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Original Paper

Local aquatic microflora as a potential source of probionts in biofloc technology for whiteleg shrimp, *Penaeus vannamei*

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Abstract

The Philippine shrimp aquaculture industry, a key supplier of *Penaeus vannamei* to domestic and international markets, faces significant disease challenges, particularly from pathogenic *Vibrio* species. Current disease management strategies often employ terrestrially-sourced probiotics, whose efficacy can be limited by environmental incompatibility with the aquatic host. This study aimed to address this limitation by isolating and characterizing putative probiotic microorganisms from the local aquatic environment of *P. vannamei*. Water samples, collected from a shrimp farm outlet pond in Negros Occidental, Philippines, were enriched with brown sugar to promote biofloc formation. Bacterial colonies were isolated on Nutrient Agar supplemented with 1% NaCl. *In vitro* antagonistic activity against the shrimp pathogen *Vibrio harveyi* was assessed via spot-on-lawn and cross-streak assays. The spot-on-lawn assay revealed a 45% inhibition rate against *V. harveyi*. Subsequent cross-streak assays confirmed inhibitory effects in five of nine isolates. These five isolates underwent morphological, biochemical, and molecular characterization using 16S rRNA gene sequencing. Three isolates were identified as putative *Vibrio alginolyticus*, while the remaining two closely matched *Pseudoalteromonas* species. Given the established use of non-pathogenic *Vibrio* and *Pseudoalteromonas* species as probiotics in aquaculture, these findings highlight the potential of local microflora as a source of probionts for biofloc-based shrimp culture. This approach may reduce reliance on external probiotic sources, contributing to enhanced industry sustainability.

Key words: aquaculture, C:N ratio, *Pseudoalteromonas*, *Vibrio*, water quality.
Abbreviations: BFT, biofloc technology; NA, nutrient agar.

Introduction

The shrimp culture industry in the Philippines has a significant impact on both the local and national economies due to its substantial contribution by means of exports to many international markets, including South Korea, Japan, the United States, and other countries (Vergel 2017; Clapano et al. 2022). *Penaeus vannamei* Boone is recognized as a highly favored shrimp species for aquaculture purposes. This particular species has gained significant recognition in tropical regions because of its favorable attributes, including its relatively short cultivation time and rapid rate of development (Rosario, Lopez 2005). Moreover, the cultivation of *P. vannamei* in the Philippines is regarded as a strategic approach aimed at increasing shrimp farming output both nationally and internationally (Vergel et al. 2019). The production of this commodity has shown significant growth in recent years due to its substantial economic profitability, but it has also been adversely affected by several illnesses (Shinn et al. 2018).

Vibrio species are a diverse group of Gram-negative, comma-shaped bacteria that reside in many aquatic settings, including freshwater, estuarine, and marine habitats. This group of species has been identified as a causative agent of mass mortality in shrimp hatcheries and is often seen in ponds used for intensive shrimp production (De Schryver et al. 2014). Various stressors, such as elevated stock density and suboptimal water quality, contribute to the occurrence of infections caused by opportunistic and pathogenic microorganisms (Aguilera-Rivera et al. 2019). Specifically, *Vibrio harveyi, Vibrio owensii, Vibrio penaecida*, and *Vibrio parahaemolyticus* are known to be pathogenic to crustaceans (Karunasagar 2018). Furthermore, a prevailing health concern in Asia pertains to the occurrence of acute hepatopancreatic necrosis disease , which is attributed to the presence of pathogenic strains of *Vibrio parahaemolyticus* (Zorriehzahra, Banaederakhshan, 2015). The disease has been shown to have a significant detrimental effect on shrimp production and is now documented in a minimum of eight Asian territories, including the Philippines (Shinn et al. 2018).

Given the limited availability of land and water resources, the long-term viability of the shrimp culture industry is expected to hinge upon the enhancement of production settings, the augmentation of productivity, the

advancement of aquaculture technology, and the reduction of production costs. The use of biofloc technology (BFT) has been proposed as an ecologically sustainable approach to aquaculture. The BFT system integrates the process of nutrient removal from water with the simultaneous creation of microbial biomass (Caipang et al. 2022). The microbial biomass generated has the potential to serve as a supplementary nutritional resource for shrimp aquaculture (Krummenauer et al. 2011). Additional cultural factors, such as the inclusion of a carbon source to maintain a suitable carbon-to-nitrogen ratio, the exchange of water, and the provision of rearing substrates, are also crucial for achieving optimum production of shrimp and prawn in biofloc systems (El-Sayed 2020).

