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Characterization of Antagonistic Bacteria from Gill Mucus of Marine Cage-Cultured Milkfish (*Chanos chanos*)

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ABSTRACT

With the rise of aquaculture, rearing intensification and irresponsible use of antibiotics have led to chemical pollution, antibiotic resistance, and proliferation of pathogens. The milkfish (*Chanos chanos*) is one of the economically important aquaculture species in the Philippines threatened by bacterial pathogens, including *Vibrio* spp., which cause mortalities leading to economic losses. To combat these pathogens, probiotics have been proposed as alternatives since they aid in the growth and health of fish. Hence, this study aimed to determine the bacterial and *Vibrio* count of the milkfish and to screen for potential probiotics from their gill mucus. Two hundred forty-five (245) isolates were collected from the gills of six milkfish samples and were tested against *Vibrio harveyi* for antagonistic activity. Out of the 245 isolates, five showed antagonistic activity against *V. harveyi* through the spot-on-lawn and the cross-streak assays. These isolates were characterized morpho-biochemically and identified through *16S rRNA* sequencing. Three isolates were identified to be closely related to either *Vibrio alginolyticus* or *Vibrio neocaledonicus*, while the other two were putative *Oceanimonas* and *Marinomonas*. Findings of this study might have a potential contribution to further characterizing the probiotic potential of the *Oceanimonas* and the *Marinomonas* species as they appear understudied in the area of probiotics research in aquaculture.

INTRODUCTION

Milkfish (*Chanos chanos*) or locally known as bangus is one of the most important species in aquaculture not just in the Philippines but also in countries like Taiwan and Indonesia **(SEAFDEC, 2022)**. According to the **Bureau of Fisheries and Aquatic Resources (2022)**, there is an increasing trend in the average annual growth rate of the milkfish, contributing to 17.9% of total fisheries production in 2020. One of the top producers of the milkfish in the country is Western Visayas region **(Espina, 2020)**.

The tremendous contribution of aquaculture, especially in the global food system, has led to its expansion resulting in adverse effects on the environment. This includes nutrient pollution, biological pollution, and chemical pollution **(Brañ*a et al.*, 2021)**. The intensification of aquaculture has become an avenue for diseases and infections to develop due to the favorable conditions in which they can proliferate **(Okocha *et al.*, 2018)**.

Antibiotics are usually incorporated with the feeds although some are directly put into the environment as this increases productivity and prevents or treats diseases. However, when antibiotics are used irresponsibly, economic and health problems can arise **(Pham *et al.*, 2015)**. A study by **Lundborg and Tamhankar (2017)** showed that antibiotic residues reported from Asian countries (Thailand, Indonesia, Bangladesh, and India) are found in aquatic products and aquaculture water which may pose harmful risks for human consumption. Additionally, the prolonged use of antibiotics in aquaculture has resulted in the occurrence of antibiotic-resistant bacteria **(Yuan *et al.*, 2023)**.

To aid in the reduction of antibiotic use, probiotics, which are live microorganisms that give health benefits to their host, are administered in sufficient amounts **(Irianto & Austin, 2002)**. They aid in the growth, feed conversion, stress resistance, disease resistance, and health of the organism **(Wuertz *et al.*, 2021)**. A study by **Allameh *et al.* (2016)** showed that supplementation of probiotics to fish diet improved the culture outcomes of the Javanese carp. An improvement in the growth performance of the fish, specifically, its effect on weight gain, feed conversion ratio, protein efficiency ratio, and growth rate were observed.

