The Potential of Sponge-Associated *Bacillus* spp. as A Biocontrol Agent to Inhibit Several Bacteria from Infected Catfish (*Clarias gariepinus* Burch)

Risky Hadi Wibowo[1,2,3]*, Sipriyadi[1,2], Welly Darwis[1], Ardinan Pribadi[4], Novi Susianti[4], Della Indah Medani[5], Nadya Rosianti[5], and Gustina Dwi Wulandari[5]

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[1] Department of Biology, Faculty of Mathematics and Natural Sciences, University of Bengkulu, Bengkulu 38371, Indonesia

[2] Master Study Program of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu, Kandang Limun, Bengkulu 38371, Indonesia

[3] Research Centre of Sumatera Natural Products and Functional Materials, University of Bengkulu, Kandang Limun, Bengkulu 38371, Indonesia

[4] Quality Control and Fishery Product Safety Agency (BKIPM) of Bengkulu, Bengkulu, 38213, Indonesia

[5] Undergraduate Student, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu, Kandang Limun, Bengkulu 38371, Indonesia

*Corresponding Author:
Riskyhadiwibowo80@gmail.com

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Abstract

Catfish farming has been increasingly threatened by outbreaks of diseases caused by bacteria which are major problems for Indonesia's catfish industry. This can lead to a decrease in the food quality of freshwater fishery products, especially catfish. In catfish farming, the occurrence of diseases can cause severe financial losses. This study aimed to identify bacteria that infected catfish and discover new potential antibacterial agents from *Bacillus* spp. isolated from a marine sponge to inhibit several bacteria from the infected catfish. Catfish samples were obtained from catfish farming with clinical observation of ulcerative lesions on the outside of the body. Several bacteria were isolated from the catfish and used sponge-associated bacterium to inhibit them in which using two methods namely spot assay and disc diffusion assay. Bacterial supernatant and pellet were used to performed antibacterial assay using disc diffusion method. These bacterial isolates were identified through their colony morphology, Gram staining, biochemical assays, and 16S rRNA gene sequence. Bacterial identification based on the 16S rRNA gene sequence showed that GL1 was 99.92% closely related to *Aeromonas jandaei*, HL1 was 100% closely to *Bacillus amyloliquefaciens*, and GL2 and HL2 was closely related to *Bacillus cereus*. The results of antibacterial assay indicated that sponge-associated *Bacillus* spp. successfully inhibited some bacteria that associated with infected catfish. In addition, *Bacillus velezensis* APD10 exhibited the most potential strain with antibacterial property which could inhibit all bacteria from the diseased catfish.

Keywords: Catfish, bacteria, Bacillus, sponge, antibacterial

Introduction

Catfish is one of the cultured freshwater fish commodities with a high consumption level and production in Bengkulu City. Based on data from the Bengkulu City Fisheries Service Statistics, the production value of catfish increased significantly from 2,859 ton in 2017 to 11,578 ton in 2020, compared to other freshwater fish (KKP, 2020). This proves the potential of catfish culture for further development. The demands of this fish encourage the farmers to increase their productivity and apply an intensive cultivation system as an effort to fulfill the requirement for fish on the market in high quantities. High stocking density of fish with rapidly changing water temperature and the chemical composition in ponds cause fish stress, resulting in a favorable condition for the disease onset and distribution (Ran et al., 2012).

Furthermore, a disease outbreak in catfish is a serious problem in several areas of Indonesia (Purwaningsih et al., 2022). *Motile Aeromonas Septicemia* (MAS) disease outbreaks in catfish in Indonesia occurred in Banjar, South Kalimantan was induced by *Aeromonas* sp. isolated from the kidney (Olga et al., 2020). In Samarinda, East Kalimantan, disease outbreaks were induced by *Aeromonas* sp., *Pseudomonas* sp., and *Enterobacter* sp. in tilapia (*Oreochromis niloticus*) and channel catfish (*Clarias*

batrachus) (Hardi et al., 2018). Meanwhile, a disease outbreak in catfish caused by *Bacillus* spp. in Indonesia still remains unreported at present, yet, there was a disease outbreak of *Clarias gariepinus* caused by *Bacillus cereus* in North Egypt (Abdelgayed et al., 2021). The disease often occurs in catfish culture activities due to bacterial infection outbreaks. Several types of pathogenic bacteria could cause disease in catfish and increase the mass mortality rate. Bacterial infection in fish is one of the diseases that can cause considerable loss and decreased fish quality. In a Korean fish culture, a mass mortality case was caused by *Edwardsiella tarda*, based on the isolate analysis from the kidney and liver in Korean catfish (Yu et al., 2009). Additionally, disease outbreaks that caused mass mortality in many catfish also occurred in Vietnam, which induced by *Aeromonas veronii* as pathogenic bacteria in catfish culture (Hoai et al., 2019).

The use of antibiotics increased the existence of antimicrobial resistant bacteria as well as drug residues in the aquaculture commodities (Subharthi, 2015). The use of biocontrol agents for aquaculture is increasing with the demand for environmental beneficials, eco-friendly alternative for sustainable aquaculture productionIn poor water quality, fish will easily experience stress. The combination of these conditions and the presence of some opportunistic bacteria in the water increases the potential risk of bacterial infection. Unfortunately, a large number of antibiotics are used in fish feeds for the purpose of preventing and treating infections in aquaculture facilities worldwide (Sapkota et al., 2008).

Our previous studies have succeeded in isolating several bacteria associated with marine sponges, such as *Bacillus* spp., with promising antimicrobial activity (Wibowo et al., 2020). It has been noted that some *Bacillus* can combat pathogenic bacteria from freshwater environment. For example, *Bacillus subtilis* from marine sponges can inhibit *Motile Aeromonas Septicemia* (MAS) that infected *Labeo rohita* (Paul et al., 2021). Another previous study indicated that *Bacillus* sp. found in marine sponges *Callyspongia diffusa* has abundant antibacterial compounds and can survive in normal saline condition (Kiran et al., 2018). This condition has been confirmed through an antibacterial assay using the spot and disc methods. Based on Li et al. (2021), *Bacillus* produced surfactin as an antibacterial compound (Li et al., 2021). This study aimed to isolate and identify bacteria from diseased catfish, then evaluate the ability of several sponge-associated *Bacillus* spp. to inhibit bacteria originating from diseased catfish.

