BIODIVERSITAS
Volume 25, Number 8, August 2024
Pages: 2602-2611

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250832

Antifungal activity of *Bacillus* sp. against *Fusarium* sp. on intensive farmed striped catfish in Vinh Long Province, Vietnam

QUACH VAN CAO THI[1], THANH TRAN[2], TRUONG QUOC TAT[3], TAN PHAT CHAU[4,♥]

[1]Faculty of Applied Biological Sciences, Vinh Long University of Technology Education. Vinh Long City, Vietnam. Tel.: +84-028-7109-9221, [♥]email: phat.ct@vlu.edu.vn
[2]Research and Development Institutes Advanced Agrobilogy, Nguyen Tat Thanh University. Ho Chi Minh City, Vietnam
[3]Faculty of Technology and Engineering, Dong Thap University. Cao Lanh City, Vietnam
[4]Faculty of Applied Technology, School of Technology, Van Lang University. Ho Chi Minh City, Vietnam

Manuscript received: 9 July 2024. Revision accepted: 15 August 2024.

Abstract. *Thi QVC, Tran T, Tat TQ, Chau TP. 2024. Antifungal activity of* Bacillus *sp. against* Fusarium *sp. on intensive farmed striped catfish in Vinh Long Province, Vietnam. Biodiversitas 25: 2602-2611.* Fungal diseases often appear and cause significant losses in aquaculture in Vietnam and many other countries around the world. Therefore, the study's objective was to identify and assess *Bacillus*'s inhibitory action against *Fusarium* sp., which causes swollen swim bladder disease in striped catfish, in order to minimize the usage of antibiotics, which may be harmful to both human health and the environment. As a result, 34 strains of *Bacillus* were obtained from samples of sludge, pond water, and the intestines of intensively farmed *Pangasius* in Vinh Long Province of the Mekong Delta. Inhibitory activity of the fungal spores of isolated *Bacillus* isolates was performed using the diffusion well method. The findings revealed that twenty out of thirty-four bacterial strains exhibited spore inhibitory activity after 3 days of incubation at 30°C. Among them, six out of twenty bacterial strains had the strongest spore inhibitory activity, with inhibition diameters ranging from 15-23 mm. Additionally, six bacterial strains with strong spore inhibitory activity also secreted extracellular enzymes such as amylase, chitinase, cellulase, and protease. Sequencing results showed that strain BC11 had a 99.37% similarity to the reference *Bacillus* on GenBank. The findings of the study serve as the foundation for additional research into the production of probiotics to manage pathogenic fungus in striped catfish, which could provide a different approach to lowering the need for antibiotics in the future.

Keywords: Antifungal activity, *Bacillus* sp., *Fusarium* sp., swollen swim bladder, striped catfish

INTRODUCTION

The striped catfish (*Pangasianodon hypophthalmus* Sauvage 1878) is a freshwater catfish with high economic value that is commonly raised in most Mekong Delta Provinces, such as An Giang, Tra Vinh, Can Tho, and Tien Giang (Phan et al. 2009). *Pangasius* is farmed on an area of about 6 thousand hectares, and the output reaches about 1.5 million tons (Pham Hai and Thanh Tung 2023). In Vinh Long Province, the area and output of intensively cultivated *Pangasius* reach about 361 hectares and 89 thousand tons (Vinh Long Department of Agriculture and Rural Development 2023). Currently, more than 140 countries and territories have imported Vietnamese *Pangasius*, with an annual export output of *Pangasius* reaching a turnover of billions of USD each year, contributing to making Vietnam one of the world's leading countries in exporting *Pangasius* (Korving 2024).

However, *Pangasius* farming in the Mekong Delta is facing many challenges and risks due to water pollution and disease outbreaks (de Silva and Phuong 2011; Anh et al. 2023). Intensification and high farming density have increased the frequency of fish diseases (Dung et al. 2015). In addition to bacterial pathogens (Oanh and Phu 2022), recent research results have identified fungi as pathogens that often appear and cause disease in aquatic animals in the Mekong Delta (Duc et al. 2010; Thy et al. 2016). Duc et al. (2015) identified the fungus *Fusarium incarnatum-equiseti* as the etiological agent of swollen swim bladder disease in *Pangasius*. Sick fish often show signs of pathology, such as lethargy, loss of appetite, and an enlarged abdomen (Duc et al. 2015). Swollen swim bladder disease in *Pangasius* often appears at a low rate of 3% (Hoa et al. 2020). Losses due to swollen swim bladder disease are often not as high as those caused by other pathogens, such as bacteria or parasites; however, many *Pangasius* farming households in the Mekong Delta reported that fungal infections on *Pangasius* have reduced growth and development ability as well as the quality of commercial fish during the farming process.

Currently, the main solutions to prevent and treat pathogenic agents in aquatic animals around the world are using drugs and chemicals, for instance, antibiotics (Rico et al. 2013; Singh and Singh 2018). However, chemicals and drugs that are not strictly controlled and managed have polluted or degraded the quality of the pond environment and negatively affected consumer health (Okocha et al. 2018; Lulijwa et al. 2020; Serwecińska 2020). Thus, the use of probiotics to control microbial pathogens, including bacteria and fungi, is one of the safe alternatives in aquaculture that is attracting the attention of many countries in the world (Sharifuzzaman and Austin 2017; Yousuf et al. 2023).

Along with lactic bacteria, most *Bacillus* species are considered a safe bacteria group, not causing disease in

humans or aquatic animals (Soltani et al. 2019; James et al. 2021). Presently, this bacterial group is commonly used in aquaculture because it produces spores and adapts to many different environmental conditions (Nemutanzhela et al. 2014). Many studies have shown that *Bacillus* has the ability to inhibit many pathogenic bacteria in aquatic animals (Lestari et al. 2016; Li et al. 2020). In *Pangasius*, reports on the antibacterial activity of *Bacillus* against pathogenic bacteria have been reported (Liaqat et al. 2024). Furthermore, *Bacillus* also has other characteristics, such as producing extracellular enzymes (Sonune and Garode 2018). This characteristic of bacteria helps improve the pond environment and strengthen the digestive system of aquatic animals (Kuebutornye et al. 2019; Hlordzi et al. 2020). Meanwhile, the fungal pathogen inhibitory activity of *Bacillus* has also been reported on many fungal species that cause disease in fish and shrimp (Veras et al. 2016; Foysal and Lisa 2018). However, there is still no information about *Bacillus*'s antifungal activity against *Fusarium* on *Pangasius* catfish in the Mekong Delta in general and Vinh Long in particular. Therefore, the study was conducted to isolate and evaluate the inhibitory activity of *Bacillus* against *Fusarium* sp., which causes swollen swim bladders.

MATERIALS AND METHODS

Isolation and identification of *Fusarium* sp.

Swollen swim bladder disease-infected catfish is used to isolate the *Fusarium* fungus. Sick fish show signs of lethargy, especially swollen abdomen bladders inside the internal organs (Figure 3.A). Diseased fish are randomly collected 2-3 fish per pond, with weights ranging from 50 to 100 g. *Fusarium* fungus was isolated from fish bladders, according to Duc et al. (2015). After being collected, *Pangasius* fish are rinsed with sterile distilled water and externally sterilized with 70% ethanol. Then, the fish was dissected, and the swim bladders were cut into thin circles about 4-6 mm. The fish bladder was then washed several times with 0.9% NaCl physiological saline. The fish bladder was placed in a petri dish containing PDA medium (Potato dextrose agar, Himedia, India). The plates were then incubated for about 3-5 days at 28°C. The fungal mycelium was then subcultured many times on the PDA medium until it reached purity. Pure fungal samples were stored in sterile distilled water for further purposes.

