* and *S. agalactiae*, using an agar well diffusion method modified from Aslim *et al*. (2005) [12]. These two pathogens were obtained from the Songkhla Aquatic Animal Health Research and Development Center. A cell density of 10^5 CFU/ml of each indicator pathogenic bacterium was poured over the TSA medium with 0.75% agar, and the mixture was allowed to dry. Once the agar solidified, a sterile borer with a diameter of 5.8 mm was used to drill holes in the agar plate. LAB isolates were cultivated in MRS broth, while *Bacillus* spp. isolates were cultivated in tryptic soy broth. All cultures were incubated at 35 °C for 18 h. Each culture broth was centrifuged at 8,000 × *g* for 5 min to obtain supernatant. Subsequently, eighty microliters of the LAB and *Bacillus* spp. supernatants were added to the agar wells. The plates were then incubated at 30 °C for 24 h. The inhibition zone was measured to evaluate the antagonistic effect.

2.4.2 Nutrient digestion test

Both LAB and *Bacillus* spp. isolates with the antagonistic effect were tested for nutrient digestion properties, including protein, carbohydrate, and fat. LAB isolates were cultivated in MRS medium and *Bacillus* spp. isolates were cultivated in tryptic soy broth medium, with both groups incubated at 35 °C for 18 h. After cultivation, the culture was washed twice with 0.85% NSS, and the cell was suspended in NSS. One loopful of each cell suspension was dropped on gelatin, starch, and tributyrin agars[13]. After incubation at 30 °C for 48-72 h, protein and fat digestion tests were observed as clear zones around colonies. The digestion of starch was examined by adding an iodine solution. No blue color around colonies indicated the positive result of starch digestion.

2.4.3 Cell surface hydrophobicity test

The adhesion properties were modified using the method of Taheri *et al.*[14]. LAB isolates were cultivated in MRS medium and *Bacillus* spp. isolates were cultivated in tryptic soy broth medium, with both groups incubated at 35 °C for 18 h. After cultivation, the culture was washed twice with 0.85% NSS. Subsequently, cells were suspended in NSS and adjusted with 0.85% (w/v) NSS to turbidity at OD600 = 0.5. Three milliliters of cell suspension were placed before adding 1 ml of xylene. The mixture was vortexed for 90 seconds and then set for 15 min for solvent separation. The cell suspension measured the turbidity at OD600 and calculated the surface cell as the following equation.

$$\text{Cell surface hydrophobicity } (\%) = ((OD_0 - OD_t)/OD_0) \times 100$$

OD_0 and OD_t are the turbidity of the cell suspension before xylene mixing and the turbidity of the aqueous layer after mixing.

2.4.4 Acid and bile salts resistance test

Isolated bacteria that passed the inhibition of fish pathogens, nutrient digestion, and the cell surface hydrophobicity tests were tested for acid and bile salt resistance. The resistance of the acid and bile salts test was according to the method applied previously by Ratanaburee *et al.* [15]. Firstly, one loopful of LAB and *Bacillus* spp. were transferred into 4 ml of MRS broth and TSB, incubated at 37 °C for 24 h to use as inoculums. A 10% culture was added to 10 ml of MRS broth and TSB medium, again incubated at 37 °C for 24 h, then pipette 1 ml of culture broth into a sterile 1.5 ml microtube. Culture broths were centrifuged at 10,000 rpm at 4 °C for 10 min, rinse the cells with 0.85% saline twice, and then resuspend the prepared cells in 0.85% saline adjusted to pH 2.5 containing pepsin 3 mg/ml, 1 ml volume, and then incubated at 37 °C for 2 h in a water bath. The surviving organisms were then counted using the drop plate method on MRS agar containing 0.004% bromocresol purple and TSA and incubated at 37 °C for 24 h. The acid-incubated suspension for 2 h was

centrifuged at 10,000 rpm at 4 °C for 10 min. The cells were washed twice with 0.85% saline solution. The cell suspension was prepared with phosphate buffer pH 8 and 1 mg/ml of pancreatin enzyme, and 1 ml of tilapia bile of 3% concentration was incubated at 37 °C for 6 h in a water bath. Surviving bacteria were also counted using the drop plate method. Bacterial strains with constant residual cells were selected for further study (adapted from Madureira *et al*. [16]).