Materials and Methods

Sampling

Experimental fish, i.e. *C. gariepinus* (±80 – 121 g) were obtained from catfish culture in Kandang Mas, Bengkulu, Indonesia. Fish were acclimatized at 25 ± 1°C. Diseased fish were sterilized with an iodine solution and dissected dorsoventrally with a sterile blade to expose kidney and liver. Two methods were carried out for bacterial isolation, namely the swab method and the serial dilution method. Bacteria were then isolated from the liver and kidney by swabbing and cultured onto *Trypticase Soy Agar* (Berati et al., 2023). These isolates were incubated at 28 °C for 24 h. In the serial dilution method, the catfish organ samples were minced with mortar and pestle before serially diluting at 10^{-1}, 10^{-3}, and 10^{-5}. After dilution, 0.1 ml of the dilution product was taken onto the TSA media and distributed evenly with a drigalski spatula (Irianto and Austin, 2002).

Morphological and Biochemical assay

Bacterial characterization was done through conventional microbiological assay methods, including Gram staining, catalase and oxidase production assays, Gram KOH 3%, Urea assay, Simmon citrate assay, gelatin assay, Triple Sugar Iron Agar assay, Lysin Iron Agar assay, Motility Indole Ornithine assay, Oxidative-Fermentative (OF) assay, Methyl Red assay, and carbohydrate assays (Al-Dhabaan, 2019). After that, the result of this characterization was compared to Bergey's Manual of Determinative Bacteriology 9th Edition (Holt et al., 1994), Bacterial Fish Pathogens 4th Ed (Austin and Austin, 2007), and Bacterial Fish Pathogens 5th Ed (Austin and Austin, 2012).

DNA Isolation and Amplification

A total of 1.5 mL of bacterial culture was put into Eppendorf and centrifuged at 8000 rpm for 10 min, STE buffer (composited with 0.3 M sucrose; 25 mM Tris-HCL; 25 mM EDTA. 2Na pH 8) used to wash the formed pellet, then centrifuged for 10 min at 8000 rpm. Pellet washing was carried out three times repeatedly. Furthermore, 200 µL of buffer STE and 45 mL of lysozyme (20 mg/mL) were added to the pellet then mixed and shaken gently. To form protoplast, it was incubated at 55 °C for 60 min. About 20 µL of proteinase-K (20 mg/mL) was added to the mixture and incubated at 55 °C for 1 h. Subsequently, 400 µL of 10% CTAB was added in a solution of 0.7 M NaCl

then incubated at 65 °C for half a h. A volume of the phenol:chloroform (25:24) was added into the solution and centrifuged at 12000 rpm for 10 min. The clear phase formed was then transferred to the new tube. Isopropanol volume was added 0.6 ml also 20 µL sodium acetate, the incubation was carried out at -20 °C overnight and centrifuged at 12000 rpm for 10 min. The supernatant was removed while 1 mL pellets was washed using 70% alcohol. DNA was dried for 1 h to remove alcohol and then dissolved in 50 µL sterile ddH2O, and the results of DNA isolation was stored at 4 °C or –20 °C (Sambrook et al., 1987). The 16S rRNA gene was amplified using the universal primer forward 27F (5'AGAGTTTGATCMTGGCTCAAG-3') and reverse 1492R (5'-ACCTTGTTA CGACTTC AC-3'). The 16S rRNA gene amplification were done in PCR thermocycler. PCR mixture contained 2,5 µL of template DNA; 0,4 µM of primer 27 F and 0,4 µM of primer 1492 R; 18,5 µL PCR-grade water, 2,5 µL 2x KAPA Taq extra hotstart ready mix with dye. PCR reactions were carried out in an thermocycler programmed for the initial denaturation at 95 °C for 3 min and 29 cycles as follows: denaturation 95 °C for 1 min, annealing at 55 °C for 1 min, extention 72 °C for 1 min and final extention 7 °C for 7 min.

DNA Sequencing, Phylogenetic Trees Reconstruction and Genetic Distance Analysis

The PCR products were sent to PT. Genetics Science Indonesia for sequencing. The sequences were analyzed using BLAST program on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees reconstruction and genetic distance were analyzed using MEGA software. The phylogenetic trees were constructed using Neighbor-Joining (NJ) method and Minimum-Evolution (ME) method. The evolutionary distances were calculated using Kimura 2-parameter method with bootstrap assay 1000 replicates (Rodiansyah et al., 2021).

Antibacterial Assay

An antibacterial assay was performed to evaluate the antibacterial potential of six bacteria from *Aplysina* sp. which collected from Enggano island (Wibowo et al. 2020) to combat the newly isolated bacteria from the diseased catfish. This assay was conducted using two methods namely spot and paper disc diffusion. The diseased catfish-associated bacteria were cultivated on *Trypticase Soy Agar* (TSA) for 24 h before assay. For the spot method, the pure *Bacillus* spp. were cultivated on *Sea Water Complete* (SWC) Agar. Then, the *Bacillus* spp. were spotted on *Trypticase Soy Agar* (TSA) that had been mixed with the liquid bacterium culture of infected catfish and then incubated during 48 hours at 28 °C. For the paper disc method, each pure isolate of *Bacillus* spp. was cultivated in SWC broth at 120 rpm for 24 hours at 25 °C. Then, the culture was centrifugated at 10000 r.p.m. for 5 min. The pellet (10 µl) and supernatant (10 µl) were inoculated into the paper disc (diameter of paper disc). Subsequently, the plates were incubated for 48 h at 28 °C. The presence of clear zone indicated a positive result of antibacterial activity.

Results and Discussion

The collection of catfish was carried out at the Kandang Mas catfish farm using a purposive sampling method. In total, two catfish were found to have symptoms of the disease. The results of observing symptoms are shown in Figure 1.

According to Thomas et al. (2013), the ulcerative lesions on the catfish have similarity with *Aeromonas* spp. infections. In addition, catfish infected with bacteria showed ventral and dorsal body ulceration and pale liver. Clinical symptoms in catfish induced by *Bacillus cereus* showed several red ulcerations on the scalp to the caudal fin. Besides, *Bacillus* spp. cause disease outbreaks and affect mass mortality in catfish farming (Abdelgayed et al., 2021). In order to confirm the causative agent of this infection, bacterial isolation was performed.