In recent years, studies in this field have focused more on native probiotics and their host specificity as these probiotics are expected to perform better than those from various sources **(Wuertz *et al.*, 2021)**. However, these studies mostly focus on fish microbiota in the skin and gut of fish while gill bacterial communities are overlooked **(Reverter *et al.*, 2017)**. Fish gills are important organs involved in various functions such as respiration, osmoregulation, and excretion of waste, among others **(Foyle *et al.*, 2020)**. These gills are exposed to the external environment, subjected to varying environmental conditions, and are in contact with water, and consequently, pathogens. A mucus layer is found in these gills which serves as the primary defence of the organism against pathogens as they are composed of ions, lipids, mucins, and proteins that aid in microbial adherence and growth and immune molecules like lysozyme and proteases **(Gomez *et al.*, 2013)**. Additionally, the mucus has a community of microorganisms that not only protects against pathogens and maintains mucus homeostasis but also produces metabolites **(Reverter *et al.*, 2017)**. A study by **Lowrey *et al.* (2015)** showed that the bacterial populations found in trout are similar to bacteria that contain antifungal properties cultured from amphibian skin.

Given the afore-mentioned data, the study aimed to explore the probiotic potential of gill mucus-associated bacteria of the milkfish. Specifically, it aimed to determine the

total bacteria and *Vibrio* count in the gill mucus of the milkfish, and screen for the presence of gill mucus-associated bacteria with probiotic potential.

MATERIALS AND METHODS

Sample collection and preparation

Six (6) samples of milkfish weighing 200 to 500 grams reared in sea cages in Guimaras, Central Philippines were collected from a fish landing site in Miagao, Iloilo, Philippines. The samples were kept in an ice box and were transported to the University of the Philippines Visayas Microbiological Laboratory for dissection and bacterial isolation. Using aseptic procedures, the gill was collected by removing the operculum with sterile scissors. The first gill arch was removed, and the second gill arch was used to extract gill mucus. The extracted gill was mixed with equal parts of normal saline solution (NSS). The solution was then serially diluted up to 10^5 for total bacteria and *Vibrio* counts.

Total viable bacteria and presumptive *Vibrio* count

Serial dilution was conducted through transferring 0.1mL of the sample mixture to each subsequent tube with 0.9mL NSS. Using the spread plate method, 0.1mL from each serially diluted sample was pipetted and spread onto duplicate plates of Nutrient Agar (NA) and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar to determine the total bacterial count and presumptive *Vibrio* count, respectively. The plates were then incubated at 30 ± 2°C for 48 hours. Colony counts were performed on plates with 25–250 colonies and were expressed as colony-forming units (CFU) per mL or gram of sample. For presumptive *Vibrio* identification, as described by **Azwai *et al*. (2016)**, round green colonies (2–3mm in diameter) indicate the presence of putative *Vibrio parahaemolyticus* or *V. vulnificus*, while yellow colonies of similar size suggest the presence of *V. cholerae*, *V. fluvialis*, or *V. alginolyticus*.

Screening for antagonistic activity

The bacterial pathogen *Vibrio harveyi* PN 9801 **(de la Pena *et al*., 2001)** was obtained from Fish Health Section of the Southeast Asian Fisheries Development Center (SEAFDEC), Aquaculture Department, Tigbauan, Iloilo, Philippines. A colony was inoculated in 10mL nutrient broth with 1% NaCl, and incubated overnight at 27-30°C with gentle shaking. The working bacterial culture was prepared by diluting the previous culture to 10^3 CFU ml^{-1} in normal saline solution (NSS).

The preliminary antagonistic screening using 245 isolates was performed through spot-on-lawn method following **Caipang *et al.* (2010)**. Isolates with observed inhibition against *V. harveyi* were used for the secondary screening.

The cross-streak method following the procedures of **Lucero-Velasco *et al.* (2018)** was implemented for secondary screening. A single streak of *V. harveyi* from the diluted bacterial culture was performed in the middle of the NA plates. Each of the thirteen isolates obtained from the preliminary screening was cross-streaked in triplicate using sterile cotton swab in the previously streaked NA plates. These are incubated for 24h at 30°C ± 2°C.

Morphological and biochemical characterization of isolates

A total of five isolates showed antagonistic activity during the first and second screening stage. These isolates were further characterized through morphological and biochemical assays including citrate utilization, gelatin hydrolysis, hydrogen sulfide production, motility, and triple sugar iron test.