After purification, *Fusarium* is preliminary identified based on morphological characteristics, mycelial color, and characteristics such as spore shape and mycelial wall for identification. In addition, the isolated fungal isolates were identified by PCR technique with primer pairs ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (Zarrin et al. 2016). Firstly, fungal DNA was extracted using the Plant/Fungi DNA Isolation Kit (Norgen Biotek, Canada). The manufacturer's instructions were followed when performing the extraction stages. Then, the PCR reaction (25 µL) was amplified with the following ingredients: 12.5 µL PCR Master Mix (Promega, USA), 1.0 µL primer ITS1 (20 pmol), 1.0 µL primer ITS4 (20 pmol), 8.5 µL double distilled water, and 2 µL sample DNA. The PCR reaction cycle and conditions include the following steps: initial denaturation at 95°C for 5 minutes, then 35 cycles including denaturation at 95°C for 50 seconds, primer annealing at 54°C for 45 seconds, extension at 72°C for 70 seconds, and final extension at 72°C for 5 minutes. Following amplification, the PCR products were electrophoresed on a 2% agarose gel, and Analytik Jena gel imaging equipment was used for the photos. Finally, PCR products (600 bp) were sent to a DNA sequencing company (Vietnam) for sequencing.

Collected samples for *Bacillus* isolation

Samples of sludge, water, and healthy *Pangasius* fish used to isolate *Bacillus* were collected directly from intensive fishponds in Long Ho and Mang Thit Districts, Vinh Long Province (Figure 1). Mud or water samples are collected at three locations in the pond. The collected samples from the same pond were then mixed and transferred to the laboratory for bacterial isolation. Meanwhile, the selected *Pangasius* samples for bacterial isolation were healthy, with no signs of disease. Fish are taken from 3-5 fish per pond, with body weight ranging from 50-250 g/fish.

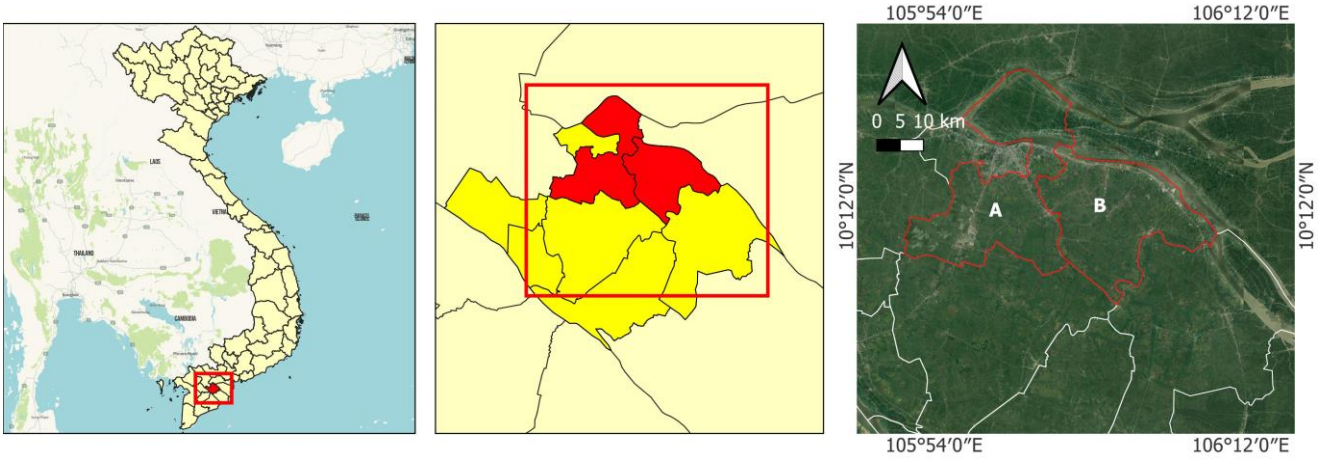

Figure 1. Sampling locations of sludges, pond water, and healthy *Pangasius* fish for *Bacillus* isolation in A. Long Ho District; B. Mang Thit District, Vinh Long Province, Vietnam

Isolation of *Bacillus*

Bacillus was isolated from fish intestines, according to Santos et al. (2021). In brief, fish were rinsed with tap water and externally sterilized with 70% ethanol. Then, the fish is dissected, and the fish intestines are cut into short segments of 1-2 cm. Fish intestines were homogenized and enriched overnight in NB medium (Nutrient broth, Himedia, India) on a shaker at 120 rpm. The enriched culture, after treatment at 80°C for 20 minutes, was diluted with physiological saline (0.85% NaCl) to 10^{-4}. The solution at each dilution concentration was spread onto a petri dish containing NA medium (Nutrient Agar, Himedia, India) and incubated at 30°C for 24-48 hours.

Similarly, sludge or pond water samples were homogenized and enriched overnight in NB medium on a shaker at 120 rpm. The sample was then diluted and processed through the same steps as the above-described intestinal sample. Suspected colonies of *Bacillus*, after incubation for 24-48 hours, were chosen for purification. Pure bacteria were tested for motility, colony morphology (size, color, shape, elevation, and edge), and basic biochemical characteristics (Gram and spore staining, oxidase, and catalase activity) (Aneja 2009).

Antifungal activity of isolated *Bacillus* strains against *Fusarium*

The fungal spore inhibitory ability of *Bacillus* against *Fusarium* was conducted using Kumar et al. (2009)'s diffusion well method. Isolated fungal spores were prepared at a density of 10^5 spores/mL. Then, the fungal spores were spread onto a Petri dish containing PDA medium. Wells were created using a 6 mm diameter cork borer. In parallel, bacterial isolates were cultured overnight in 5 mL of TSB medium (Himedia, India) on a shaker at 120 rpm. Then, the bacterial culture was centrifuged at 10,000 rpm for 15 minutes. The supernatant (100 µL) after centrifugation was added into the wells prepared above. After 24-48 hours of incubation at 28°C, the isolated bacterial strains showed inhibitory activity against *Fusarium*, and an inhibition zone around the wells was recorded. The diameter of the inhibition zone is calculated according to the formula D - d, in which D is the diameter of the antifungal ring (mm) and d is the diameter of the well (6 mm). Antifungal ring diameter of 0: isolated bacteria have no antifungal activity against *Fusarium*; inhibition zone diameter >0: isolated bacteria have inhibitory activity against *Fusarium*. Each bacterial strain in this experiment was repeated three times.

Extracellular enzyme activity

We evaluated the extracellular enzyme production capacity of isolated *Bacillus* strains according to Harley et al. (2001). The liquid LB medium (Luria-Bertani, Himedia, India) was used to enrich the *Bacillus* isolates for 24 hours at 37°C. Following the incubation period, LB agar plates enriched with particular substrates were inoculated with the bacterial growth solution to determine the presence of cellulase, amylase, protease, chitinase, and lipase. For the enzymes cellulase, amylase, protease, chitinase, and lipase, the substrates were 0.5% carboxymethyl cellulose (CMC), 1% starch, 1% gelatin, 5% colloidal chitin, and 1% (v/v) olive oil. After an overnight incubation period at 37°C, the colonies on the plates developed transparent halos, which suggested the presence of enzyme activity.