Two methods were applied to conduct bacterial isolation from the diseased catfish. According to

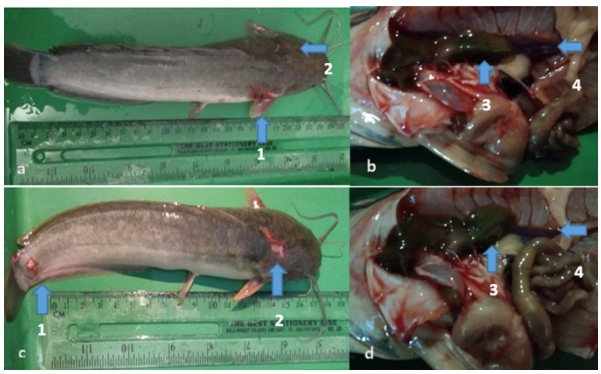

Figure 1. External morphology of catfish 1 (a): ulcerative lesions on the pectoral fin (blue arrow 1), ulcerative lesions on the dorsal (blue arrow 2). Internal morphology of catfish 1 (b): pale yellow color on the liver (blue arrow 3), and pale white color on the kidney (blue arrow 4). External morphology of the catfish 2 (c): ulcerative lesions on the caudal fin (blue arrow 1), ulcerative lesion on the head (blue arrow 2), Internal morphology of catfish 2 (d) Pale yellow color on the liver (blue arrow 3), and pale white color on the kidney (blue arrow 4).

Indonesian National Standard, the swab method is carried out by wiping the digestive organs of sick catfish using a sterile inoculation loop on Trypticase Soy Agar (TSA) (SNI 7663:2011, ICS 65.150). Moreover, the serial dilution method was performed to isolate bacteria in the fish, following the standard protocols applied by some researchers. The bacteria isolate from *Clarias gariepinus* used serial dilution, which was mixed with NaCl sterile until homogenous. The serial dilution method could reduce the bacterial density abundance (Novita et al., 2015).

In total, four bacterial strains were selected for further investigation. Two bacteria isolated from the kidney and two bacteria isolated from the liver. The morphological characteristics of these bacteria are shown by Figure 2 and 3.

Figure 2, shows the colony morphology of the four selected strains from the diseased catfish. Strain GL1 had yellowish color, strain GL2 had cream color, strain HL1 and HL2 had white color. Figure 3 shows the bacterial morphology under microscope after gram staining. It is noted that strains GL2, HL1, and HL2 were identified as Gram-positive bacteria, while strain GL1 was identified as a Gram-negative bacterium. Gram-positive bacteria have a thick peptidoglycan layer, whereas Gram-negative bacteria only have a thin peptidoglycan layer covered by lipopolysaccharides and lipoproteins. Upon decolorization with alcohol or acetone, only Gram-positive bacteria remain purple (Beveridge, 2001). It is also shown that strain GL1

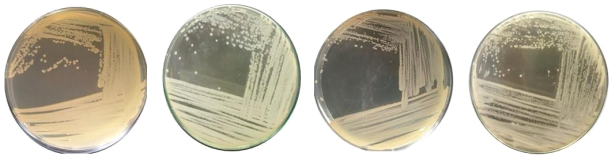

Figure 2. The morphology of bacterial colonies isolated from GL1: catfish kidney 1 (a), GL2: catfish kidney 2 (b), HL1: catfish liver 1 (c), and HL2: catfish liver 2 (d) were grown on TSA media which were incubated for 24 hours at 28 °C.

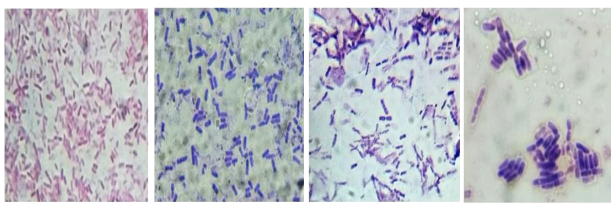

Figure 3. Gram staining on bacteria isolated from GL1 (a), GL2 (b), HL1 (c), and HL2 (d)

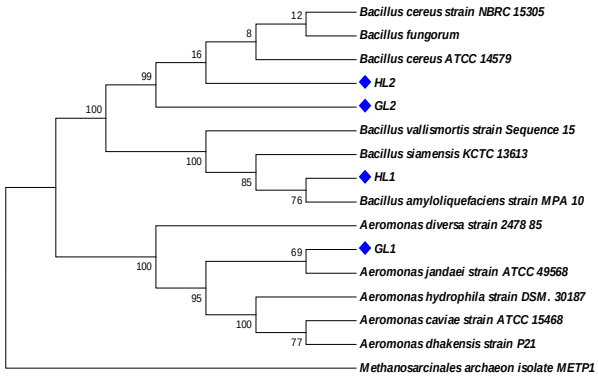

Figure 4. Phylogenetic tree results of isolated catfish from GL1: kidney 1 (a), GL2: kidney 2 (b), HL1: liver 1 (c), and HL2: liver 2 (d) isolates based on 16S rRNA gene analysis.

had a rod cell shape, while strains GL2, HL1 and HL2 had bacilli cell shapes. Further characterization was performed through a molecular approach. The result of 16S rRNA gene sequence analysis is presented by Figure 4.

The result of BLAST analysis discovered that strain GL1 was identified as *Aeromonas jandaei* with similarity 99.92%, strain HL1 as *Bacillus amyloliquefaciens* with similarity 100%, strain GL2 and HL2 as *Bacillus cereus* with similarity 100%. In Figure 2, a phylogenetic tree was successfully reconstructed based on 16S rRNA gene sequences of the isolated strains and their homologues species (Adegbesan, 2019). Based on BLAST analysis and phylogenetic tree reconstruction, three species of pathogenic bacteria that associated with the diseased catfish were successfully identified. In the study by Chandra et al. (2015), the isolated bacteria from catfish grown on TSA at 28 °C showed certain clinical symptoms such as ulcers on the skin which gradually grew in size and progressed to form ulcerous dermatitis reported that *B. cereus* was pathogenic showed and caused mass mortality in fish culture activities in India.