2.5 Identification of selected bacterial strains

Five bacterial strains from morphological inspections were all in Gram-positive bacteria. Phenotypic characterization of bacterial isolates on biochemical property was conducted using commercial test kits, API 50 CHL (Ref 50410, BioMérieux, France) and 50 CHB/E (Ref 50430, BioMérieux, France) for *Bacillus* spp., respectively. The procedure was tested according to manufacturing instructions. More specifically, molecular identification was applied [17]. In brief, 3 ml of bacterial suspension (OD660 = 1.0) extracted the DNA by following the protocol (TIANamp bacteria DNA kit, Tiangen, PR China) to obtain 30 µl of genomic DNA solution finally. 16s rRNA region was applied for amplification with both specific primers. 70 µl of amplified cocktail (TopTaq Master Mix Kit, Qiagen, Germany) was included each 1.4 µl of 10 µM of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') as a forward and a reverse primer, 35 µl of 2x master mix, 3 µl of DNA template, and 29.2 µl of RNase-free water, and then applied for 3 min at 94 °C of initial activation, 30 s at 94 °C of denaturation, 30 s at 46.4 °C of annealing, 1 min at 72 °C of extension, and 10 min at 72 °C of final extension with 35 cycle amplification. The PCR product was purified by kit protocol (TIANquick Mini Purification Kit, Tiangen, PR China) to obtain a final volume of 25 µl. The purified PCR product was sequenced using the Sanger sequencing method and submitted to 1st Base Company (Malaysia). After receiving the results, the sequences were analyzed by combining the forward and reverse sequences using BioEdit version 7.6.2.1. software. The sequences were identified with Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to receive the most similar species [18]. After identification, the Submission Portal submitted the sequences to the NCBI database (https://submit.ncbi.nlm.nih.gov/). The accession number of isolated bacteria was composed of OL365735 for strain AQ1/1B, OL365736 for HW3B, OL365737 for RN3B, OL365738 for DW5L, and OL365739 for LS7L. A phylogenetic tree was constructed to estimate the relationship between strains by the Bootstrap method of phylogeny with 1,000 replication tests in the Jukes-Cantor nucleotide model in a neighbor-joining statistical method by MEGA11 software. *Clostridium perfringens* DSM 756^T (NR_121697.2) was used as the out-group in the phylogenetic tree.

2.6 Statistical analysis

Data on the probiotic properties from *in vitro* tests were analyzed for statistical differences in variables by One Way Analysis of Variances (ANOVA) and comparing differences in means of variables using Duncan's multiple range test at a significance level of $p < 0.05$. Results are given as mean of three replicates ± standard deviation (S.D.).

2.7 Permission to use animals

The experimental procedures in this study followed the Institute of Animals for Scientific Purposes Development (IAD) National Research Council of Thailand (NRCT), permit's date: 26-2/2018, and license number: U1-07693-2561.

3. Results and Discussion

3.1 Sample collection and isolation of potential probiotic strains

Bacteria with different colony characteristics were isolated using TSA and MRS media for *Bacillus* and LAB, respectively (Table 1). 14 LAB isolates were found on MRS medium and 30 *Bacillus* spp. isolates were found on the TSA medium. All LAB isolates were obtained from the tilapia body, including the digestive tract, fish scale, and mucus, while *Bacillus* spp. isolates were obtained from fish pond water and the fish body (digestive tract, fish scale, and mucus). The detection of isolated strains in this study is correlated with

Worananthakij [4], who selected probiotics from the gastrointestinal tract of tilapia, which mostly found *Bacillus* spp. and LAB. Based on this data, 44 bacterial isolates were further studied for their probiotic properties.