Biofloc has the potential to function as probiotics, therefore enhancing immune systems, mitigating illness outbreaks, and stimulating digestive enzyme functions (El-Sayed 2020). It has been shown that probiotics created locally or manufactured in a laboratory exhibit superiority over commercial probiotics due to their precise isolation from the target host and equivalent outcomes (Sohel et al. 2023). In contrast, the viability and efficiency of commercially available probiotics, which replace the originally given probiotic bacteria derived from the gastrointestinal tract of the host species, exhibit variability based on the specific strains and manufacturers involved (Sohel et al 2023). Despite the presence of several imported probiotic medicines available on the market, there is a lack of scientific evidence pertaining to their efficacy.

The use of exogenous bacteria from the surrounding environment as probiotics has been a prominent subject of investigation in recent years (Lazado, Caipang, 2014; Jamal et al. 2019). There is a lack of clear evidence suggesting that potential probiotics obtained from the surrounding environment exhibit superior performance compared to bacteria of terrestrial origin or those coming from varying environments (Lazado, Caipang 2014). Nevertheless, it is an undeniable reality that microbes exhibit optimal performance within their native habitats (Ninawe, Selvin 2009). Minimizing the problems related to physicochemical and biological needs that impact probiotic characteristics may be achieved by isolating the strain from the environment in which it will be later delivered (Lazado, Caipang 2014).

The goal of this study was to conduct a screening process to identify potential probiotic candidates from the aquatic microorganisms present in the local aquatic microflora of a shrimp farm to enhance the biofloc technology utilized in the culture of whiteleg shrimp, *Penaeus vannamei*. Additionally, the study aimed to classify these potential probiotic candidates through morphological, biochemical and molecular assays. After the identification and characterization of these probiotic strains, the study further assessed the *in vitro* antagonistic activity of the selected probiotic candidates against a bacterial pathogen in shrimp.

Materials and methods

Collection of water samples and isolation of potential probionts

The aquatic samples were collected from one of the inlet ponds of whiteleg shrimp at a commercial semi-intensive shrimp farm (10°50'7.332" N, 122°58'5.9628" E) located in Negros Occidental, Philippines. The water sample was placed into two 2-L bottles and filled up to 80%. The bottle cap of each bottle was punctured where the hose connected to a portable aerator was inserted to ensure that the microorganisms were suspended at the surface. For the production of biofloc, brown sugar was added to the two bottles at a ratio of one teaspoon brown sugar to every liter of water sample. The samples were transported immediately to the Microbiology Laboratory of the National Institute of Molecular Biology and Biotechnology of the University of the Philippines Visayas, Miagao, Iloilo and provided with vigorous aeration for 5 days to stimulate biofloc production.

After biofloc maintenance with no water exchange, a ten-fold serial dilution of the treated water sample was prepared and 100 µL from each dilution was spread plated onto Nutrient Agar (NA) plates with 1% NaCl in three replicates for each dilution. The plates were incubated at 28 °C for 24 h. To minimize sampling and counting errors (Ben-David, Davidson 2014), a viable range of 25 to 200 colonies were set. The distinct colonies were re-streaked onto NA-1% NaCl plates and incubated at 28 °C for another 24 h. The isolated colonies were stored at 8 °C for subsequent assays.

Spot-on-lawn and cross-streak assays

An overnight culture of *Vibrio harveyi* PN 9801 (de la Peña et al. 2001) in Nutrient Broth with 1% NaCl was prepared, adjusted to a concentration of 1×10^3 CFU mL^{-1}, plated onto NA-1% NaCl agar plates, and incubated for 1 h at 28 °C. This was followed with a spot-on-lawn microbial assay using the isolated bacteria from the brown sugar-treated water following the procedures of Caipang et al. (2023). The plates were incubated at 28 °C for 24 h, and the zones of inhibition were recorded. The isolates were stored at 8 °C until the screening of their antagonistic activities against the *V. harveyi* using a cross-streak assay.