Molecular characterization

The five isolates exhibiting the greatest antagonistic effect were selected for genomic DNA extraction using the Invitrogen™ PureLink™ Genomic DNA Mini Kit following the manufacturer's protocol. The isolates were grown overnight in 10mL nutrient broth and were used for the DNA extraction. Genomic DNA of the isolates were then sent for *16S rRNA* sequencing (Macrogen Inc., Korea).

Data analysis

The colonies counted for the total bacteria and *Vibrio* count after 24 hours of incubation were expressed in colony-forming units (CFU). For the phylogenetic analysis, *16s rRNA* sequences were compared to their close matches from the sequences available in the National Center for Biotechnology GenBank database through BLASTn results. *Salinivibrio costicola* subsp. *costicola* LT722673 was used as outgroup for the phylogenetic tree of *Vibrio* sp. isolates, while *Zobellella* sp. KR063567 was used as outgroup for the *Marinomonas* and *Oceanimonas* isolates. BioEdit **(Hall, 2004)** was used to assemble the sequences, and MAFFT (v.7) (https://mafft.cbrc.jp/alignment/server/) was used for alignment **(Katoh & Standley, 2013)**. To trim the sequences, Trim AI (http://phylemon.bioinfo.cipf.es/) was used **(Capella-Gutierrez *et al.*, 2009)**. Then, Alignment Transformation EnviRonment (ALTER) (https://www.sing-group.org/ALTER/) was utilized to convert the sequences to a PHYLIP file. Then IQTree

was used to construct an evolutionary tree which was later visualized using Figtree (v1.4.4). The tree was annotated in Microsoft PowerPoint 2024.

RESULTS

Screening for anti-*Vibrio harveyi* isolates

The gill-mucus layer of the milkfish was extracted where total bacteria and presumptive *Vibrio* counts were determined. Table (1) shows the results of the count. A total of 245 bacterial isolates were obtained and re-streaked on NA-1% NaCl. From this total number of isolates, 196 bacterial isolates, or 80% exhibited antagonistic activity against *V. harveyi* during the first screening, while 13 isolates were tested against the same bacterial pathogen using the cross-streaking technique during the second screening. Isolates 6, 8, 11, 12, and 13 showed consistent antagonistic activity against the bacterial pathogen in all replicates (Table 2). Isolates 5, 7, and 9 showed antagonistic activity against the bacterial pathogen in one of the replicates, while isolates 1, 2, 3, 4 and 10 did not exhibit any antagonistic activity against *V. harveyi* in all replicates, as shown in Fig. (1).

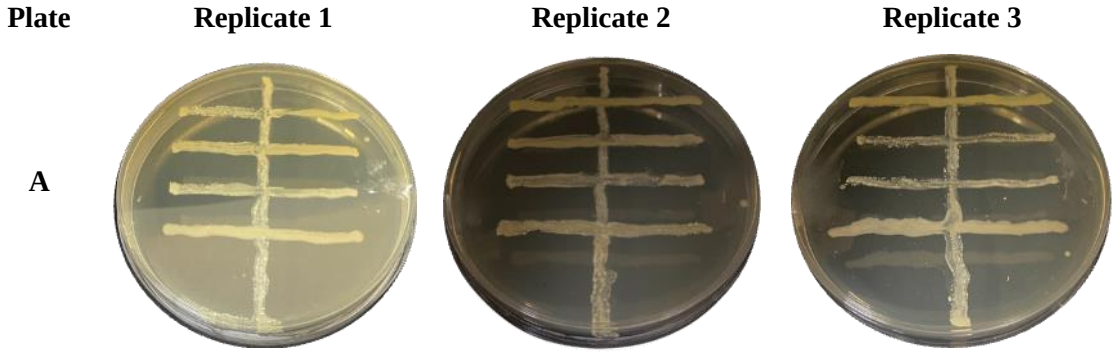
Table 1. Bacterial and presumptive *Vibrio* count of six milkfish samples

