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A Comparative Study of Antibiotics and Probiotics against Pathogens Isolated from Coastal Shrimp Aquaculture System

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Abstract

Bacterial diseases are increasing at an alarming rate in the shrimp aquaculture production systems. To control microbial diseases, a number of antimicrobial agents including antibiotics are used in shrimp farms which led to problems such as antibiotic resistance. Therefore, the use of natural bacterial isolates or probiotics as an alternative method for the control of pathogenic bacterial strains is gaining popularity. In this study, seven shrimp pathogens were isolated from a coastal shrimp aquaculture system. Then, some common antibiotics and commercially available probiotics such as *Bacillus*. spp, *Pediococcus*. spp. were applied against the pathogens. For antibiotics, the disc diffusion method was used, whereas the well diffusion method was used for probiotics. Two common pathogens of shrimp hatcheries, namely *V. parahaemolyticus* and *V. vulnificus* showed resistance against antibiotic cephalosporin and streptomycin. On the other hand, both probiotic bacteria exhibited good results against all the pathogens including *V. parahaemolyticus* and *V. vulnificus* except probiotic *Bacillus* spp against *Bacillus fastidiosus.* These results demonstrated that the use of probiotic bacteria within the shrimp aquaculture could be a good solution for decreasing pathogenic microorganisms and reducing the antibiotic resistance problem in shrimp hatcheries.

Keywords: Antibiotic resistance, Probiotics, Shrimp pathogens.

1. Introduction

The aquaculture industry is considered as one of the major contributors to global food production. The growth of the aquaculture industry is hampered by unpredictable mortalities, many of which are caused by pathogenic microorganisms. Bacterial diseases have been attributed to biological production bottlenecks in intensive aquaculture, hence necessitating the use of chemicals such as drugs and antibiotics in health management strategies (Newaj-Fyzul *et al*., 2015). The application of antibiotics had been an effective strategy only at the beginning, but the residuals remaining in the rearing environment exert selective pressures for long periods of time, and this has become a big challenge for health management (Lakshmi *et al*., 2013). The indiscriminate use of antibiotics resulted in the emergence of antibiotic-resistant bacteria in aquaculture environments, the increase of antibiotic resistance in fish pathogens, transfer of these resistance determinants to the bacteria of land animals and to human pathogens, and in alterations of the bacterial flora both in sediments and in the water column (Verschuere *et al.*, 2000). An alternative method for controlling pathogenic bacterial strains in shrimp cultures could be the supplementation with pure cultures of natural bacterial isolates (biocontrol or use of probiotics) which might produce chemical substances inhibiting the growth of pathogens. The approach basically employs the activity of microorganism that could suppress or inhibit the growth of *V. harveyi*without causing a bad impact on the equilibrium system in a particular microbial community. (Ohira *et al.*, 1996). This research is an attempt to present a comparative study of the efficacy of conventional antibiotics and probiotics against some pathogens isolated from coastal shrimp aquaculture systems.

2. Materials and Methods

2.1. Sample Collection

Water, soil, raw water, treated water, and water from post-larva culture were collected from a total of seven shrimp hatcheries and grow-out ponds of Cox's Bazar, Bangladesh. The samples were taken in sterile containers, and were immediately transferred to the laboratory.

2.2. Enumeration and Isolation of Bacteria

A Nutrient agar medium and a Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar medium were used for the enumeration of bacteria. Serial dilution up to 10^6, pour plate and spread plate (Sanders *et al*., 2012) methods were applied for the total count. The inoculated media were incubated at 37^0C for twenty-four to forty-eight hours. After incubation, the plates having well-spaced colonies

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were placed on a colony counter (Stuart Scientific U K). The colonies were counted and calculated by multiplying the average number of colonies per plate by reciprocal of the dilution factor. The calculated results were expressed as colony forming units (CFU) per mL of the sample. The colonies were selected for isolation on the basis of colony morphology including elevation, margin, and surface. The colonies were then transferred to nutrient agar slants and purified through the streak plate method. The pure cultures of the isolates were coded and kept in polythene bags and preserved as a stock culture in the refrigerator at 4^0C for further study.

2.3. Identification of Selected Isolates

The selected isolates were subjected to biochemical tests, and the results were compared with the standard descriptions given in "Bergey's Manual of Determinative Bacteriology", 8th ed. (Buchanon and Gibson 1974) and 9th ed. (Halt *et al*., 2000). The tests included Gram-staining, spore staining, acid-fast staining, starch hydrolysis, Voges Proskauer (V-P) test, production of H_2S, gelatin liquefaction, nitrate reduction, indole, deep glucose agar, catalase reaction, methyl-red, carbohydrate fermentation, urease , motility , oxidase. Cultural and physiological studies were also done.