Table 1. Isolation of *Bacillus* and lactic acid bacteria (LAB) with Gram staining from Nile tilapia and fish pond water

Location	Aquatic Science and Innovative Management Division (AQ)	Klong Rian Market (RN)	Songkhla Inland Fishery Research and Development Center (KH)	Water from AQ pond	Water from KH pond	Total
Bacillus Isolates (TSA)	4	7	6	10	3	30
LAB Isolates (MRS)	6	4	4	0	0	14

Data are the number of different colony forms.

3.2 Probiotic properties test

It was found that from the 44 isolates of isolated bacteria, there were only 14 isolates that were able to inhibit fish pathogens. Four bacterial isolates could inhibit only *Aeromonas hydrophila*, and only 4 isolates inhibited *Streptococcus agalactiae*. Furthermore, only 6 bacterial isolates inhibited both types of pathogens, consisting of DW5L, LS7L, AQ1/1B, RN3B, RX1B, and HW3B. They were separated into 2 LAB (DW5L, LS7L) isolates and 4 *Bacillus* spp. isolates (AQ1/1B, RN3B, RX1B and HW3B). Table 2 shows their inhibition zones of potential probiotics against the growth of fish pathogens. This is consistent with many studies reporting that *Bacillus* spp. and LAB can inhibit both pathogens. For example, bacteria that can inhibit and resist *A. hydrophila*, including *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus fermentum* [5], *B. subtilis* [6], and *B. pumilus* [7], whereas *B. subtilis* inhibited *S. agalactiae* [8]. This was due to the probiotics producing bioactive compounds, i.e., hydrogen peroxide, bacteriocins, lysozymes, siderophores, and proteases, to inhibit pathogens in fish [19, 20]. Some bacteria can also produce organic acids (acetic, butyric, lactic, and propionic acids) to lower the pH, making it an unsuitable condition for pathogenic growth [20].

Then, all 6 isolates of bacteria were tested for their ability to digest nutrients, including protein (gelatin), carbohydrates (starch), and fat (tributyrin agar). It was found that 3 isolates could digest protein, namely bacilli strains (RN3B, A/1/1B, and HW3B). Some isolates could digest carbohydrates, namely *Bacillus* strain AQ1/1B and *Bacillus* strain HW3B, and there were no isolates that could digest the medium mixed with tributyrin in the fat digestion test. This means that they have only enzymes to digest protein and carbohydrates. They can secrete the enzyme protease to cut protein peptide bonds into amino acids. As a result, probiotics can be absorbed and used for growth by probiotics, which benefits aquatic animals by allowing them to use nutrients better [21, 22]. Both bacilli (AQ1/1B and HW3B) also digested starch. Consistent with the study of Hayashida *et al.* [23], it was found that the bacterium *B. subtilis* produced the enzyme alpha-amylase to be used in the digestion of raw starch. *Bacillus* strain RN3B and two lactobacilli strains showed no digestion on the nutrients test. However, both LAB could inhibit both serious fish pathogens at a high level and were included for further study.

Table 2. Inhibitory effect of potential probiotic bacteria against the growth of *Aeromonas hydrophila* and *Streptococcus agalactiae*

Bacterial isolate	Inhibition zone (mm)		Bacterial isolate	Inhibition zone (mm)	
	A. hydrophila	*S. agalactiae*		*A. hydrophila*	*S. agalactiae*
B5L	12.5 ± 0.4	-	RX3L	-	13.6 ± 0.3
DW5L	15.5 ± 0.3	17.2 ± 0.3	RX4L	-	14.4 ± 0.2
B9L	-	13.3 ± 0.3	NN12L	-	12.7 ± 0.3
LS7L	10.1 ± 0.3	12.6 ± 0.2	AQ1/1B	10.5 ± 0.3	12.7 ± 0.3
RN1B	11.6 ± 0.3	-	AQ1/3B	10.5 ± 0.4	-
RN3B	12.4 ± 0.5	14.9 ± 0.3	HW2B	14.9 ± 0.2	-
RX1B	10.6 ± 0.2	12.0 ± 0.2	HW3B	15.8 ± 0.2	14.2 ± 0.3

The values presented are expressed as Mean of triplicate ± SD. "**-**" stands for no inhibition.