Sample	Bacterial count *(CFU/g)*	Presumptive *Vibrio* count *(CFU/g)*
1	3.0×10^{-5}	2.8×10^{-3}
2	4.6×10^{-4}	3.2×10^{-3}
3	8.4×10^{-5}	2.6×10^{-3}
4	4.0×10^{-6}	7.11×10^{-4}
5	6.0×10^{-6}	2.23×10^{-5}
6	3.8×10^{-6}	2.5×10^{-5}

Table 2. Cross-streaking against *Vibrio harveyi*

Isolate	***Replicate 1***	***Replicate 2***	***Replicate 3***
B1	-	-	-
B2	-	-	-
B3	-	-	-
B4	-	-	-
B5	-	+	-
B6	+	+	+
B7	+	-	-
B8	+	+	+
B9	-	+	-
B10	-	-	-
B11	+	+	+
B12	+	+	+
B13	+	+	+

$^{+}$ inhibition; $^{-}$ no inhibition



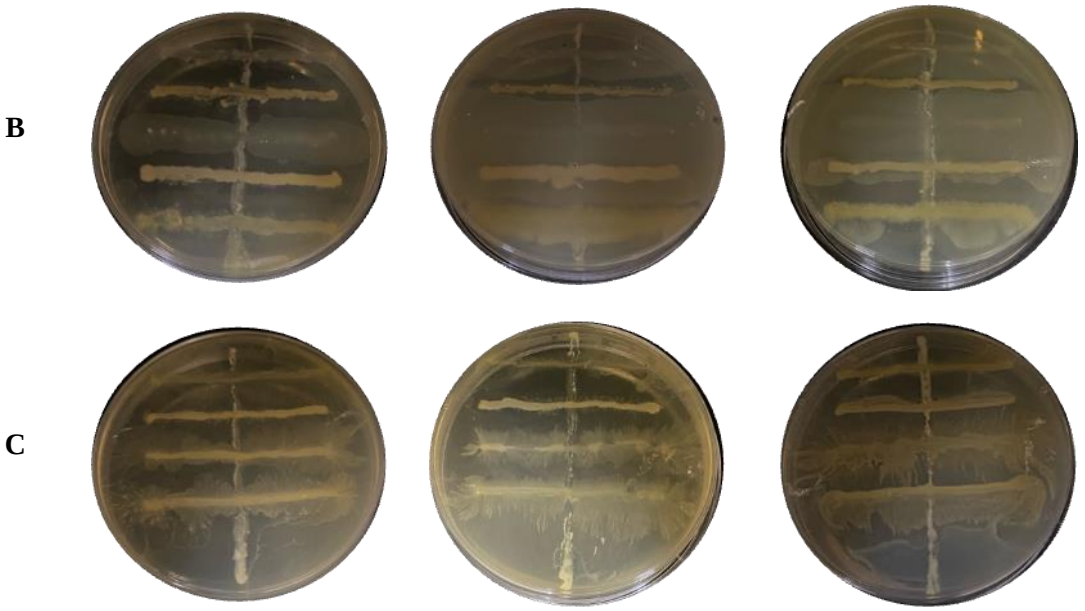


Fig. 1. Cross-streaking in NA plates to determine the antagonistic activity of selected isolates (horizontal streak) against *Vibrio harveyi* PN 9801(vertical streak). **(A)** Isolates 1-4; **(B)** Isolates 5-9; **(C)** Isolates 10-13

Morphological and biochemical characterization

The morphology of the isolates with antagonistic activity against *V. harveyi* was observed by culturing the isolates in NA supplied with 1% of NaCl. Cultural characteristics including shape, elevation, color, and Gram stain were determined. Biochemical identification of isolates was done using various tests for motility, H_2S production, gelatinase, TSI, and citrate utilization.

All obtained isolates were Gram-negative exhibiting creamy to yellow round colonies (Table 3). In addition, all bacterial isolates were motile and tested positive H_2S production, and gelatinase activity. In the citrate utilization test, three isolates tested negative while the other two isolates were positive. For the TSI test, no gas was produced and they exhibited alkaline/acid (K/A) reaction.