Identification of *Bacillus* by the 16S rRNA gene

Bacillus bacteria (the strain with the highest fungal inhibitory activity) were selected and identified by PCR and sequencing of the 16S rRNA gene segment with primer pairs 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGACTT-3' (Heuer et al. 1997). Isolated bacterial DNA was extracted using the TopPURE® GENOMIC DNA EXTRACTION KIT (ABT, Vietnam). Based on the manufacturer's instructions, the extraction steps were carried out. Reaction ingredients (25 µL) include: 10.0 µL MyTaq™ DNA Polymerase (Bioline, Germany), 12.0 µL double distilled water, 0.5 µL primer 27F (20 pmol), 0.5 µL primer 1492R (20 pmol), and 2 µL sample DNA. The PCR reaction cycle and conditions include the following steps: initial denaturation at 95°C for 5 minutes, then 35 cycles including denaturation at 95°C for 1 minute, primer annealing at 58°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 5 minutes. Following amplification, 2% agarose gel electrophoresis of the PCR products was conducted, and Analytik Jena gel imaging equipment was used for the photos. PCR products (1.500 bp) were sent for sequencing at DNA Sequencing Company (Vietnam). Also, bacteria were identified using the API 20E kit (Biomerieux, France) with the manufacturer's instructions.

Data analysis

The mean values and standard deviations were computed using descriptive statistical techniques. An ANOVA was used with the MiniTab 20 software to examine the difference in inhibitory activity between bacterial strains at a significance level of 5%. Using the BLASTn tool, the sequencing results of several bacterial strains were evaluated for similarity with reference *Bacillus* sequences in the NCBI database. The CLUSTAL W was used to multialign the DNA sequences of Bacillus (Thompson et al. 1997). The neighbor-joining algorithm (Saitou and Nei 1987) was used to build the phylogenetic tree that shows the genetic relationships between bacterial strains using MEGA X software and a bootstrap value of 1,000 replications (Tamura 2013).

RESULTS AND DISCUSSION

Isolation of *Fusarium* isolates

After 5-7 days of culture on PDA medium, *Fusarium* colonies have pale pink or white cottony mycelium (Figures 2.B and 2.C). Microscopic observation results showed that the fungal hyphae have septa and branches (Figure 2D). Macroconidia are crescent-shaped, slightly curved, small at both ends and have 4-6 septa (Figure 2.E).

Identification of *Fusarium* isolates

The findings demonstrated that all selected fungal strains amplified the internal transcribed spacer (ITS) gene

segment, with a 600-bp DNA band appearing (Figure 3). Sequencing results showed that strain FSVL1 is 100% similar to *Fusarium equiseti* strain FTer13 (MT764832.1), 99.80% similar to *Fusarium equiseti* isolate JG22 (KJ412501.1), *Fusarium* sp. strain LB5 (MN944920.1), *Fusarium ipomoeae* isolate LXYB20 (MT928727.1), 99.61% similar to *Fusarium incarnatum* strain JL3-4-1 (MT563419.1), *Fusarium chlamydosporum* isolate TWD11 (MT448890.1), and *Fusarium oxysporum* strain FSOT (KY100124.1) in the NCBI database. The phylogenetic tree (Figure 4) shows that strain FSVL1 belongs to the same group as *Fusarium equiseti* isolate JG22 (KJ412501.1) on GenBank.

Isolation of *Bacillus* bacteria

A total of 34 bacterial strains were obtained from water, mud, and *Pangasius* fish samples (Table 1). Among the isolated bacterial strains, the highest proportion was 15/34 strains (44.12%) isolated from pond water, followed by mud samples (12/34 strains, 35.29%), and the lowest were bacterial strains isolated from fish intestines (7/34 strains, 20.59%).

Morphological, physiological, and biochemical characteristics of isolated bacterial strains

In general, after 24 hours of incubation on NA medium, most isolated bacterial strains have round colonies, an opaque white color, smooth or slightly wrinkled surfaces, and sizes ranging from 2-3 mm (Figure 5.C). Gram staining results showed that the bacterial isolates were Gram-positive, long-rod (Figure 5.D). All bacterial isolates were positive for catalase (Figure 5.E) and oxidase activity and had endospores (Figure 5.F). Most bacterial isolates were capable of growing in a wide temperature range (20°C to 60°C) and pH range (4.0 to 10.0), but they could grow at salt concentrations up to 8% and temperatures up to 65°C.

The colony, morphological, physiological, and biochemical characteristics of the isolated bacteria are presented in detail in Table 3.

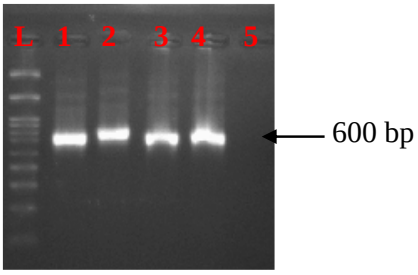

Figure 3. Amplification results of the internal transcribed spacer (ITS) gene segment of fungal strains. M: 100 bp plus standard ladder; Lanes 1-4: Fungal strains FSVL1, FSVL2, FSVL3, and FSVL4; Lane 5: Negative control

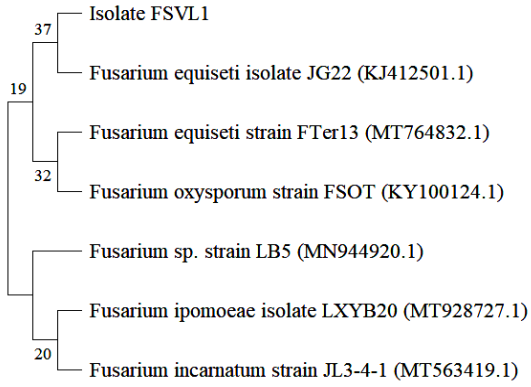

Figure 4. The phylogenetic tree shows the relationship between *Fusarium* isolates (the numbers in the branches on the phylogenetic tree are bootstrap values of 1,000 replicates)

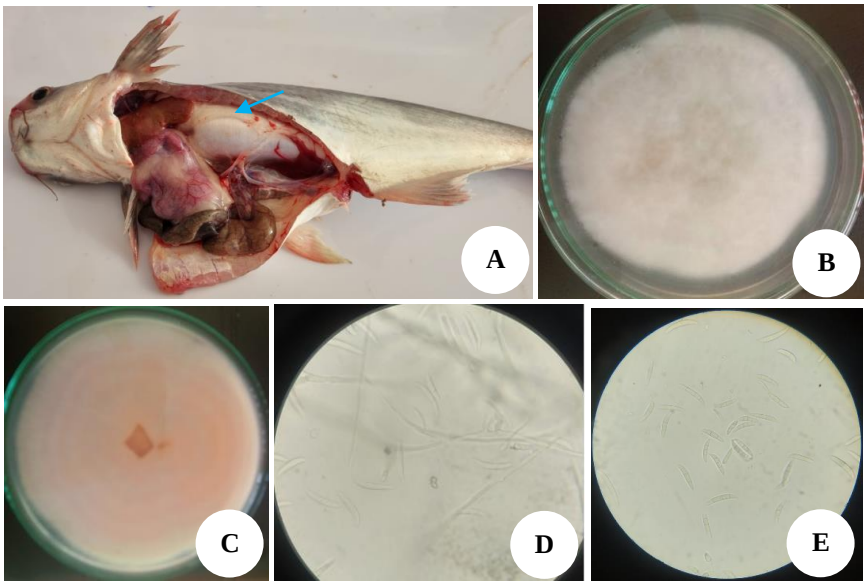

Figure 2. *Fusarium* isolate obtained from swollen swim bladder disease-infected striped catfish. A. Swollen swim bladder (blue arrow) of *Fusarium* infected striped catfish; B. Surface of *Fusarium* fungal colony on PDA after 5 days at 28°C; C. Reverse of *Fusarium* fungal colony on PDA after 7 days at 28°C; D. Septum in *Fusarium* mycelium; D. Macroconidia are crescent-shaped, slightly curved, and small at both ends