For the cross-streak assay, a broth culture of the *Vibrio* pathogen was prepared 24 h prior to the assay. In NA plates supplemented with 1% NaCl, a loopful of the bacterial pathogen was streaked in a vertical line at the center of the agar plate. The pathogen was allowed to adhere to the medium for 1 h. The test isolates were then streaked perpendicularly with the pathogen, forming a right angle. The plates were incubated at 28 °C for 24 to 48 h and observed for antagonistic effect. The assay was done in triplicate.

Morphological and biochemical characterization

Bacterial isolates were subjected to Gram staining, motility and morphology using light microscopy following the procedures described by Nguyen et al. (2007) and Kopermsub and Yunchalard (2010). Biochemical tests including catalase activity, hydrolysis of casein, starch, lipid, and gelatin were conducted following methods described in the Bergey's Manual of Systematic Bacteriology (Holt et al. 2000).

Molecular characterization

The isolates were subjected to molecular identification by extracting bacterial genomic DNA from an overnight culture of the isolates in 5 mL Nutrient Broth supplemented with 1% NaCl in accordance with the manufacturer's instructions using a commercial kit (Purelink Genomic DNA Mini, Thermo Fisher Scientific, California, USA). The 16S rRNA was amplified via polymerase chain reaction (PCR) in a 25 µL PCR reaction using the eubacterial universal primers (forward: GAGAGTTTGATCCTGGCTCAG; reverse: CTACGGCTACCTTGTTACGA) (Bianciotto et al. 2003). For the amplification, the PCR reactions and amplification conditions described by Caipang et al. (2010) were utilized. The PCR products were cleaned and sequenced (Macrogen, Korea). The obtained sequences were aligned and analyzed using data from the NCBI GenBank (blast.ncbi.nlm.nih.gov) in order to identify the closest match of bacterial isolates. MEGA 7.0 (Tamura et al. 2013) was used for the alignment of DNA sequences together with the reference sequences in the construction of phylogenetic trees. Identification of the species was inferred using the neighbor-joining method with 1000 bootstrap replications (Tamura et al. 2013).

Results

Spot-on lawn assay

In a series of spot-on-lawn assays to determine the inhibitory effects of various isolates against *Vibrio harveyi*, a total of 135 colonies or 45% out of 300 bacterial colonies exhibited antagonistic activity against *V. harveyi* (Table 1).

Table 1. Spot-on lawn assay of three hundred bacterial isolates

Test plate No.	Total No. of tested colonies	No. of colonies that tested positive	Percentage (%)
1	30	30	100.0
2	30	22	73.3
3	30	6	20.0
4	30	16	53.3
5	30	15	50.0
6	30	7	23.3
7	30	5	16.7
8	30	20	66.7
9	30	8	26.7
10	30	6	20.0
Total	300	135	45.0

Table 2. Cross streak assay of the five bacterial isolates against *V. harveyi*. (+), the isolate inhibited the growth of the *V. harveyi*; (N), *V. harveyi* inhibited the growth of the bacterial isolate; (n), *V. harveyi* and bacterial isolate co-existed

Isolate No.	24 h	48 h
FASN-A1	+	+
FASN-A2	N	+
FASN-A3	N	+
FASN-A4	+	+
FASN-A5	N	n

Cross streak assay

From the 135 isolates that tested positive in the spot-on-lawn assay, five isolates were selected as strong antagonists against *V. harveyi* and were further subjected to the cross-streak assay. Results are shown in Table 2. Isolates FASN-A1 and FASN-A4 inhibited the pathogen at 24 and 48 h of incubation. Isolates FASN-A2 and FASN-A3, showed antagonistic effects against *V. harveyi* at 48 h. Lastly, FASN-A5 showed negligible inhibition of *V. harveyi*.