Table 3. Morphological and biochemical characteristics of bacterial isolates obtained from gill mucus of cage-cultured milkfish

Isolate	**B6**	**B8**	**B11**	**B12**	**B13**
Form	Round	Round	Round	Round	Round
Color	Yellow	Yellow	Cream yellow	Creamy white	Yellow
Elevation	Flat	Flat	Flat	Convex	Flat
Optic	Shiny	Shiny	Shiny	Shiny	Not shiny
Margin	Entire	Entire	Entire	Entire	Entire
Gram stain	negative	negative	negative	negative	negative
Motility	+	+	+	+	+
H_2S production	+	+	+	+	+
Gelatinase	+	+	+	+	+
TSI	K/A; no gas	K/A; no gas	K/A; no gas	K/A; no gas	K/A; no gas
Citrate	-	-	-	+	+

$^{+}$ positive result for biochemical test

$^{-}$ negative result for biochemical test

^{K}/A alkaline slant, acid butt

Molecular characterization

To identify the species further, *16S rRNA* sequencing was done on the five isolates. The identities of the five isolates are shown in Table (4). Phylogenetic analyses

showed that three of the isolates are clustered with the genus *Vibrio* – *Vibrio alginolyticus* and *Vibrio neocaledonicus* (Fig. 2), while the other two isolates are clustered with *Oceanimonas* sp. and *Marinomonas* sp. (Fig. 3), all of which are Gram-negative microorganisms.

Table 4. Molecular identities of bacterial isolates obtained from gill mucus of cage-cultured milkfish

Isolate Code	*Scientific Name of Closest Species*	*Query Coverage (%)*	*Percent Identity (%)*	*Accession Number*
B6	*Vibrio alginolyticus* strain CAPL-B-VA1	84%	96%	KX904708.1
B8	*Vibrio alginolyticus* strain K09K1	94%	99%	CP017919.1
B11	*Vibrio neocaledonicus* strain NC470	86%	99%	KT989844.1
B12	*Oceanimonas* sp. 33	98%	93%	DQ386136.1
B13	*Marinomonas* sp. RP-2F	80%	76%	GU592212.1