Inhibitory activity of isolated *Bacillus* strains against *Fusarium*

The results showed that 20 of the 34 bacterial strains isolated in the study showed inhibitory activity against *Fusarium* (Figure 6). Among them, strain BC11 has the strongest antifungal activity (diameter 23±0.58 mm), followed by strains BC5 and BC8 with antifungal diameters of 22.23±0.29 mm and 21.67±0.58 mm, respectively. Strains BĐ7 and BN8 have the weakest fungal inhibitory activity, with the smallest antifungal diameters of 2.17±0.29 and 2.33±0.58 mm, respectively.

Extracellular enzyme activity

The findings demonstrated that the four bacterial strains BC5, BC7, BC8, and BC11 exhibited extracellular protease, cellulase, chitinase, and amylase enzyme activities (Figure 7). However, the four bacterial strains did not exhibit lipase enzyme activity (Table 2).

Identification of isolated *Bacillus* isolates using the API 20E kit

Four bacterial strains, such as strains BC5, BC7, BC8, and BC11, were tested by the API 20E kit. All bacterial strains showed negative reactions to the following criteria: orthonitrophenyl galactosidase, arginine, lysine, ornithine, citrate, H_2S, urease, indole, Voges-Proskauer (sodium pyruvate), gelatin, glucose, inositol, sorbitol, rhamnose, saccharose (sucrose), melibiose, amygdalin, and arabinose. Meanwhile, bacterial strains only showed positive reactions to tryptophane and mannitol. The findings of identifying *Bacillus* using the API 20E kit, along with characteristics of colony morphology and bacterial biochemistry, are presented in detail in Table 3.

Table 1. Origin of isolated *Bacillus* strains

Source of bacterial isolation	Farm	Number of samples	Number of isolates
Striped catfish	5	17	7
Sludge	4	9	12
Pond water	5	10	15
Total	14	36	34

Table 2. Activities of isolated *Bacillus* isolates' extracellular enzymes

Extracellular enzymes	Isolate BC5	Isolate BC7	Isolate BC8	Isolate BC11
Amylase	+	+	+	+
Cellulase	+	+	+	+
Protease	+	+	+	+
Lipase	-	-	-	-
Chitinase	+	+	+	+

Notes: +: Positive; -: Negative

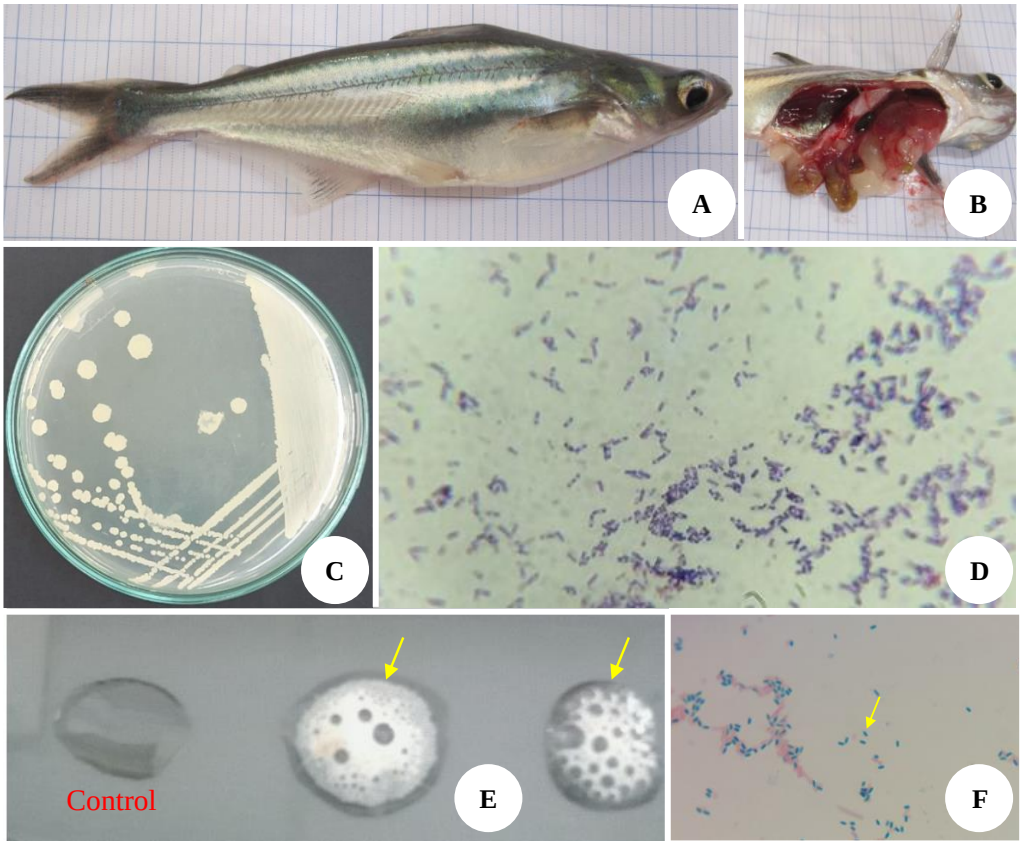

Figure 5. Properties of an isolated *Bacillus* isolated in terms of morphology and biochemistry. A. Healthy striped catfish samples with normal body color; B. Normal internal organs of healthy *Pangasius;* C. *Bacillus* bacterial colonies on NA medium; D. Gram stain (100×); E. Positive catalase activity (bubble gas, yellow arrow); F. Spore staining (blue spores, yellow arrow)

Table 3. Colony morphological, physiological, and biochemical characteristics of isolated *Bacillus* isolates

Characteristics	Isolate BC5	Isolate BC7	Isolate BC8	Isolate BC11
Gram staining	Gram-positive	Gram-positive	Gram-positive	Gram-positive
Cell shape	Long-rod	Short-rod	Short-rod	Long-rod
Motility	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
Grown on NaCl medium:				
0.5%	+	+	+	+
1%	+	+	+	+
2%	+	+	+	+
4%	+	+	+	+
6%	+	+	+	+
8%	-	-	-	-
Grown on pH medium:				
2	-	-	-	-
4	+	+	+	+
6	+	+	+	+
8	+	+	+	+
10	+	+	+	+
12	-	-	-	-
Grown on temperature:				
10°C	-	-	-	-
20°C	+	+	+	+
30°C	+	+	+	+
40°C	+	+	+	+
50°C	+	+	+	+
60°C	+	+	+	+
65°C	-	-	-	-
ONPG	-	-	-	-
Arginine	-	-	-	-
Lysine	-	-	-	-
Ornithine	-	-	-	-
Citrate	-	-	-	-
H_2S production	-	-	-	-
Urease	-	-	-	-
Tryptophane	+	+	+	+
Indole	-	-	-	-
Voges-Proskauer	-	-	-	-
Gelatin	-	-	-	-
Glucose	-	-	-	-
Mannitol	+	+	+	+
Inositol	-	-	-	-
Sorbitol	-	-	-	-
Rhamnose	-	-	-	-
Saccharose	-	-	-	-
Melibiose	-	-	-	-
Amygdalin	-	-	-	-
Arabinose	-	-	-	-