Morphological colony characteristics observation (Gram stain, mobility, motility, shape, and color) and biochemical tests (catalase, urease, oxidase activities, nitrate reduction, indol production, acid/gas production from carbohydrates, and fermentation of sugars) were considered as a conventional approach to identify pathogenic bacteria in aquaculture (Smibert and Krieg, 1994; Udgire et al., 2015). However, this study added a molecular approach to strengthen the result of our bacterial identification. Strain GL1 was allegedly closely related to the genus *Aeromonas* based on the clinical symptoms of catfish, bacterial colony morphology,

Table 1. The result of biochemical characterization of the isolated bacteria from diseased catfish

Biochemical Test	Isolated Code				*Aeromonas* (Austin and Austin, 2012)	*Bacillus* (Austin and Austin, 2012)
	GL1	GL2	HL1	HL2		
Gram KOH 3%	Gram -	Gram+	Gram+	Gram +	Gram -	Gram +
Catalase test	+	+	+	+	+	+
Urease test	-	+	+	+	-	+
Lysine Iron Agar Test	+	+	+	+	+	+
MIO:						
• Motility	+	+	+	+	+	+
• Indole	+	+	+	+	+	+
• Ornithine	-	+	+	+	-	+
Triple Sugar Iron Agar Test	A/A	K/A	K/K	K/A	A/A	K/A
Slunt/Butt						
• Gas	-	-	-	-	-	-
• H_2S	-	+	-	-	-	+
Oxidative-Fermentative Test	F	F	F	F	F	F
Gelatin Hydrolysis Test	+	+	-	-	+	+
Methyl Red test	+	+	+	+	+	+
Simmons Citrate Agar Test	+	+	+	+	+	+
Carbohydrate Tests						
• Glucose	+	+	+	+	+	+
• Lactose	-	-	-	+	-	+
• Maltose	+	+	+	+	+	+
• Mannitol	+	+	+	+	+	+
• Sorbitol	+	+	+	+	+	+

Gram staining, and biochemical profile. *Aeromonas* bacteria are Gram-negative bacteria with yellowish colonies with opaque, straight-rod shapes and are motile. The biochemical characteristics of *Aeromonas* are positive oxidase and catalase assays, producing gas and acid, fermenting glucose and sucrose, and acid butt with gas on triple sugar iron agar (Workgegn et al., 2021). This finding was in line with the result of 16S rRNA gene analysis which confirmed GL1 as *Aeromonas jandaei*. Furthermore, a positive result for oxidase assay and negative for catalase, indole, and urease assays were exhibited in GL2, HL1, and HL2. Thus, these three strains were confirmed as the member of *Bacillus*. This finding also suitable with the result of 16S rRNA gene analysis which suggested these strains as *Bacillus* spp. Nonetheless, strain GL1 and HL2 had negative oxidase, indole, and urease assays, and positive catalase assay. The same biochemical characteristics strengthen the results of the 16S rRNA gene analysis that both come from the same species, namely *Bacillus cereus*. *Bacillus* has been reported to infect African catfish in the Vietnam area through the oral tract and infect the kidneys of catfish with high levels of pathogenicity (Austin and Austin, 2012).

Aeromonas is bacteria that causes disease in catfish and other aquatic animals. *Aeromonas* causes significant catfish mortality and economic losses in the freshwater fish culture industry (Hossain, 2021). Based on observations, catfish infected with *Aeromonas* showed red ulceration on the pectoral and dorsal fins (Nawaz et al., 2006). *Aeromonas jandaei* cause fish disease and increased stress to death. Previous studies reported *Aeromonas jandaei* cause 100% mortality within 24 hours after infection (Dong et al., 2017). A prior study showed that *Aeromonas jandaei* possessed an enterotoxin gene (Pablos et al., 2010). Therefore, these results are concurrent with the hemolytic activity of these bacteria, thus, providing potent pathogens. The positive detection of the aerolysin gene (aerA) was confirmed in *Aeromonas jandaei* from catfish (Bondi et al., 2000).

In order to overcome bacterial infection in catfish without antibiotic, this study aimed to find a biocontrol agent from sponge-associated bacteria. One of the most important features of biocontrol agents is the ability to inhibit the pathogenic microorganisms. Therefore, antibacterial assay was conducted in this study to combat the newly isolated bacteria from the diseased catfish. The result of this assay is presented by Figure 5, 6 and 7.

In the antibacterial assay using the spot method (Figure 5), all *Bacillus* spp. have the potential to inhibit the newly isolated bacteria from the diseased catfish. *Bacillus velenzensis* APD10 was the best isolate to inhibit the growth of the HL2 bacteria with an inhibition zone diameter of 8.5 mm. *Bacillus velezensis* has the potential as a probiotic for animals and fish. *Bacillus*

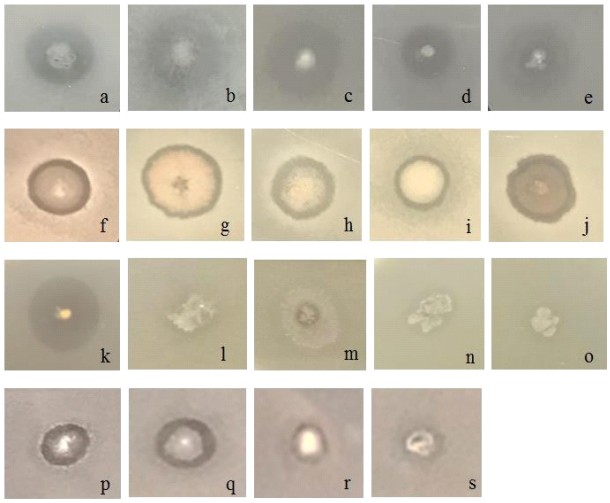

Figure 5. Inhibition zone is formed from *Bacillus* spp. against newly isolated bacteria from diseased catfish using the spot method. *B. velezensis* APD10 (a), *B. subtilis* APD11 (b), *B. subtilis* APD15 (c), *B. subtilis* APD3 (d), *B. subtilis* APD4 (e) against HL1 isolate; *B. subtilis* APD4 (f), *B. velezensis* APD10 (g), *B. subtilis* APD3 (h), *B. subtilis* APD15 (i), *B. subtilis* APD11 (j) against GL1 isolate; *B. velezensis* APD10 (k), *B. paralicheniformis* APD9 (l), *B. subtilis* APD4 (m), *B. subtilis* APD11 (n), *B. subtilis* APD3 (o) against HL2 isolate; *B. velezensis* APD10 (p), *B. subtilis* APD3 (q), *B. subtilis* APD11 (r), and *B. subtilis* APD4 (s) against GL2 isolate.