Morphological characteristics of the putative probionts

Table 3 shows the morphological characteristics of the five bacterial isolates with antagonistic activities against

Table 3. Morphological characteristics of the potential probionts

Characteristic	FASN-A1	FASN-A2	FASN-A3	FASN-A4	FASN-A5
Motility	+	+	+	+	+
Gram-stain	–	–	–	–	–
Cell shape	bacillus; curved	bacillus	bacillus; curved	bacillus; curved	bacillus
Arrangement	single	single	single	single	single
Color	white	white	white	white	yellow
Margin	smooth	smooth	smooth	smooth	smooth
Elevation	convex	convex	convex	convex	slightly raised
Shape	round	round	round	round	round
Texture	moist	moist	moist	moist	moist

Table 4. Biochemical characterization of the potential probionts

Enzyme	FASN-A1	FASN-A2	FASN-A3	FASN-A4	FASN-A5
Catalase	+	+	+	+	+
Gelatinase	+	+	+	+	–
Protease	+	+	+	+	–
Amylase	+	–	–	–	+
Lipase	–	–	–	–	–

V. harveyi. All bacterial isolates were Gram-negative, rod-shaped, motile, and had singular cell arrangement. The colony colour was mostly white, except for FASN-A5, which was yellowish in colour.

Biochemical characterization

Table 4 shows the biochemical characteristics of the bacterial isolates. Catalase activity was detected in all isolates. Gelatinase and protease activities were observed in all isolates except in FASN-A5. Amylase activity was detected in isolates FASN-A1 and FASN-A5. No lipase activity was observed in the isolates.

Molecular characterization

Isolate FASN-A1 had 98.32% similarity with *Vibrio alginolyticus*, while FASN-A3 and FASN-A4 demonstrated high similarities of 99.48 and 98.97% to the same species, respectively, suggesting a strong genetic connection (Table 5). On the other hand, FASN-A2 was closely related to *Pseudoalteromonas mariniglutinosa* with 96.93% identity, and FASN-A5 shared 98.94% identity with both *Pseudoalteromonas piscicida* and *Pseudoalteromonas flavipulchra*. Fig. 1 shows the phylogenetic tree of *Vibrio* species with *Bacillus aquaemaris* strain TF-12 as the outgroup. The tree categorized isolates FASN-A1, FASN-A3, and FASN-A4 into distinct groups based on their genetic similarities, supported by a bootstrap analysis of 1000 replicates. FASN-A1 clustered with *Vibrio alginolyticus* strains and *Vibrio campbellii*, showing a close genetic relationship. FASN-A3 formed a clade with *Vibrio alginolyticus* strains and *Vibrio azureus*, while FASN-A4 clustered with *Vibrio alginolyticus* strains and *Vibrio orientalis*. Fig. 2 shows the phylogenetic tree of *Pseudoalteromonas* species with *Bacillus hwajinpoensis* strain CCMM B646 as the outgroup, showing FASN-A2 clustered with *Pseudoalteromonas lipolytica* and *Pseudoalteromonas nigrifaciens*, while FASN-A5 grouped with *Pseudoalteromonas flavipulchra* and *Pseudoalteromonas piscicida*.

Discussion

Inhibition assays

The isolated bacterial colonies exhibited varying degrees of inhibitory effects against *V. harveyi*, some of which did not result in visible clear zones or inhibition. It was found that complex interactions involving biofilm-related toxins and enzymes allow bacteria to compete for space and nutrients, revealing mechanisms of competition not visible in traditional assays (Kobayashi, Ikemoto 2019). It was demonstrated that extracellular enzymes, such as proteases, facilitated toxin movement through biofilm layers, showcasing sophisticated intercellular competition. Certain bacterial species, such as *Corynebacterium propinquum*, could hinder the growth of Staphylococcus epidermidis by producing siderophores, which are compounds that bind to iron (Stubbendieck et al. 2019). This type of competition, which involves sequestering nutrients, is likely similar to how the bacterial isolates outcompeted *V. harveyi* without an instant observable inhibition, ultimately improving its survival within the microbial community over time. Furthermore, the significance of taking into account the competitive interactions between the potential probiotic candidates and the bacterial pathogen was emphasized, as conventional approaches might underestimate these because of the suppressing impact of competition (Neves et al. 2020).