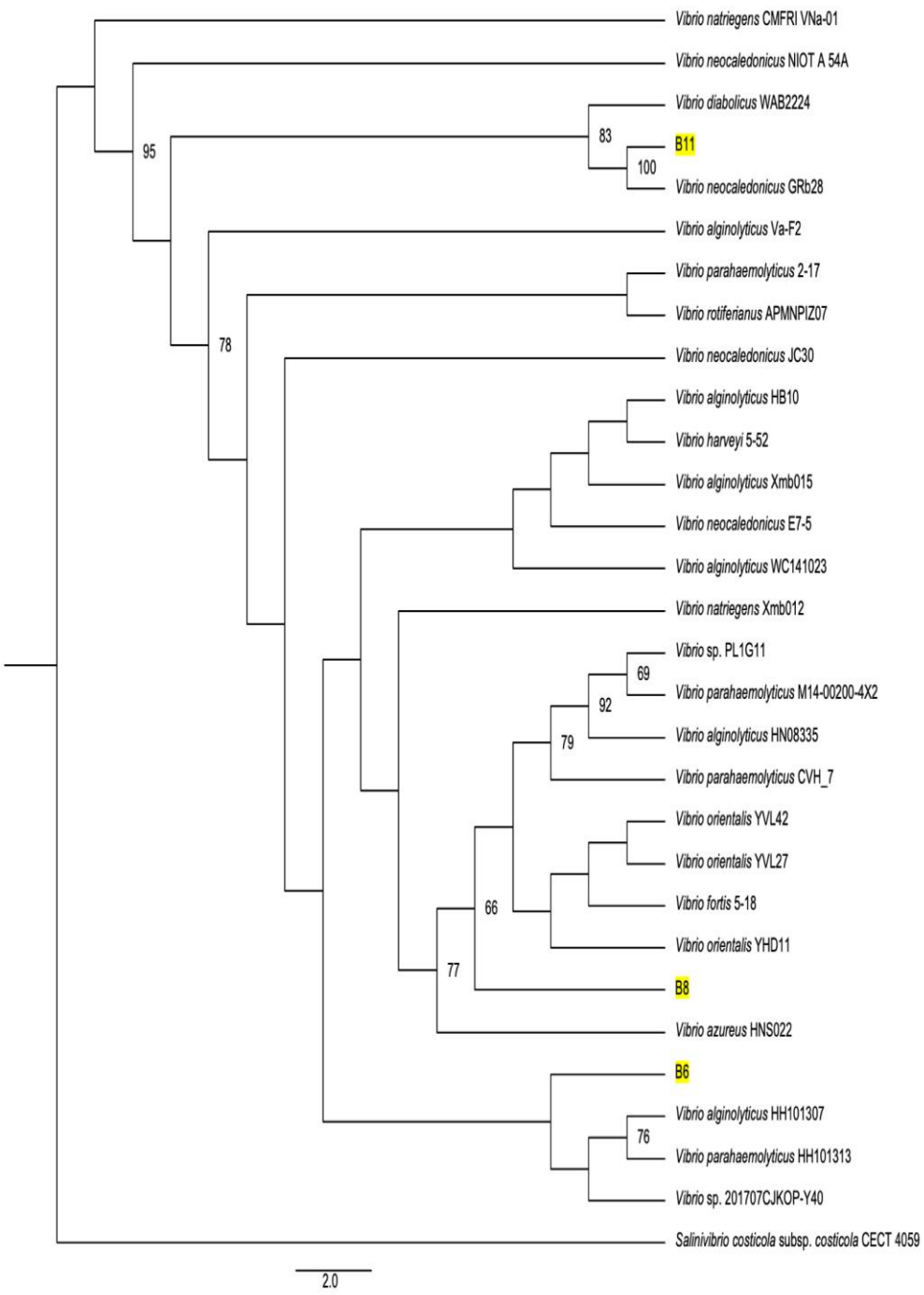


Fig. 2. Phylogenetic relationship of *Vibrio* isolates from milkfish gill mucus with 26 *Vibrio* spp. based on *16s rRNA* partial sequence reads