velezensis has been studied and applied more widely recently because it can inhibit bacteria and become a potential biocontrol agent. Systematically, the secondary metabolites and molecular research of antimicrobial are available in the bacteria. Previous studies stated that lipopeptides from *Bacillus velezensis* have strong cytotoxic properties against pathogenic microbes (Meena et al., 2018; Ye et al., 2018). Bioactive compounds in these bacteria can inhibit microbial growth and decrease any disease outbreak (Ngalimat et al., 2021). *Bacillus subtilis* APD3, APD4, and APD11 also inhibited all newly isolated bacteria from the diseased catfish with moderate and weak inhibition zones. Based on previous studies, *Bacillus subtilis* is one of the most common bacterial groups associated with antimicrobial metabolite products (Caulier et al., 2019). Van et al. (2020) reported that *B. subtilis* can inhibit the growth of *Edwardsiella* and *Aeromonas* pathogenic bacteria in catfish. According to Nair et al., (2021), the ability of *Bacillus subtilis* was proven by assaying the spot method and the supernatant on the disc diffusion method with antibacterial activity against *Aeromonas*.

In the antibacterial assay using the disc method with the supernatant (Figure 6), 4 isolates of *Bacillus* spp. shown to have the potential as an antimicrobial agent in catfish. *Bacillus velenzensis* (APD10) inhibited the GL2 bacterial isolate with an inhibition zone diameter of 17.6 mm. Phelan et al. (2013), reported that the cell-free supernatant of *Bacillus velezensis* strains was also used for antimicrobial screenings against several Gram-positive and Gram-negative bacteria. All *Bacillus velezensis* strains showed broad-spectrum inhibitions across different groups of other bacteria. *Bacillus velezensis* CPA1-1 carries the gene encoding the

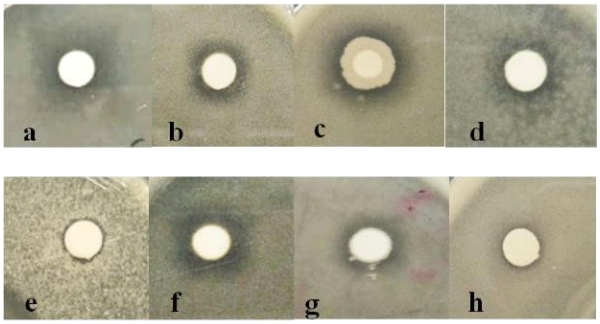

Figure. 6. Inhibition zone formed from supernatant of *Bacillus* spp. against newly isolated bacteria from diseased catfish using the disc method. *B. subtilis* APD3 (a), *B. subtilis* APD4 (b), *B. velezensis* APD10 (c) against GL2 isolate, *B. velezensis* APD10 (d) against HL2 isolate, *B. subtilis* APD3 (e) against GL1 isolate, *B. subtilis* APD3 (f), *B. paralicheniformis* APD9 (g), *B. velezensis* (APD10) against HL1 isolate (h).

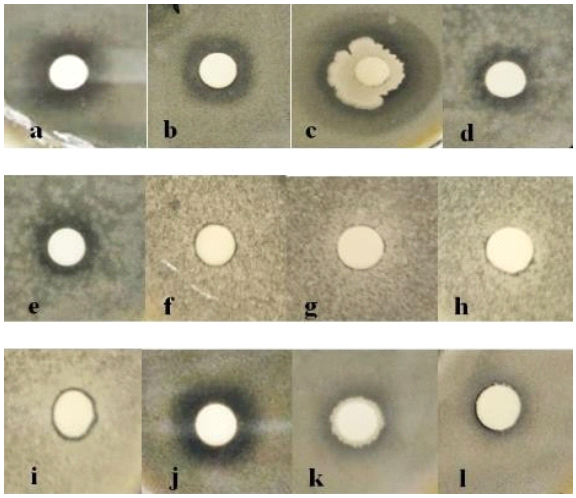

Figure. 7. Inhibition zone formed from pellets of *Bacillus* spp. against newly isolated bacteria from diseased catfish using the disc method. *B. subtilis* APD3 (a), *B. subtilis* APD4 (b), *B. velezensis* APD10 (c) against GL2 isolate, *B. subtilis* APD3 (d), *B. velezensis* APD10 (e) against HL2 isolate, *B. subtilis* APD3 (f), *B. subtilis* APD4 (g), *B. subtilis* APD11 (h), *B.* subtilis APD15 (i) against GL1 isolate, *B. subtilis* APD3 (j), *B. paralicheniformis* APD9 (k), *B. velezensis* APD10 (l) against HL1 isolate

biosynthesis of various peptidoglycans, polyketide antimicrobial compounds, and proteins, which inhibiting *Aeromonas*, *Bacillus*, and *Vibrio*. *Bacillus velezensis* is a good antipathogen in fish and has potential for further prospects to prevent and control various aquatic animal diseases (Li, X et al., 2020). According to Sipriyadi et al. (2021), the bacterial isolate of *Bacillus* spp. from *Haliclona* sp. sponges from Enggano island can inhibit several pathogenic bacteria.

In the antibacterial assay results of the disc method using pellets (Figure 7), there were six isolates of *Bacillus* spp. which has the potential to inhibit bacteria in catfish. Again, *Bacillus velenzensis* APD10 exhibited the best antibacterial property by inhibiting GL2 with an inhibition zone diameter of 22.15 mm. According to Wibowo et al. (2020), the bioactive compound as a pellet and supernatant in *Bacillus* spp. from *Aplysina* sp. can inhibit *Candida albicans*. This strain has great potential as a biocontrol agent, which can be developed commercially to improve disease control in fish culture. Barale et al. (2022) reported that *Bacillus velezensis* can grow on *Trypticase Soy Agar* (TSA) at a normal temperature within 24-48 hours. The inhibition zone produced by *Bacillus velezensis* was reported a crucial role in the biocontrol agent (Li, Z et al., 2020).