Note: +: Positive; -: Negative

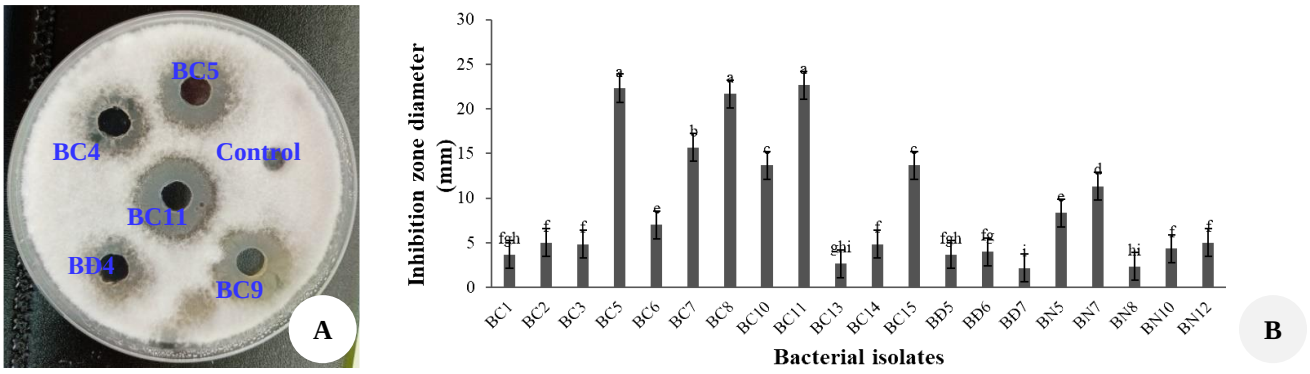

Figure 6. The antifungal ability of isolated *Bacillus* strains against *Fusarium.* A. Antifungal activity of isolated *Bacillus* strains by using the well-diffusion agar method; B. Differences in the inhibitory activity of *Bacillus* strains against *Fusarium* (different letters on each bar indicate significant differences between treatments ($p<0.05$)

Identification of isolated *Bacillus* isolates by 16S rRNA gene fragment

The results showed that the 16S rRNA gene fragment was amplified in all of the chosen bacterial strains, with a 1,500-bp-sized DNA band emerging (Figure 8).

According to sequencing results, strain BC11 is 99.37% similar to *Bacillus paramycoides* strain ZS3 (OR394251.1), *Bacillus thuringiensis* strain AB37-SW1 (MG890230.1), *Bacillus* sp. strain XAAS.x250 (MN187270.1), *Bacillus tropicus* strain CSN-15 (ON422091.1), and *Bacillus albus* strain CZW028 (MW435385.1) on the NCBI database, and 98.48% similar to *Bacillus cereus* strain ATCC 14579T.52 (MN543842.1), *Bacillus thuringiensis* strain AB37-SW1 (MG890230.1). Strain BC11 is classified in the same group as *Bacillus thuringiensis* strain AB37-SW1 (MG890230.1) in GenBank, according to the phylogenetic tree (Figure 9).

Discussion

In this study, *Bacillus* isolates were isolated from fish intestines, water, and pond bottom mud. This result is consistent with previous studies showing that *Bacillus* bacteria are commonly present in different types of environments (Mandic-Mulec et al. 2015). In particular, *Bacillus* bacteria are isolated from the digestive systems of aquatic animals (Chen et al. 2016). In Vietnam, research by Trung et al. (2022) revealed that 194 different bacterial isolates were found in the digestive tracts of fish, clams, and shrimp. Another study by Kavitha et al. (2018) demonstrated the probiotic activity of *Bacillus* spp. derived from the Indian freshwater fish (*Labeo calbasu* Hamilton 1822) digestive system. Similarly, the potential probiotic *Bacillus* spp. that originated from Nile tilapia's intestine (*Oreochromis niloticus* Linnaeus 1758) was found in the investigation of Nakharuthai et al. (2023). According to Jlidi et al. (2023), *Bacillus* strains isolated from the intestine of shrimp have shown high antibacterial activity against *Vibrio anguillarum* and *Vibrio harveyi*, two bacteria that cause vibriosis. The existence of *Bacillus* in different conditions can be explained by the fact that they have endospores (James et al. 2021). Endospores from this species can withstand extreme temperatures, radiation, freezing, acidity and UV rays, droughts, and rising oxygen levels. In the present study, the findings indicated that all isolated bacterial strains had endospores. The presence of endospores is one of the advantages of *Bacillus* compared to other beneficial bacteria for the production of probiotics in the future (Ramlucken et al. 2020; Dumitru and Ciurescu 2022). In this study, isolated *Bacillus* strains can survive at pH 4-10. This investigation is in agreement with the research by Nakharuthai et al. (2023), showing that isolated *Bacillus* can tolerate pH 2-9. Similarly, the study by Sánchez-Ortiz et al. (2015) investigated the growth ability of the bacterial strain collected from pustulose ark (*Anadara tuberculosa* G.B.Sowerby I 1833) in an environment with a pH varying from 4-10. Additionally, the study also showed that isolated bacteria can survive in environments with NaCl salt concentrations of up to 10%.

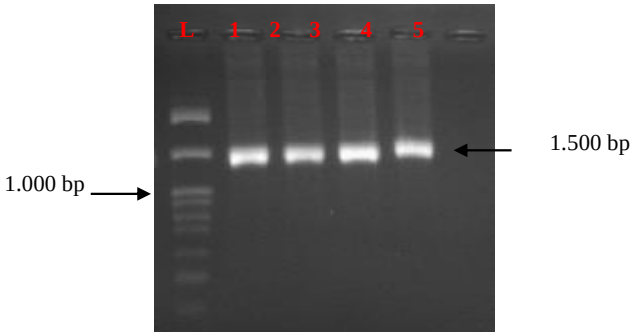

Figure 8. Amplification results of the 16S rRNA gene segment of *Bacillus*. M: 100 bp standard ladder; Lanes 1-4: Bacterial strains BC5, BC7, BC8, and BC11; Lane 4: Negative control

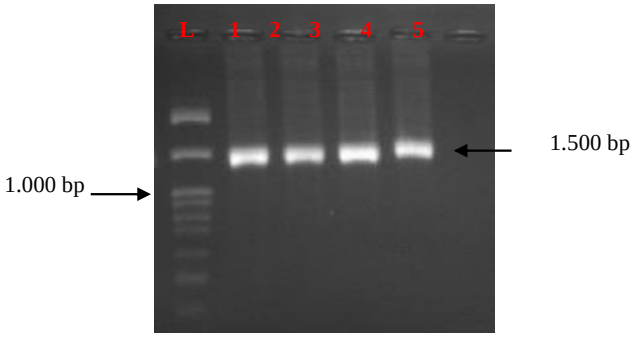

Figure 8. Amplification results of the 16S rRNA gene segment of *Bacillus*. M: 100 bp standard ladder; Lanes 1-4: Bacterial strains BC5, BC7, BC8, and BC11; Lane 4: Negative control