Potential probiotic strains could compete for resources, eliminate harmful substances, and inhibit pathogen growth (Verschuere et al. 1999). Similarly, the present study found that the bacterial isolates showed varying antagonistic interactions with *V. harveyi*, from initial coexistence and later inhibition of the bacterial pathogen, which likely suggests that the interactions might involve

Table 5. Closest match and percent identity of the bacterial isolates

Isolate No.	Closest match	Percent identity (%)	Accession number
FASN-A1	*Vibrio alginolyticus* strain 5-50	98.32	MW080024
FASN-A2	*Pseudoalteromonas mariniglutinosa* strain DSW7	96.93	HM055757
FASN-A3	*Vibrio alginolyticus* strain CAPL-B-VA1	99.48	KX904708
FASN-A4	*Vibrio alginolyticus* strain 4-14	98.97	MN938360
FASN-A5	*Pseudoalteromonas flavipulchra* strain TBZ-S12	98.94	MK480664

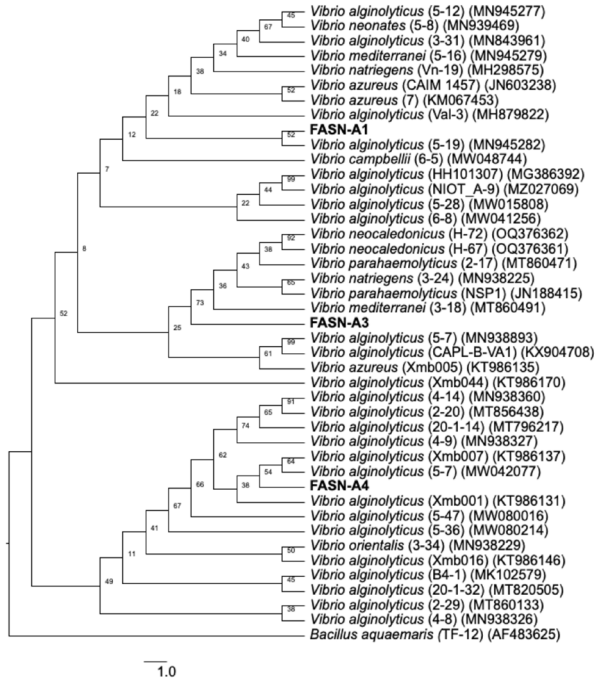

Fig. 1. Phylogenetic tree of *Vibrio* species. The relationship of *Vibrio* spp. with FASN-A1, FASN-A3, and FASN-A4 using *Bacillus aquaemaris* strain TF-12 as the outgroup.

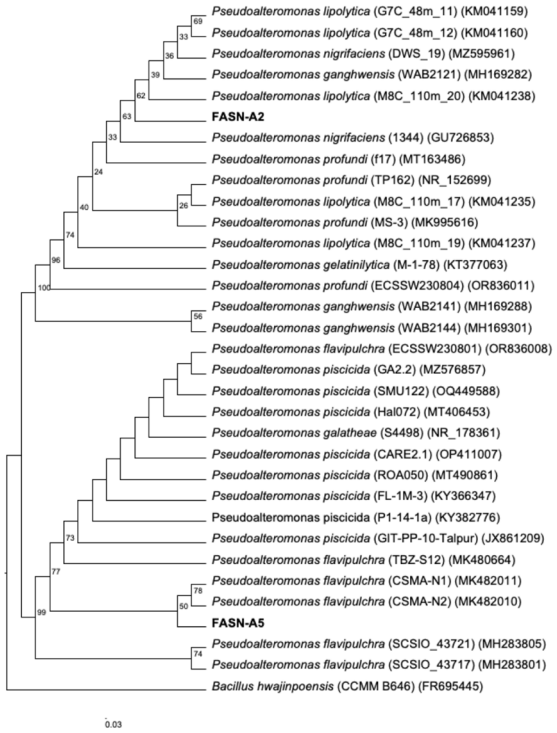

Fig. 2. Phylogenetic tree of *Pseudoalteromonas* species. The relationship of *Pseudoalteromonas* spp. with FASN-A2, and FASN-A5 using *Bacillus hwajinpoensis* strain CCMM B646 as the outgroup.

resource competition over time rather than immediate harmful effects. In another study, no inhibitory effects of the bacterial pathogen in cross-streak tests were observed, suggesting that competition for nutrients and space, rather than direct antagonistic interactions, was the primary control mechanism exerted by the probiotic candidates (Siladan et al. 2013). It is possible that there was a delayed production of inhibitory compounds or there was a gradual improvement in the competitive capability for essential resources.