2.4. Antibiotic Susceptibility Test (Bauer et al. 1966)

The isolates were subjected to the discs diffusion method for antibiotic susceptibility against common antibiotics. The test was performed on Mueller Hinton agar plates. The suspension of the isolates was prepared using sterile distilled water, and was adjusted to 0.5 McFarland standards. A 100µL suspension of freshly-grown bacterial cultures was spread on Mueller Hinton agar plates. The antibiotic discs were placed on the surface of the agar and kept at 4^0C for thirty minutes. Then, the plates were incubated at 37ºC for twenty-four to forty-eight hours. Chloramphenicol (30µg), Penicillin G (10 Units), Erythromycin (15µg), Nitrofuran (30 µg), Rifampicin (5µg), Cephalosporin (30 µg), and Streptomycin (10µg) (Manufacturer: Oxoid) were used to observe the susceptibility pattern of the isolates.

2.5. Probiotic Efficacy Test (Vijayan et al. 2006)

Overnight culture filtrates of two probiotic bacteria *Bacillus* spp. and *Pediococcus* spp (Manufacturer: Lactospore) were used in the well diffusion method (Magaldi *et al*. 2004) for the probiotic efficacy test. The selected isolates were heavily seeded in the nutrient agar plate. Then a hole was made in media by a sterile cork borer in aseptic condition, and one drop of the malted agar was poured into the hole to make a base layer. 0.1 mL culture filtrates of probiotic bacteria (*Bacillus* spp. and *Pediococcus* spp.) were poured into two separate holes. The culture plates were kept at a low temperature (4ºC) for two-four hours for a maximum diffusion. The plates were then incubated at 37ºC for twenty-four hours. The efficacy of the probiotic was determined by measuring the zone of inhibition expressed by the diameter in millimeter. The experiment was carried out more than once, and the mean of reading was taken.

3. Results

3.1. Enumeration of Total Count:

The total bacterial count and *Vibrio* load count of the collected samples are shown in Table 1. There is a variation in the bacterial count and *Vibrio* load count among different types of samples on the Nutrient agar medium and TCBS agar medium (Figure 1)

Figure 1. *Vibrio* Load Count on TCBS Agar Medium

Table 1. Total bacterial count and *Vibrio* load count of the collected samples at selected sampling sites.

Sl. No.	Location	Type of Sample	Total Bacterial Count (CFU/mL)	*Vibrio* Load Count (CFU/mL)
1.	Mixing water zone at Kolatali, Cox's Bazar	Water sample	2.25×10^3	2.17×10^2
		Soil sample	6.24×10^3	4.14×10^3
2.	Pioneer Shrimp Hatchery Limited	Raw water	3.19×10^4	2.01×10^4
		Treated water	2.31×10^2	3.76×10^3
3.	Golden Shrimp Hatchery Limited	Water sample	2.56×10^4	3.22×10^4
		Soil sample	3.18×10^4	4.54×10^4
4.	Mixing water zone at Sonapara, Cox'sBazar	Water sample	5.54×10^2	2.91×10^2
		Soil sample	2.37×10^2	4.55×10^3
5.	United Hatchery Limited, Cox's Bazar	Raw water	2.37×10^3	2.55×10^3
		Treated water	4.6×10^2	2.32×10^2
		Water from algal culture	2.09×10^2	4.61×10^2
6.	Modern Hatchery Limited, Cox's Bazar	Raw water	3.18×10^3	3.54×10^3
		Treated water	2.15×10^2	0
		Water from post larval culture	2.03×10^3	3.29×10^3
7.	Baley Shrimp Hatchery	Raw water	4.31×10^3	2.43×10^3

3.2. Identification of Selected Isolates

During the period of the study, a total of twenty bacterial colonies were isolated according to their morphological characteristics. Seven isolates (Coded as AM1 to AM7) were finally selected from seven groups for a detailed examination. The bacterial isolates were

characterized according to their morphological characteristics including the size and shape of the organism, the arrangement of the cells, presence or absence of the spores, regular or irregular forms, gram reaction etc. The cultural and physiological characteristics include temperature tolerance, salt tolerance, IMViC test, H_2S production, nitrate reduction, deep glucose agar test, fermentation of different carbohydrates etc. (Table 2). All these characteristics were then compared with the standard descriptions of ''Bergey'sManual of Determinative Bacteriology'', 8th ed. (Buchanon and Gibson 1974) and were found to be closely-related to the species given below. (Table 3**)**

Table 2. Morphological and biochemical test results of selected isolates.