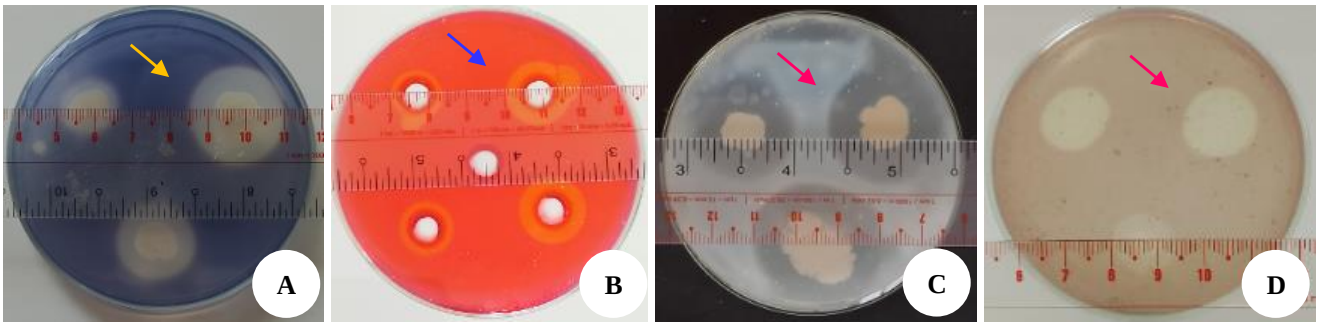

Figure 7. Extracellular enzyme activity of isolated *Bacillus* strains. A. Amylase activity (arrow); B. Cellulase activity (arrow); C. Protease activity (arrow); D. Chitinase activity (arrow)

According to Franco et al. (2016), *Bacillus licheniformis* strain CIGBC-232, isolated from white-leg shrimp intestine (*Litopenaeus vannamei* Boone 1931), is capable of growing in environments with salt concentrations of 2-7% and does not grow in environments with high salt concentrations of 7.5%. Besides, the bacterial strains in the study can grow well at temperatures ranging from 20-60°C. This finding is similar to Liu et al. (2009) finding that *Bacillus subtilis* E20 was able to survive at a wide range of temperatures on NA at a range of 10-50°C. Currently, some intensive *Pangasius* farming areas in Vietnam are suffering from saltwater intrusion, affecting *Pangasius* farming in the Mekong Delta. Therefore, selecting *Bacillus* strains that are able to adapt to environmental conditions, such as salt resistance and survival at different pH levels, can produce probiotics for *Pangasius* farming.

In this study, isolated bacterial strains were able to inhibit *Fusarium* fungal spores. This result is similar to previous studies showing that *Bacillus* isolates also have fungal spore inhibitory activities. Crude lipopeptides isolated from *B. amyloliquefaciens* YN201732's fermentation broth demonstrated antagonistic effects against the indicator pathogen *F. solani* in a study by Jiao et al. (2021). Al-Mutar et al.'s study from 2023 also found that the cell-free supernatant from *B. subtilis* DHA41 culture exhibited antifungal efficacy against *Fusarium oxysporum*. However, the results of this observation showed that *Bacillus* isolates exhibited different spore inhibitory activities. This may be due to different antifungal compounds produced by different species and strains of *Bacillus*, such as extracellular lipopeptides (iturins, surfactins, and fengycins) (Zhao et al. 2016; Al-Mutar et al. 2023). In the current finding, however, the antifungal compounds of isolated bacterial isolates were not studied. Hence, the antifungal compounds of the bacteria in the study need to be determined to understand the antifungal mechanism of these bacteria better.

Potential *Bacillus* bacteria often have probiotic characteristics such as pathogen inhibition, bile salt tolerance, low pH survival, and adhesion ability (Kavitha et al. 2018; Zhang et al. 2022). Additionally, the ability to produce extracellular enzymes is also an important criterion when selecting bacterial strains as probiotics. In this study, all four strains (BC5, BC7, BC8, and BC11) with the strongest antagonistic activity against *Fusarium* had extracellular enzyme activities like amylase, cellulase, and protease. These findings are in line with those of Kieu et al. (2023), who demonstrated that two *Bacillus* B18 and B23 isolated from striped fish, pond water, and sediment samples in Dong Thap Province of the Mekong Delta, Vietnam, exhibited amylase, cellulase, and protease extracellular enzyme activities. Extracellular enzymes play an important role in enhancing food digestion, helping food to be easily absorbed and animals to gain weight well (Dat et al. 2019; Truong et al. 2021). In addition, extracellular enzymes play a role in treating and maintaining the quality of the pond environment (Omar et al. 2024). Research by Monier et al. (2023) showed that supplementing commercial *Bacillus* species probiotics improved the water quality by increasing dissolved oxygen and decreasing total ammonia, nitrite, and unionized ammonia in the rearing ponds, as well as the resistance of white-leg shrimp to *F. solani* infection. Therefore, to reach this objective in the future, further research must be done.

In conclusion, *Bacillus* strains were isolated from samples of sludge, pond water, and intestines of intensively farmed *Pangasius* in Vinh Long Province of the Mekong Delta, Vietnam. The findings revealed that twenty out of thirty-four bacterial strains exhibited spore inhibitory activity after 3 days of incubation at 30°C by using the diffusion well method. With inhibition diameters ranging from 15 to 23 mm, six of the twenty bacterial strains they exhibited the strongest spore inhibitory efficacy. Extracellular enzymes, including cellulase, protease, and amylase, were also released by six bacterial strains that exhibited high spore inhibitory action. According to sequencing data, strain BC11 was 99.37% comparable to the reference *Bacillus* found in GenBank. The study's conclusions form the basis for more investigation into the cultivation of probiotics to control pathogenic fungus in striped catfish, which may offer an alternative strategy for reducing the need for antibiotics.

REFERENCES

Al-Mutar DMK, Noman M, Alzawar NSA, Qasim HH, Li D, Song F. 2023. The extracellular lipopeptides and volatile organic compounds of *Bacillus subtilis* DHA41 display broad-spectrum antifungal activity against soil-borne phytopathogenic fungi. J Fungi 9: 797. DOI: 10.3390/jof9080797.

Aneja KR. 2009. Experiments in Microbiology, Plant Pathology and Biotechnology. New Age International, Delhi.

Anh PT, Kroeze C, Bush SR, Mol AP. 2010. Water pollution by *Pangasius* production in the Mekong Delta, Vietnam: Causes and options for control. Aquac Res 42 (1): 108-128. DOI: 10.1111/j.1365-2109.2010.02578.x.

Chen Y, Li J, Xiao P, Zhu W, Mo Z. 2016. The ability of marine *Bacillus* spp. isolated from fish gastrointestinal tract and culture pond sediment to inhibit growth of aquatic pathogenic bacteria. Iran J Fish Sci 15 (2): 701-714.

Dat TTH, Tam VTT, Dung TTK, Bui LM, Anh HLT, Oanh PTT. 2019. Isolation and screening of cellulose and organic matter degrading bacteria from aquaculture ponds for improving water quality in aquaculture. IOP Conf Ser: Earth Environ Sci 266: 012002. DOI: 10.1088/1755-1315/266/1/012002.

de Silva SS, Phuong NT. 2011. Striped catfish farming in the Mekong Delta, Vietnam: A tumultuous path to a global success. Rev Aquac 3 (2): 45-73. DOI: 10.1111/j.1753-5131.2011.01046.x.