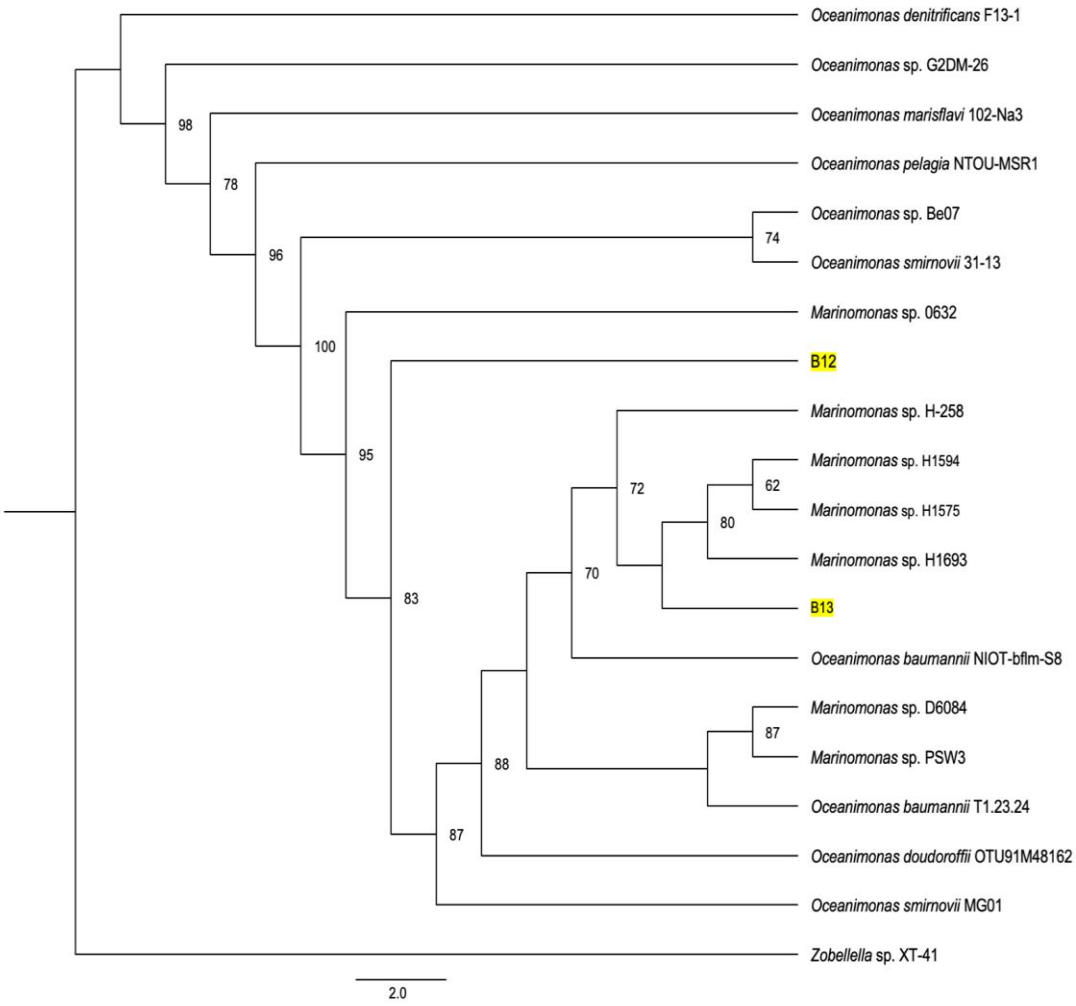


Fig. 3. Phylogenetic relationship of putative *Oceanimonas* sp. and *Marinomonas* sp. isolates from milkfish gill mucus with 17 previously reported isolates based on *16s rRNA* partial sequence reads

DISCUSSION

Fish are prone to diseases caused by pathogens since they directly interact with aquatic environments. To protect them from these pathogens, they have complex defense mechanisms, and at the forefront is the epidermal mucus, which acts as a stable physical and chemical barrier and enables the exchange of gametes, gases, hormones, nutrients, odorants, and water **(Wang *et al*., 2011; Esteban, 2012; Dash *et al*., 2018)**. Furthermore, these mucosal surfaces are colonized by commensal microorganisms that serve as

biological reinforcement against pathogens **(Lazado & Caipang, 2014)**. In a study by **Ringø and Holzapfe (2000)**, it was found that the gills of the Atlantic salmon have *Carnobacterium piscicola*-like bacteria that inhibited the growth of *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio anguillarum* and *Vibrio salmonicida.* Hence, it can be inferred that gill mucus can be a potential source of probiotics.

In this study, the first screening – spot-on-lawn assay, showed that 80% of the isolates exhibited antagonistic activity against the selected pathogen, *V. harveyi*. For the second screening, 13 isolates were cross-streaked against the same pathogen and 5 isolates showed consistent antagonistic activity. This activity can be attributed to various means, such as competition, production of inhibitory substances, and competitive exclusion **(De *et al.*, 2014; Zorriehzahra *et al.*, 2016; El-Saadony *et al.*, 2021)**.

The remaining 5 isolates were subjected to morphological, biochemical, and molecular analysis. The first and second isolates were determined to be associated with *V. alginolyticus.* It tested positive in motility, and gelatin hydrolysis, and negative in gas production following literature **(Zhou *et al.*, 2021)**. The third isolate was closely related to *V. neocaledonicus*, with positive tests for citrate and gelatinase, which can also be observed in this article **(Geng *et al.*, 2022)**. The fourth isolate was identified as putative *Marinomonas* sp. Various species-level literature showed that they have a positive result for motility and variable reactions on their gelatinase reaction **(Ivanova *et al.*, 2005; Lucas-Elio *et al.*, 2005; Yu *et al.*, 2020; Butt *et al.*, 2024)**. Similar to *Marinomonas* sp., the fifth isolate, closely linked to *Oceanimonas* sp., also has species-specific literature for biochemical and morphological tests. A study by **Yang *et al.* (2024)** isolated a member of the *Oceanimonas* genus (*Oceanimonas pelagia*) from marine sediment on the coast of Taiwan and their findings showed positive results in motility and variable results for gelatin hydrolysis.