In Table 2, all of the antibacterial assays using two methods consisting of spot method and disc diffusion using supernatant and pellet present the inhibitory

Table 2. The results of the antibacterial assay of the spot method and disc method antibacterial assay using the supernatant and pellet

No	Isolate code of *Bacillus* spp.	Bacteria of Infected catfish	Diameter of Ihibitory Zone Activity (mm) ± SD		
			Pure Culture	Supernatant	Pellet
1.	APD 3	HL1	5.75 ± 0.25	0.2 ± 0.1	5.3 ± 0.8
		GL1	3.1 ± 0.15	0.7 ± 0.1	0.35 ± 0.15
		HL2	2.2 ± 0.45	-	3.3 ± 0.2
		GL2	2.3 ± 0.1	1.85 ± 0.05	5.6 ± 0.1
2.	APD 4	HL1	5.9 ± 0.15	-	-
		GL1	3.35 ± 0.25	-	0.15 ± 0.05
		HL2	1.85 ± 0.1	-	-
		GL2	1.9 ± 0.55	2.4 ± 0.2	5.25 ± 0.15
3.	APD 9	HL1	-	-	-
		HL2	1.1 ± 0.05	2.65 ± 0.05	2.15 ± 0.05
4.	APD 10	HL1	6.65 ± 0.15	0.3 ± 0.2	0.3 ± 0.1
		GL1	6.4 ± 0.4	-	-
		HL2	8.5 ± 0.05	0.35 ± 0.15	4.6 ± 0.1
		GL2	6.9 ± 0.05	17.6 ± 0.3	22.15 ± 0.05
5.	APD 11	HL1	6.05 ± 0.05	-	-
		GL1	6.15 ± 0.05	-	-
		HL2	4.35 ± 0.25	-	-
		GL2	4.4 ± 0.1	-	-
6.	APD 15	HL1	4.8 ± 0.3	-	-
		GL1	1.7±0.1	-	-

activities for each method. The great assay inhibition zone diameter was obtained from the disk dilution method with pellet, followed by disk dilution with supernatant and pure bacterial culture. The spot method presented an inhibition zone diameter that was smaller than supernatant and pellet, however, inhibition zones from the spot method were moderate because almost all *Bacillus* spp. from marine sponges showed a clear zone. Phelan et al. (2013) reported that the pure bacteria culture of *Bacillus* spp. from marine sponge produced a variety composition of several antimicrobial peptides, and the most important antibacterial substance was from the supernatant, namely subtilomycin. Meanwhile, the pellet contained some enzyme that stimulated the establishment of secondary metabolite as an antibacterial compound. The secondary metabolite of marine sponges associated bacteria can inhibit bacteria that could reduce the disease outbreak in fish culture (Mohan et al., 2016).

Conclusion

This study successfully isolated four bacteria from two diseased catfishes which collected from Kandang Mas, Bengkulu, Indonesia. Morphological and biochemical profiling indicated these bacteria belongs to *Aeromonas* and *Bacillus*. The result of 16S rRNA gene sequence analysis discovered that strain GL1 was identified as *Aeromonas jandaei* with similarity 99.92%, strain HL1 as *Bacillus amyloliquefaciens* with similarity 100%, strain GL2 and HL2 as *Bacillus cereus* with similarity 100%. Furthermore, all sponge-associated bacteria exhibited antibacterial potential against all newly isolated bacteria from diseased catfish. Moreover, *Bacillus velezensis* APD10 was suggested as the best candidate due to its antibacterial potential.

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Supplementary Materials

Supplementary materials is not available for this article.

References

Adegbesan, S. I. (2019). Nutrigenomics and nutrigenetics-effect of dietary supplementation of chromolaena odorata leave the paste on the inassayinal bacteria of African mud catfish (*Clarias gariepinus*) fingerlings using 16s rRNA gene sequencing. International Journal of Bioprocess and Biotechnological Advance, 5(1),151-168. https://doi.org/10.1016/j.btre.2016.06.001

Abdelhamed, H., Ibrahim, I., Baumgartner, W., Lawrence, M. L., & Karsi, A. (2017). Characterization of histopathological and ultrastructural changes in channel catfish experimentally infected with virulent *Aeromonas hydrophila*. Frontiers in Microbiology, 8, 15-19. https://doi.org/10.3389/fmicb.2017.01519

Albert, M. J., Ansaruzzaman, M., Talukder, K. A., Chopra, A. K., Kuhn, I., Rahman, & M., Mollby, R. (2000). Prevalence of enterotoxin genes in *Aeromonas* spp. isolated from children with diarrhea, healthy controls, and the environment. Journal Of Clinical Microbiology, 38(1), 3785-3790. https://doi.org/10.1128/JCM.38.10.3785-3790.2000

Abdelgayed M. Younes, Alkhateib Y. Gaafar, Alaa Eldeen Z. Abu-Bryka, Attia A. Abou Zaid & Ahmed A. Askora. (2021). Isolation and pathogenicity determination of *Bacillus cereus* associated with the ulcer, 15(1), 10-18. 10.3923/ajas. 2021. 10.18

Al-Dhabaan, F. A. (2019). Morphological, biochemical, and molecular identification of petroleum hydrocarbon biodegradation bacteria isolated from oil-polluted soil in Dhahran, Saud Arabia. Saudi Journal of Biological Sciences, 2(6),1247-1252. https://doi.org/10.1016/j.sjbs.2018.05.029

Austin, B., & Austin, DA. (2007). Bacterial fish pathogens: Disease in farmed and wild fish (4th ed.). Springer, USA.

Austin, B. & Austin, DA. (2012). Bacterial Fish Pathogens: Disease of farmed and wild fish. (5th ed.). Springer, Netherlands.

Altsschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J., (1990). Basic Local Alignment search Tool. J. Mol. Biol. 215, 403-410.

Beveridge, T. J. (2001). Use of the gram stain in microbiology. Biotechnic and Histochemistry, 76(3), 111-118. https://doi.org/10.1080/bih.76.3.111.118

Bondi, M., Messi, P., Guerrieri, E., Bitonte, F. (2000). Virulence profiles and other biological characters in water isolated *Aeromonas hydrophila*. The New Microbiological, 23(4), 347–356.

Barati-Deak, B., Da Costa Arruda, G. C., Perjessy, J., Klupacs, A., Zalan, Z., Mohacsi-Farkas, C., & Belak, Á. (2023). Inhibition of foodborne pathogenic bacteria by excreted metabolites of *Serratia marcescens* strains isolated from a dairy-producing environment. Microorganisms, 11(2), 403.