Duc PM, Nguyen TP, Tran NTT. 2010. Overview fungal diseases in aquatic animals. CTU J Sci 16b: 88-97.

Duc PM, Thy DTM, Hatai K, Muraosa Y. 2015. Infection of striped catfish (*Pangasianodon hypophthalmus*) in Viet Nam caused by the fungus *Fusarium incarnatum-equiseti*. Bull Eur Ass Fish Pathol 35 (6): 208-216.

Dumitru M, Ciurescu G. 2022. The beneficial effect of *Bacillus* spp. as probiotics in poultry nutrition-a review. Sci Papers Ser D Anim Sci 65 (2): 2285-5750.

Dung TT, Oanh DTH, Duc PM. 2015. Diseases and disease management in farmed striped catfish (*Pangasianodon hypophthalmus*). In: Striped Catfish (*Pangasianodon hypophthalmus*) Culture in The Mekong Delta: Successes and Challenges in Sustainable Development. Nguyen TP, Nguyen AT (eds). Can Tho University Publishing House, Can Tho.

Foysal MJ, Lisa AK. 2018. Isolation and characterization of *Bacillus* sp. strain BC01 from soil displaying potent antagonistic activity against plant and fish pathogenic fungi and bacteria. J Genet Eng Biotechnol 16 (2): 387-392. DOI: 10.1016/j.jgeb.2018.01.005.

Franco R, Martín L, Arenal A, Santiesteban D, Sotolongo J, Cabrera H, Mejías J, Rodríguez G, Moreno AG, Vázquez EP, de Estrada NMCD.

2017. Evaluation of two probiotics used during farm production of white shrimp *Litopenaeus vannamei* (Crustacea: Decapoda). Aquac Res 48 (4): 1936-1950. DOI: 10.1111/are.13031.

Heuer H, Krsek M, Baker P, Smalla K, Wellington EM. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63: 3233-3241. DOI: 10.1128/aem.63.8.3233-3241.1997.

Hlordzi V, Kuebutornye FK, Afriyie G, Abarike ED, Lu Y, Chi S, Anokyewaa MA. 2020. The use of *Bacillus* species in maintenance of water quality in aquaculture: A review. Aquacult Rep 18: 100503. DOI: 10.1016/j.aqrep.2020.100503.

Hoa TTT, Boerlage AS, Duyen TTM, Thy DTM, Hang NTT, Humphry RW, Phuong NT. 2020. Nursing stages of striped catfish (*Pangasianodon hypophthalmus*) in Vietnam: Pathogens, diseases and husbandry practices. Aquac 533: 736114. DOI: 10.1016/j.aquaculture.2020.736114.

Kieu NTT, Hiep NC, Tran T. 2023. Isolation and characterization of indigenous microorganisms for antagonizing *Fusarium incarnatum* and preventing swollen swim bladder disease in *Pangasius hypophthalmus*. Materials Today: Proceedings. DOI: 10.1016/j.matpr.2023.04.584. [officially unpublished]

James G, Das BC, Jose S, VJRK. 2021. *Bacillus* as an aquaculture friendly microbe. Aquac Intl 29: 323-353. DOI: 10.1007/s10499-020-00630-0.

Jiao R, Cai Y, He P, Munir S, Li X, Wu Y, Wang J, Xia M, He P, Wang G, Yang H, Karunarathna SC, Xie Y, He Y. 2021. *Bacillus amyloliquefaciens* YN201732 produces lipopeptides with promising biocontrol activity against fungal pathogen *Erysiphe cichoracearum*. Front Cell Infect Microbiol 11: 598999. DOI: 10.3389/fcimb.2021.598999.

Jlidi M, Akremi I, Ibrahim AH, Brabra W, Ali MB, Ali MB. 2022. Probiotic properties of *Bacillus* strains isolated from the gastrointestinal tract against pathogenic vibriosis. Front Mar Sci 9: 884244. DOI: 10.3389/fmars.2022.884244.

Kavitha M, Raja M, Perumal P. 2018. Evaluation of probiotic potential of *Bacillus* spp. isolated from the digestive tract of freshwater fish *Labeo calbasu* (Hamilton, 1822). Aquac Rep 11: 59-69. DOI: 10.1016/j.aqrep.2018.07.001.

Korving I. 2024. Report: Aquaculture Farmer's Needs in Mekong River Delta, Viet Nam. Agricultural Department, Netherlands Embassy in Hanoi and The Netherlands Enterprise Agency, Ha Noi.

Kuebutornye FK, Abarike ED, Lu Y. 2019. A review on the application of *Bacillus* as probiotics in aquaculture. Fish Shellfish Immunol 87: 820-828. DOI: 10.1016/j.fsi.2019.02.010.

Kumar A, Saini P, Shrivastava J. 2009. Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*. Indian J Exp Biol 47 (01): 57-62.

Lestari NW, Budiharjo A, Pangastuti A. 2016. Aerobic heterotrophic bacteria from the digestive tract of eel (*Anguilla bicolor bicolor*) and its potential as probiotics. Bioteknologi 13: 9-17. DOI: 10.13057/biotek/c130102. [Indonesian]

Li X, Gao X, Zhang S, Jiang Z, Yang H, Liu X, Jiang Q, Zhang X. 2020. Characterization of a *Bacillus velezensis* with antibacterial activity and inhibitory effect on common aquatic pathogens. Aquaculture 523: 735165. DOI: 10.1016/j.aquaculture.2020.735165.

Liaqat R, Fatima S, Komal W, Minahal Q, Kanwal Z, Suleman M, Carter CG. 2024. Effects of *Bacillus subtilis* as a single strain probiotic on growth, disease resistance and immune response of striped catfish (*Pangasius hypophthalmus*). PloS One 19 (1): e0294949. DOI: 10.1371/journal.pone.0294949.

Liu C-H, Chiu C-S, Ho P-L, Wang S-W. 2009. Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease-producing probiotic, *Bacillus subtilis* E20, from natto. J Appl Microbiol 107: 1031-1041. DOI: 10.1111/j.1365-2672.2009.04284.x.

Lulijwa R, Rupia EJ, Alfaro AC. 2020. Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. Rev Aquacult 12 (2): 640-663. DOI: 10.1111/raq.12344.

Mandic-Mulec I, Stefanic P, van Elsas JD. 2015. Ecology of *Bacillaceae*. Microbiol Spectr 3 (2): 59-85. DOI: 10.1128/microbiolspec.TBS-0017-2013.

Monier MN, Kabary H, Elfeky A, Saadony S, El-Hamed NNA, Eissa ME, Eissa ESH. 2023. The effects of *Bacillus* species probiotics (*Bacillus subtilis* and *B. licheniformis*) on the water quality, immune responses, and resistance of whiteleg shrimp (*Litopenaeus vannamei*) against *Fusarium solani* infection. Aquac Intl 31 (6): 3437-3455. DOI: 10.1007/s10499-024-01503-6.

Nakharuthai C, Boonanuntanasarn S, Kaewda J, Manassila P. 2023. Isolation of potential probiotic *Bacillus* spp. from the intestine of nile tilapia to construct recombinant probiotic expressing cc chemokine and its effectiveness on innate immune responses in Nile Tilapia. Animals 13: 986. DOI: 10.3390/ani13060986

Nemutanzhela ME, Roets Y, Gardiner N, Lalloo R. 2014. The use and benefits of *Bacillus* based biological agents in aquaculture. Sustain Aquac Technique 19: 1-34. DOI: 10.5772/57375.