Hence, both LAB strains and 3 bacilli strains were selected to test probiotic properties *in vitro*, consisting of an acid tolerance test, bile salt tolerance test, and adhesion test by cell surface hydrophobicity. Table 3 presents the results of the characteristics of probiotics, including an acid tolerance test, bile salt tolerance test, and adhesion test by cell surface hydrophobicity. They all survived in simulated gastric juice (pH 2.0) and simulated intestinal juice (pH 8.0 + tilapia bile salt). Both strains of LAB (DW5L and LS7L) survived in simulated gastric juice at 0 and 2 h incubation more than the group of *Bacillus* spp. However, the bacilli (AQ1/1B, HW3B, and RN3B) survived in the simulated intestinal juice and grew at a longer incubation time than LAB (at 6 h). In adhesion tested by cell surface hydrophobicity method using xylene droplets, the isolates DW5L and RN3B were the best to adhere at 69.50 ± 12.41% and 18.08 ± 1.74%, respectively (Table 3). This suggests that both bacterial strains have the potential to be used as probiotics.

All of them survived in acidic pH. Normally, the pH of the tilapia's digestive system is between 2.5-8, and the physiological concentration of bile in the tilapia's digestive system is between 0.4 and 1.3% [5]. In acidic conditions, lactobacilli (DW5L and LS7L) grew better than the bacilli group because the former group already produces acid and grows in low-pH environments. This agrees with the experiment of Worananthakij [4], *Lactobacillus* sp. strain M202 could grow well in the test medium with a pH of 1-4. Moreover, Chowdhury *et al.* [24] reported the acid tolerance of *L. plantarum* as it grew well at pH 4-8. On the other hand, *Bacillus* spp. (AQ1/1B, HW3B, and RN3B) had a higher ability to survive and grow in simulated intestinal juice (pH 8.0 + tilapia bile salt). This might be because they can form endospores in unsuitable environments. There are also reports that *Bacillus* spp. can develop biofilms to increase resistance to acid and bile in the digestive tract of fish [25].

3.3 Identification of isolated bacterial strains

Five isolated bacteria were identified by phenotypic characterization using API test kits and molecular techniques using 16s rRNA genes. Based on phenotypic results, a high percentage (99.24-100) of close similarity was received from this comparison to type strains (Table 4). The relationship amongst five strains was illustrated in Figure 1. by a phylogenetic tree which explained that three strains were arranged in *Bacillus* genus which included AQ1/1B (OL365735), HW3B (OL365736), RN3B (OL365737) strains, and identified to *Bacillus pumilus* DSM 27^T (NR_112637.1), *Bacillus subtilis* DSM 10^T (NR_027552.1), *Bacillus cereus* DSM 31^T (NR_115526.1). Meantime, the rest strains named DW5L (OL365738) and LS7L (OL365739) were categorized

into LAB as *Lactiplantibacillus plantarum* DSM 20174^T (NR_115605.1) and *Limosilactobacillus fermentum* DSM 20052^T (NR_104927.1). Both phenotypic and phylogenetic methods gave the same result for each bacterial strain.

Table 3. Probiotic properties of LAB (DW5L, LS7L) and *Bacillus* spp. (AQ1/1B, HW3B, and RN3B)