Barale, S. S., Ghane, S. G., & Sonawane, K. D. (2022). Purification and characterization of antibacterial surfactin isoforms produced by *Bacillus velezensis* SK. Amb Express, 12(1), 1-20. https://doi.org/10.1186/s13568-022-01348-3

Benga, L., Benten, W. P. M., Engelhardt, E., Köhrer, K., Gougoula, C., & Sager, M. (2014). 16S ribosomal DNA sequence-based identification of bacteria in laboratory rodents: a practical approach in laboratory animal bacteriology diagnostics. Laboratory animals, 48(4), 305-312.

Caulier, S., Nannan, C., Gillis A., & Licciardi F. (2019). Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Front Microbiol, 10(302), 1–19. https://doi.org/10.3389/fmicb.2019.00302

Chandra, G., Bhattacharjee, I., & Chatterjee, S. (2015). *Bacillus cereus* infection in stinging catfish, Heteropneustes fossilis (*Siluriformes: Heteropneustidae*) and their recovery by *Argemone mexicana* seed extract. *Iranian Journal of Fisheries Sciences*, *14*(3), 741-753. 10.22092/IJFS.2018.114479

Dong, H. T., Techatanakitarnan, C., Jindakittikul, P., Thaiprayoon, A., Taengphu, S., Charoensapsri, W., & Senapin, S. (2017). *Aeromonas jandaei* and *Aeromonas veronii* caused disease and mortality in Nile tilapia, *Oreochromis niloticus* (L.). Journal of Fish Diseases, 40(10), 1395-1403. https://doi.org/10.1111/jfd.12617

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., & Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology. 9th ed. William & Wilkins. Department of Microbiology, Gltner Hall, (pp. 824-1101). Michigan State University, East Lansing, MI, USA.

Hawaz, P. (2014). Isolation and identification of probiotic lactic acid bacteria from curd and in vitro evaluation of its growth inhibition activity against pathogenic bacteria. African Journal Microbiology Research, 8(13), 1419-1425. https://doi.org/10.5897/AJMR2014.6639

Hossain, S., & Heo, G. J. (2021). Ornamental fish: a potential source of pathogenic and multidrug resistant motile *Aeromonas* spp. Applied Microbiology, 72(1), 2-12. https://doi.org/10.1111/lam.13373

Hardi, E. H., Nugroho, R. A., Saptiani, G., Sarinah, R. I. A., Agriandini, M., & Mawardi, M. (2018). Identification of potentially pathogenic bacteria from tilapia (*Oreochromis niloticus*) and channel catfish (*Clarias batrachus*) culture in Samarinda, East Kalimantan, Indonesia. Biodiversitas Journal of Biological Diversity, 19(2), 480-488. https://doi.org/10.13057/biodiv/d190215

Hoai, T. D., Trang, T. T., Van Tuyen, N., Giang, N. T. H., & Van Van, K. (2019). *Aeromonas veronii* caused disease and mortality in channel catfish in Vietnam. Aquaculture, 513, 734-425. https://doi.org/10.1016/j.aquaculture.2019.734425

Irianto, A., & Austin, B. (2002). Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Disease, 25, 333–342. https://doi.org/10.1046/j.13652761.2002.00375

Kiran, G. S., Priyadharsini, S., Sajayan, A., Ravindran, A., & Selvin, J. (2018). An antibiotic agent pyrrole [1, 2-a] pyrazine-1, 4-dione, and hexahydro isolated from a marine bacteria *Bacillus tequilensis* MSI45 effectively controls multi-drug resistant *Staphylococcus aureus*. RSC advances, 8(32), 17837-17846. 10.1039/C8RA00820E

Li, Z., Song, C., Yi, Y., & Kuipers, O. P. (2020). Characterization of plant growth-promoting rhizobacteria from perennial ryegrass and genome mining of novel antimicrobial gene clusters. BMC Genomics, 21(1), 1-11. https://doi.org/10.1186/s12864-020-6563-7

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

Mohan, G., Thangappanpillai, A. K. T., & Ramasamy, B. (2016). Antimicrobial activities of secondary metabolites and phylogenetic study of sponge endosymbiotic bacteria, *Bacillus* sp. at Agatti Island, Lakshadweep Archipelago. *Biotechnology reports*, *11*, 44-52.

Meena, K. R., Tandon, T., Sharma, A., & Kanwar, S. S. (2018). Lipopeptide antibiotic production by *Bacillus velezensis* KLP2016. Journal of Applied Pharmaceutical Science, 8(3), 091-098. DOI: 10.7324/JAPS.2018.8313

Ngalimat, M. S., Yahaya, R. S. R., Baharudin, M. M. A. A., Yaminudin, S. M., Karim, M., Ahmad, S. A., & Sabri, S. (2021). A review of the biotechnological applications of the operational group

B. amyloliquefaciens. Microorganisms, 9(3), 614. https://doi.org/10.3390/microorganisms9030614

Nair, A. V., Antony, M. L., Praveen, N. K., Sayooj, P., Swaminathan, T. R., & Vijayan, K. K. (2021). Evaluation of in vitro and in vivo potential of *Bacillus subtilis* MBTDCMFRI Ba37 as a candidate probiotic in fish health management. Microbial Pathogenes 152, 104-610. https://doi.org/10.1016/j.micpath.2020.104610

Nawaz, M., Sung, K., Khan, S. A., Khan, A. A., & Steele, R. (2006). Biochemical and molecular characterization of tetracycline-resistant Aeromonas veronii isolates from catfish. *Applied and environmental microbiology*, *72*(10), 6461-6466. : https://doi.org/10.1128/AEM.00271-06

Novita, H., Rusmana, I., Yuhana, M., & Pasaribu, F. H. (2015). Characterization of anti-quorum sensing (AQS) bacteria as an inhibitor of disease virulence in African catfish (*Clarias gariepinus*). Aquaculture Research Journal, 10(1), 89-98. http://dx.doi.org/10.15578/jra.10.1.2015.89-98

Olga, O., Aisiah, S., A Tanod, W., Risjani, Y., Nursyam, H., & Maftuch, M. (2020). Immunogenization of heat-killed vaccine candidate from *Aeromonas hydrophila* in catfish (*Pangasius hypothalamus*) using a strain of Banjar, South Kalimantan, Indonesia. Egyptian Journal of Aquatic Biology and Fisheries, 24(4), 1-13. https://doi.org/10.21608/EJABF.2020.93528

Pablos, M., Remacha, M. A., Rodríguez-Calleja, J. M., Santos, J. A., Otero, A., García-López, M. L. (2010). Identity,

virulence genes, and clonal relatedness of Aeromonas isolates from patients with diarrhea and drinking water. European Journal of Clinical Microbiology and Infectious Diseases, 29(9),1163-1172.