Although Gram-positive bacteria, such as LAB, are mainly used as probiotics, various studies showed that Gram-negative bacteria also show potential as probiotics. A study by **Kescarcodi-Watson *et al.* (2012)** tested four strains of bacteria - *Alteromonas macleodii* 0444, *Neptunomonas* sp. 0536, *Phaeobacter gallaeciensis, Pseudoaltermonas* sp. D41 against pathogens through a pathogen-challenge bioassay for mollusk larvae. Their results showed that these strains provided the larvae with protection against their respective pathogens. *Vibrio* species were also tested for their probiotic potential in a study by **Thompson *et al.* (2010)**. The study determined that *Vibrio gazogenes* were able to cause a decline in *Vibrio*-like bacteria situated in the gut of the shrimp implying that this species can control diseases caused by bacteria in shrimp.

Marine organisms such as those belonging to family *Vibrionaceae* are known for their pathogenicity – *V. parahaemolyticus, V. cholerae,* and *V. vulnificus.* However, some species under this family are capable of producing bioactive compounds including those with anticancer and antiviral activities **(Mansson *et al.*, 2011)**. *V. neocaledonicus* was observed to have an inhibiting effect on metal corrosion and was further studied for the

determination of inhibitory enzymes that they produce. In a study by **Gómez-Betancur *et al.* (2019)**, they observed that one of the major compounds was indole which was responsible for the inhibition of acetylcholinesterase (AChE) and alpha-glucosidase (AG). A strain of *V. alginolyticus* was identified for its potential as probiotics against a *V. parahaemolyticus,* a shrimp-pathogenic bacterium in a study by **Balcázar *et al.* (2007)**. They evaluated the potential of the isolate through supplemental feeding of the probiotic to the shrimp for 28 days. These shrimps were then infected with *V. parahaemolyticus* to determine whether the strain could protect the organism from vibriosis. Their results showed that the mortality of probiotic-treated shrimp ranged from 17 to 22% while those untreated shrimps had a 33% mortality **(Balcázar *et al*., 2007)**.

Studies on *Oceanimonas* sp. and *Marinomonas* sp. regarding their probiotic potential remain scarce. Current studies explore the potential of these microorganisms for industrial use. For example, a study by **Yoo and Park (2015)** noted that a species from the genus *Marinomonas* is capable of producing serine protease which can be produced and used for biological fermentation. Additionally, a study on biodegradation studied *Oceanimonas* sp. and its ability to treat phenolic wastewater. This was explored by **Tan *et al.* (2016)** by adding immobilized cells to synthetic wastewater with phenol. Their results showed that the cells were able to treat up to 1500mg/ l of phenol in saline wastewater.

CONCLUSION

This study addressed the gill mucus of the milkfish to explore the probiotic potential of their microbial community. Two hundred forty-five (245) isolates were collected and tested against a pathogen *Vibrio harveyi*, a pathogen of the milkfish that causes fin rot, through spot-on-lawn assay and cross-streaking. Two percent, or five isolates exhibited antagonistic activity against the pathogen which underwent molecular characterization *16S rRNA* sequencing, and three of these isolates were determined to have a close relation to *Vibrio alginolyticus* and *Vibrio neocaledonicus*. The other two isolates were closely related to *Oceanimonas* sp. and *Marinomonas* sp. The results show the potential of these isolates for further study on their probiotic potential, especially the *Oceanimonas* sp. and *Marinomonas* sp. which are understudied in probiotic research.

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