Isolated bacteria	DW5L	LS7L	AQ1/1B	HW3B	RN3B
Acid tolerance test /Survival (CFU/ml)					
Simulated gastric juice pH 2.0 (0 h incubation)	$4.0x10^{3a}$	$1.5x10^{4b}$	$1.2x10^{3a}$	$3.0x10^{2c}$	$5.0x10^{2c}$
Simulated gastric juice pH 2.0 (2 h incubation)	$1.1x10^{4a}$	$2.9x10^{4a}$	$8.5x10^{2b}$	$1.5x10^{2b}$	$3.5x10^{2b}$
Bile salt tolerance test /Survival (CFU/ml)					
Simulated intestinal juice pH 8.0 (0 h incubation)	$7.0x10^{3a}$	$3.3x10^{4b}$	$3.5x10^{4b}$	$4.2x10^{4b}$	$3.5x10^{4b}$
Simulated intestinal juice pH 8.0 (3 h incubation)	$4.0x10^{3a}$	$1.8x10^{4b}$	$5.6x10^{4b}$	$3.2x10^{4b}$	$3.6x10^{4b}$
Simulated intestinal juice pH 8.0 (6 h incubation)	$4.5x10^{3a}$	$1.9x10^{3a}$	$2.2x10^{6b}$	$1.0x10^{4c}$	$1.2x10^{5d}$
Adhesion test					
Cell surface hydrophobicity (%)	69.50 ± 12.41^{a}	7.11 ± 0.75^{bc}	6.36 ± 0.62^{b}	7.64 ± 1.85^{bc}	18.08 ± 1.74^{c}

Means sharing the same superscript are not significantly different ($p > 0.05$).

Table 4. Identification of 5 probiotic bacterial isolates by apiweb™ identification software with database (V5.1)

Isolate	Bacterial identification	% Similarity
AQ1/1B	*Bacillus pumilus* DSM 27^T	99.72
HW3B	*Bacillus subtilis* DSM 10^T	99.93
RN3B	*Bacillus cereus* DSM 31^T	100.00
DW5L	*Lactiplantibacillus plantarum* DSM 20174^T	99.58
LS7L	*Limosilactobacillus fermentum* DSM 20052^T	99.24

Fig. 1 shows the relationship amongst five strains of all Gram's positive isolated bacteria, which can be divided into three strains for *the Bacillus* genus and two strains for LAB, and an out-group that also listed in Gram's positive bacteria was used to distinguish between *Bacillus* and LAB sections. All strains were more significant than 97% similarity, which is used as the threshold percent for the novel species [26]. Strains RN3B, DW5L, and LS7L were isolated from the intestine of Nile tilapia, while strains AQ1/1B and HW3B were found in natural fish ponds close to their habitats. Therefore, 5 bacterial strains were considered to be applied for promoting fish. However, based on their probiotic properties and identification, results suggested that *B. subtilis* HW3B and *LB. plantarum* DW5L should be potential probiotics for application in fish cultivation. This is because the former strain could digest protein and starch, while the latter showed the highest cell surface

hydrophobicity. It should be noted that *B. cereus* strain RN3B showed the highest cell surface hydrophobicity among tested bacilli strains. However, this strain could digest only protein, and some strains of *B. cereus* are usually foodborne pathogens. *B. pumilus* strain AQ1/1B was not considered due to its lower ability to inhibit both fish pathogens than *B. subtilis* strain HW3B. It has long been known that both *Bacillus subtilis* and *Lactiplantibacillus plantarum* have been applied as probiotics and food additives, indicating their well-established safety profile in humans and fish [2, 8, 17, 27, 28, 29]. However, further experiments should be conducted to confirm the safety and effectiveness of both isolated probiotic bacteria in fish. Therefore, the experiments for this purpose were carried out to use both probiotics for promoting fish growth and maintaining water quality in tilapia cultivation.