Paul, S. I., Rahman, M. M., Salam, M. A., Khan, M. A. R., & Islam, M. T. (2021). Identification of marine sponge-associated bacteria of Saint Martin's island of the Bay of Bengal emphasizing the prevention of *motile Aeromonas septicemia* in *Labeo rohita*. Aquaculture, 545, 737156. https://doi.org/10.1016/j.aquaculture.2021.737156

Phelan, R. W., Barret, M., Cotter, P. D., O'Connor, P. M., Chen, R., Morrissey, J. P.,& Barbosa, T. M. (2013). Subtilomycin: a new lantibiotic from *Bacillus subtilis* strain MMA7 isolated from the marine sponge *Haliclona simulans*. Marine drugs, 11(6), 1878-1898.

Purwaningsih, U., Sukenda, S., Lusiastuti, A. M., Alimuddin, A., Widanarni, W., & Nuryati, S. (2022). The phenotypic, genotypic, and pathogenicity comparison of *Edwardsiella ictaluri* Indonesian local isolates causing enteric septicemia of catfish. *Aquaculture Research*, *53*(10), 3608-3625. https://doi.org/10.1111/are.15865

Ran, C., Carrias, A., Williams, M. A., Capps, N., Newton, J. C.,& Liles, M. R. (2012). Identification of *Bacillus* strains for biological control of catfish pathogens. Plos One, 7(9),1-9. https://doi.org/10.1371/journal.pone.0045793

Rodiansyah, A., Mahmudah, A. F., Ulfah, M. M., Rohmawati, U., Listyorini, D., Suyono, E. A., & Prabaningtyas, S. (2021). Identification Of Potential Bacteria On Several Lakes In East Java, Indonesia Based On 16S RRNA Sequence Analysis. *HAYATI Journal Of Biosciences*, *28*(2), 136-136. https://doi.org/10.4308/hjb.28.2.136

Sambrook, E.F. Fritsch, T. Maniatis. (1987). Moleculer cloning: a laboratory manual. New york: Cold Spring Harbor Laboratory Press

Sipriyadi, Cahlia, U., Darwis, W., Wibowo, R.H., Nugraheni, E., & Sariyanti, M. (2021). Identification of bacteria associated with *Haliclona* sp. sponges from Enggano island, Indonesia with antimicrobial activity against human pathogens. Malaysian Journal of Microbiology, 17(2),178-189. http://dx.doi.org/10.21161/mjm.200843

Smibert, R.M., & Krieg, N.R. (1994). In: Phenotypic characterization methods for general and Molecular bacteriology. American Society for Microbiology.

SNI 7663. (2011). Identifikasi Edwardsiella tarda secara morfologis, fisiologis dan biokimia. Badan Standarisasi Nasional. Jakarta.

Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., & Lawrence, R. (2008). Aquaculture practices and potential human health risks: current knowledge and future priorities. Environment international, 34(8), 1215-1226. https://doi.org/10.1016/j.envint.2008.04.009

Subharti, P. (2015). Phage Therapy an alterate disease control in aquaculture: A review on recent advencements. IOSR Journal of Agriculture and Veterinary Science, 8(9), 68-81. https://doi.org/10.9790/2380-08916881

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA 6: molecular evolutionary Genetics analysis version 6.0. Molecular Biology and Evolution, 30(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Thomas, J., Madan, N., Nambi, K. S. N., Majeed, S. A., Basha, A. N., & Hameed, A. S. (2013). Studies on ulcerative disease caused by *Aeromonas caviae*-like bacterium in Indian catfish, *Clarias batrachus* (Linn). Aquaculture, 376, 146-150. https://doi.org/10.1016/j.aquaculture.2012.11.015

Udgire, M., Shah, N., Jadhav, M., (2015). Enrichment, isolation, and identification of hydrocarbon-degrading bacteria. International Journal of Current Microbiology and Applied Science 4(6):708–713. member 6 (2015) pp. 708-713. http://www.ijcmas.com

Van Minh, N., Duy, N. H. T., Ni, T. T. A., Duc, T. K., & Linh, D. N. (2020). Isolation and selection of *Bacillus* strains with high-potential probiotics that are used in catfish farming (*Pangasianodon* hypophthalmus). Engineering and Technology, 10(1), 3-16. 10.46223/HCMCOUJS. tech.en. 10.1.355.2020

Wibowo, R.H., Sipriyadi, Darwis, W., Yudha, S.P., Pertiwi, H.P., & Pertiwi, R. (2020). Antifungal activity of bacteria associated with *Aplysina* sp. sponge collected from Enggano island, north Bengkulu, Indonesia against *Candida albicans* advances in biological sciences research. Proceedings of the International Seminar on Promoting Local Resources for Sustainable Agriculture and Development 13, 326-331. 10.2991/absr.k.210609.051

Workagegn, B., K. Lema, B., Natarajan, P., & Prabadevi, L. (2021). *Aeromonas* spp. infection in farmed nile tilapia, *Oreochromis Niloticus*. Journal of Aquaculture Research and Development, 12(4),16. https://hal.science/hal-03635085

Ye, M., Tang, X., Yang, R., Zhang, H., Li, F., Tao, F., & Wang, Z. (2018). Characteristics and application of a novel species of *Bacillus: Bacillus velezensis*. ACS Chemical Biology, 13(3), 500-505. https://doi.org/10.1021/acschembio.7b00874

Yu, J. H., Han, J. J., Park, K. S., Park, K. H., & Park, S. W. (2009). *Edwardsiella tarda* infection in Korean catfish, *Silurus asotus*, in a Korean fish farm. Aquaculture Research, 41(1), 19-26. https://doi.org/10.1111/j.1365-2109. 2009. 02296.x