Oanh DTH, Phu TQ. 2022. Occurrence of diseases and selected water quality parameters in cultured striped catfish (*Pangasianodon hypophthalmus*) ponds in the Mekong Delta of Vietnam. AACL Bioflux 15 (3): 1333-1343.

Okocha RC, Olatoye IO, Adedeji OB. 2018. Food safety impacts of antimicrobial use and their residues in aquaculture. Public Health Rev 39: 1-22. DOI: 10.1186/s40985-018-0099-2.

Omar AA, Marzouk MS, Mahfouz NB, Massoud AM, Shukry M, Farrag F, Zayed MM, Alaziz MAA, Moustafa EM. 2024. Effects of the putative probiotics *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus subtilis* on white leg shrimp, *Litopenaeus vannamei*, immune response, gut histology, water quality, and growth performance. Open Vet J 14 (1): 144-153. DOI: 10.5455/OVJ.2024.v14.i1.13.

Pham H, Thanh T. 2023. The *Pangasius* industry in the Mekong Delta aims for a circular economy and reduced emissions. Tridge. https://www.bsc.com.vn/tin-tuc/tin-chi-tiet/1080551-nganh-hang-ca-tra-vung-dbscl-huong-den-kinh-te-tuan-hoan-giam-phat-thai

Phan LT, Bui TM, Nguyen TT, Gooley GJ, Ingram BA, Nguyen HV, Nguyen PT, de Silva SS. 2009. Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus* in the Mekong Delta, Vietnam. Aquac 296 (3-4): 227-236. DOI: 10.1016/j.aquaculture. 2009.08.017.

Ramlucken U, Lalloo R, Roets Y, Moonsamy G, van Rensburg CJ, Thantsha MS. 2020. Advantages of *Bacillus*-based probiotics in poultry production. Livest Sci 241: 104215. DOI: 10.1016/j.livsci.2020.104215.

Rico A, Phu TM, Satapornvanit K, Min J, Shahabuddin AM, Henriksson PJG, Murray FJ, Little DC, Dalsgaard A, Van den Brink PJ. 2013. Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. Aquaculture 412: 231-243. DOI: 10.1016/j.aquaculture.2013.07.028

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4 (4): 406-425. DOI: 10.1093/oxfordjournals.molbev.a040454.

Sánchez-Ortiz AC, Luna-González A, CampaCórdova AI, Escamilla-Montes R, FloresMiranda MC, Mazón-Suástegui JM. 2015. Isolation and characterization of potential probiotic bacteria from pustulose ark (*Anadara tuberculosa*) suitable for shrimp farming. Lat Am J Aquat Res 43 (1): 123-136. DOI: 10.3856/vol43-issue1-fulltext-11.

Santos RA, Oliva-Teles A, Pousão-Ferreira P, Jerusik R, Saavedra MJ, Enes P, Serra CR. 2021. Isolation and characterization of fish-gut *Bacillus* spp. as source of natural antimicrobial compounds to fight aquaculture bacterial diseases. Mar Biotechnol 23: 276-293. DOI: 10.1007/s10126-021-10022-x.

Serwecińska L. 2020. Antimicrobials and antibiotic-resistant bacteria: A risk to the environment and to public health. Water 12 (12): 3313. DOI: 10.3390/w12123313.

Sharifuzzaman SM, Austin B. 2017. Probiotics for disease control in aquaculture. Diagnosis and control of diseases of fish and shellfish. In: Austin B, Newaj-Fyzul (eds). Diagnosis and Control of Diseases of Fish and Shellfish. Wiley, New Jersey. DOI: 10.1002/9781119152125.ch8

Singh M, Singh P. 2018. Drugs and chemicals applied in aquaculture industry: A review of commercial availability, recommended dosage and mode of application. J Entomol Zool Stud 6 (6): 903-907.

Soltani M, Ghosh K, Hoseinifar SH, Kumar V, Lymbery AJ, Roy S, Ringø E. 2019. Genus *Bacillus*, promising probiotics in aquaculture: Aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. Rev Fish Sci Aquac 27 (3): 331-379. DOI: 10.1080/23308249.2019.1597010.

Sonune N, Garode A. 2018. Isolation, characterization and identification of extracellular enzyme producer *Bacillus licheniformis* from municipal wastewater and evaluation of their biodegradability. Biotechnol Res Innov 2 (1): 37-44. DOI: 10.1016/j.biori.2018.03.001.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30 (12): 2725-2729. DOI: 10.1093/molbev/mst197.

Thompson J, Weaver MA, Lupatsch I, Shields RJ, Plummer S, Coates CJ, Rowley AF. 2022. Antagonistic activity of lactic acid bacteria against pathogenic vibrios and their potential use as probiotics in shrimp (*Penaeus vannamei*) culture. Front Mar Sci 9: 240. DOI: 10.3389/fmars.2022.807989.

Thy DTM, Duc PM và Hoa TTT. 2016. Identification of filamentous fungi effecting striped catfish fingerling (*Pangasianodon hypophthalmus*). CTU J Sci 44b: 48-57. DOI: 10.22144/ctu.jvn.2016.486.

Trung DQ, Vu VH, Luu TA. 2022. Isolation and characterization of native probiotics for aquaculture farming in Bach Long, Nam Dinh. CTU J Sci 58 (2): 94-101. DOI: 10.22144/ctu.jvn.2022.125.

Truong QP, Phan TCT, Vu HH, Pham TTN, Huynh TG, Vu NU. 2021. Isolation of potential probiotic *Bacillus subtilis* CM3.1 and its effects on water quality and growth performance of the whiteleg shrimp *Litopenaeus vannamei* in the Mekong Delta, Vietnam. AACL Bioflux 14 (6): 3347-3357.

Veras FF, Correa APF, Welke JE, Brandelli A. 2016. Inhibition of mycotoxin-producing fungi by *Bacillus* strains isolated from fish intestines. Intl J Food Microbiol 238: 23-32. DOI: 10.1016/j.ijfoodmicro.2016.08.035.

Vinh Long Department of Agriculture and Rural Development. 2023. One year of efforts to develop stable and sustainable *Pangasius* farming. https://nongnghiep.vinhlong.gov.vn/xem-chi-tiet-tin-tuc/id/219733.

Yousuf S, Tyagi A, Singh R. 2023. Probiotic supplementation as an emerging alternative to chemical therapeutics in finfish aquaculture: A review. Probiotics Antimicro 15 (5): 1151-1168. DOI: 10.1007/s12602-022-09971-z.

Zarrin M, Ganj F, Faramarzi S. 2016. Analysis of the rDNA internal transcribed spacer region of the *Fusarium* species by polymerase chain reaction-restriction fragment length polymorphism. Biomed Rep 4 (4): 471-474. DOI: 10.3892/br.2016.615.

Zhang Y, Wu Z, Yu M, Zhang D, Wang Q, Lin L, Wang G, Elsadek MM, Yao Q, Guo Z. 2022. Evaluating the probiotic potential and adhesion characteristics of *Bacillus* spp. isolated from the intestine of Rhynchocypris lagowskii Dybowski. Aquac Intl 30 (2): 747-772. DOI: 10.1007/s10499-022-00840-8.

Zhao Q, Mei X, Xu Y. 2016. Isolation and identification of antifungal compounds produced by *Bacillus* Y-IVI for suppressing *Fusarium* Wilt of Muskmelon. Plant Prot Sc 52: 167-175. DOI: 10.17221/70/2015-PPS.