Morphological and biochemical characteristics of the putative probionts

The consistent motility and Gram-negative features of the bacterial isolates obtained in this study were in line with results from other research on probiotic bacteria. Motility is often linked to competition for space, particularly in the gut of the host, thereby improving the probiotic capabilities of these bacteria (Chauhan, Singh 2018; Soltani et al. 2019; Adel, Dawood 2021). The Gram-negative characteristics and rod-shaped or curved cell structures seen in the strains are common attributes of many probiotics used in aquaculture (Hai 2015). One notable difference was the yellowish colour of one of the bacterial isolates (FASN-A5). This variation in colony colour could be a result of diverse pigment production, a trait that is observed in some *Pseudoalteromonas* strains that produce bioactive substances, which have crucial roles in preventing fouling in aquaculture systems (Huang et al. 2011).

Probiotics that produce catalase are important in improving the resistance to oxidative stress in aquatic organisms, thus leading to improved health and survival rates (Hoseinifar et al. 2018; Sharma et al. 2019). The existence of gelatinase and protease functions in the majority of isolates suggested a noteworthy ability for breaking down proteins, which was advantageous for absorbing nutrients and digesting food (Van Spaendonk et al. 2017; García-Márquez et al. 2023). Studies showed that the presence of protease in probionts could enhance the digestion efficiency and uptake of nutrients in aquatic organisms, resulting in improved growth performance and overall health (Lazado, Caipang 2011; Anee et al. 2021). Moreover, the presence of amylase activity in some of the isolates is crucial in breaking down starch (Usakova Na et al. 2015; Amenyogbe et al. 2024), which could notably enhance the growth and feeding efficiency of fish and shrimp (Mardani et al. 2018; Sumon et al. 2018; Amenyogbe et al. 2024).

Molecular characterization

The majority of the probiotic candidates were closely related to *V. alginolyticus*; thus, they hold great potential as probiotics in aquaculture. *V. alginolyticus* can effectively combat diseases caused by *Aeromonas salmonicida, V.*

anguillarum, and *V. ordalii* (Austin et al. (1995). The study highlighted how *V. alginolyticus* could create antibacterial substances that hinder these pathogens; thus, lowering fish mortality rates (Austin et al. 1995). The same study showed that *V. alginolyticus* produced enzymes including proteases, which helped in inhibiting fish pathogens. These results align with the observed biochemical activities of the isolates, specifically protease production, which could be essential for nutrient absorption and digestion. It was studied how *V. alginolyticus* could help manage bacterial infections in Pacific whiteleg shrimp and it was shown that *V. alginolyticus* had a positive impact by inhibiting harmful *Vibrio* species, which led to lower shrimp mortality rates and a better immune system (Thompson et al. 2010). Furthermore, a specific strain of *V. alginolyticus* was isolated that inhibited pathogenic bacteria in shrimp farms, leading to a decrease in disease occurrence and contributing to the improved health and growth of the shrimp (Gomez-Gil et al. 2002).

In another study, *Pseudoalteromonas* species enhanced the survival rates of *P. vannamei* following infection with *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (Wang et al. 2018). In their study, some strains of *Pseudoalteromonas* possessed potent antagonistic effects against *Vibrio parahaemolyticus* through the production of antibacterial compounds. Other studies also reinforced the probiotic effects of *Pseudoalteromonas* species against *Vibrio* spp. and *Bacillus* spp. (Wang et al. 2021; Eze et al. 2023; Gustilatov et al. 2024). The probiotic actions are likely due to the formation of biofilms or the production of antibacterial substances that combatt the growth of the bacterial pathogen (Wang et al. 2021).

Conclusions

Taken together, the study demonstrated that enrichment of water samples obtained from a commercial semi-intensive shrimp farm, with brown sugar, enabled the isolation of potential probiotic isolates, found thorough morphological, biochemical, and molecular analysis to belong to *V. alginolyticus* and *Pseudoalteromonas* species. These bacterial isolates displayed significant inhibitory effects against *V. harveyi*, confirming their effectiveness as putative probionts. Furthermore, the biochemical activities of the isolates showed promising characteristics as probiotics as they produce enzymes that assist in the digestion of the host. The study emphasizes the importance of using environmental and host-associated probiotics, which can offer more tailored solutions compared to terrestrial probiotics. By successfully identifying and characterizing these local strains, the study underscores the benefits of utilizing native microbial communities for sustainable shrimp aquaculture. Future studies shall focus on the beneficial effects of these probiotic candidates in the host through feeding experiments and subsequent challenge with bacterial pathogens.

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