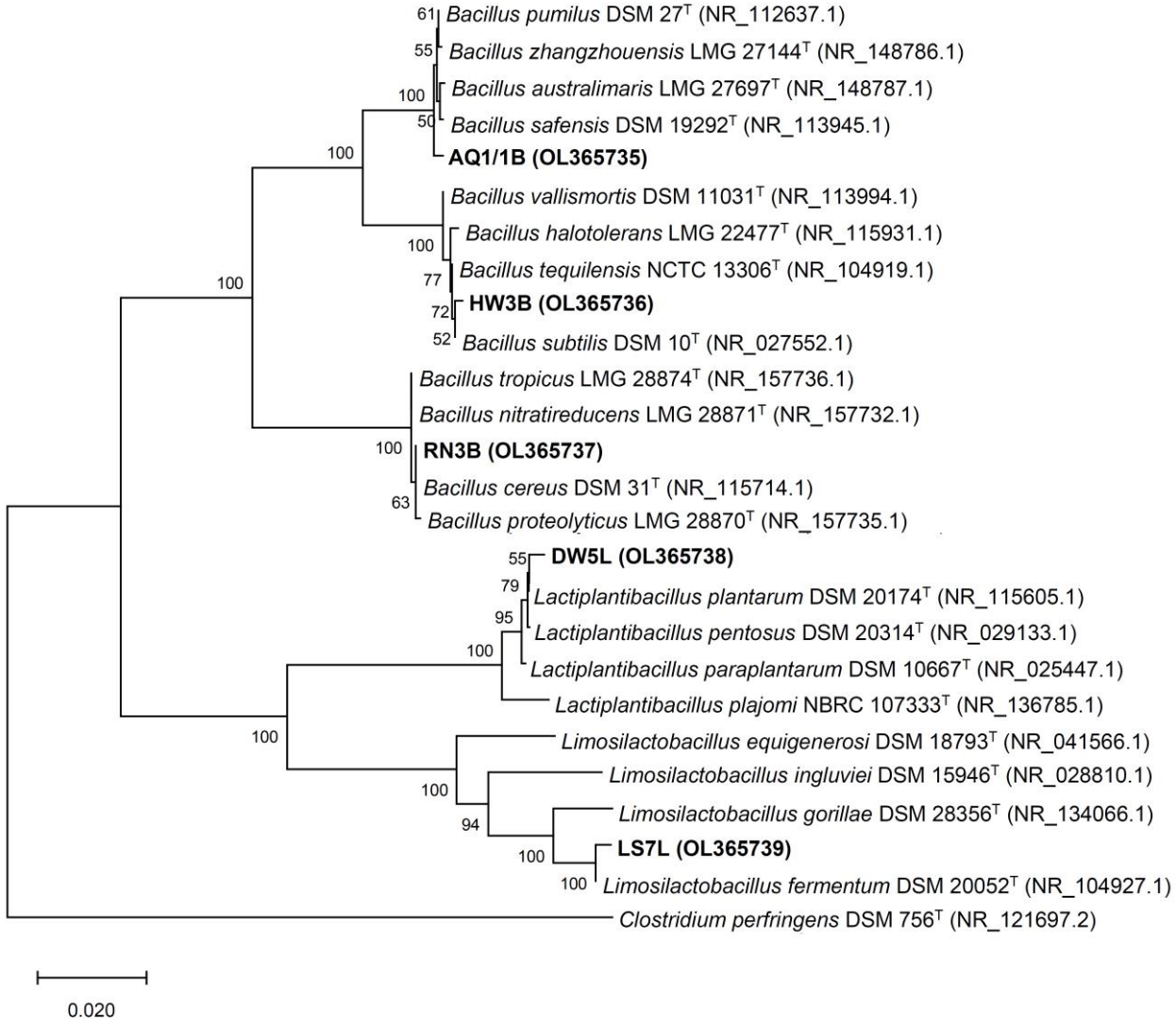

Figure 1. The relationship amongst five bacterial strains, including AQ1/1B (OL365735), HW3B (OL365736), RN3B (OL365737), DW5L (OL365738), and LS7L (OL365739) described by a phylogenetic tree of 16s rRNA gene identification using Jukes-Cantor nucleotide model in the neighbor-joining statistical analysis. The figure's annotation comprised taxonomical name, strain code, and accession number in the parentheses. *Clostridium perfringens* DSM 756T (NR_121697.2) was used as an out-group strain, and evolutionary distance appeared by the bar = 0.020.

4. Conclusions

Focus on isolating and selecting probiotic bacteria (*Bacillus* and LAB) against serious fish pathogens (*Streptococcus agalactiae* and *Aeromonas hydrophila*) from tilapia in Songkhla province of Thailand, 5 bacterial isolates had the desirable probiotic properties. Nevertheless, once 5 isolates were selected and consideration of potential safety for both tilapia and humans, only two isolates, *Bacillus subtilis* HW3B and *Lactiplantibacillus plantarum* DW5L, were promising probiotics for further studies.

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