Parameters	AM1	AM2	AM3	AM4	AM5	AM6	AM7
Vegetative cells	Short rod (0.3-1.0 µm	Curved rod (0.5-0.8 µm	Short rod (1.75-2.63µm	Curved rod (0.5-0.8 µm	Straight rod (1.1-1.5 µm	Curved rod (1.1-1.5 µm	Straight rod (0.5-0.8 µm
Cell arrangement	Single or in pair	Single	Single, pair, short chain.	Single	Single or in pair	Single	Single or in pair
Gram staining	Gram -ve	Gram -ve	Gram +ve	Gram -ve	Gram -ve	Gram –ve	Gram -ve
Spore staining	Non-spore former	Non-spore former	Spore former	Non-spore former	Non-spore former	Non-spore former	Non-spore former
Motility test	Motile	Motile	Non motile	Motile	Motile	Motile	Motile
Catalase test	+	+	+	+	+	-	-
Glucose broth	Turbid growth	Turbid growth	Turbid growth	Turbid growth	Turbid growth	Turbid growth	Turbid growth
Deep glucose agar test	Facultative Anaerobic	Facultative Anaerobic	Aerobic	Facultative Anaerobic	Facultative Anaerobic	Facultative Anaerobic	Facultative Anaerobic
Casein hydrolysis	-	+	-	+	+	+	-
Starch hydrolysis	-	+	+	+	+	+	-
Egg albumin test	-	-	+	-	+	+	-
Gelatin liquefaction	+	+	-	+	-	+	+
Growth in synthetic media	-	-	-	-	-	-	-
Growth in inorganic salt	+	+	-	+	-	+	+
Citrate utilization	-	+	-	+	-	+	Variable
Voges-Proskauer test	-	-	-	-	-	-	Variable
Methyl red test	+	+	-	+	+	+	+
Nitrate reduction test	+	+	+	+	+	+	+
H2S production	-	-	+	-	-	-	-
Indole test	-	Variable	-	+	+	+	+
Urease test	-	-	+	-	-	+	-
Oxidase test	+	+	-	+	-	+	+
glucose,	Acid and gas	+	No acid and gas	Acid without gas	Acid from	Acid but no gas	Acid and gas
Fructose	Acid and gas	+	Acid without gas	Acid and gas	Acid from	Alkali without gas	Alkali without gas
Galactose	Acid and gas	+	Alkali without gas	Alkali without gas	Acid from	Acid and gas	Acid and gas
Sucrose	Alkali without gas	-	No acid and gas	Acid and gas	Alkali without gas	Acid and gas	Acid but no gas
Lactose	Alkali without gas	-	Alkali without gas	Acid without gas	Acid from	Alkali without gas	Acid and gas
Xylose,	Alkali without gas	-	No acid and gas	Alkali without gas	Alkali without gas	Alkali without gas	Alkali without gas
Arabinose	Alkali without gas	+	No acid and gas	Acid and gas	Acid from	Alkali without gas	Acid but no gas
Maltose	Alkali without gas	+	No acid and gas	Acid without gas	Alkali without gas	Acid but no gas	Acid but no gas
Mannitol	Alkali without gas	+	Alkali without gas	Acid without gas	Acid from	Acid and gas	Acid but no gas
pH 4.5	++++	++	-	-	+++	+	+
pH 6.5	++++	+++	++++	+++	+++	++	++
pH 7.5	+++	+++	+++	+++	+++	+++	+++
pH 8.5	+++	++	-	-	++	++	+++
Temperature (5 ^{0}C)	-	-	+	+	-	-	-
Temperature (10 ^{0}C)	-	+	+	+	-	-	-
Temperature (27 ^{0}C)	+++	+++	+++	+++	+++	+++	+++
Temperature (37 ^{0}C)	+++	+++	+++	+++	+++	+++	+++
Temperature (45 ^{0}C)	-	-	-	-	-	-	-

Note: Positive (+ =Scanty, ++ = Moderate, +++ = Heavy), - = Negative

Table 3. Species Name of Selected Isolates.

Code of Isolates	Name of Species
AM1	*Aeromonas salmonicida*
AM2	*Vibrio parahaemolyticus*
AM3	*Bacillus fastidiosus*
AM4	*Vibrio vulnificus*
AM5	*Escherichia. Coli*
AM6	*Vibrio harveyi*
AM7	*Aeromonas bestiarum*

3.3. Antibiotic Susceptibility Test and Probiotic Efficacy Test

The antibiotic susceptibility test of the selected isolates was performed by the disc diffusion method using the standard discs (Figure 2). The probiotic efficacy tests (Figure 3) were done by the well diffusion method. Figure 4 presents comparative results of antibiotic susceptibility and probiotic efficacy against the identified pathogens.

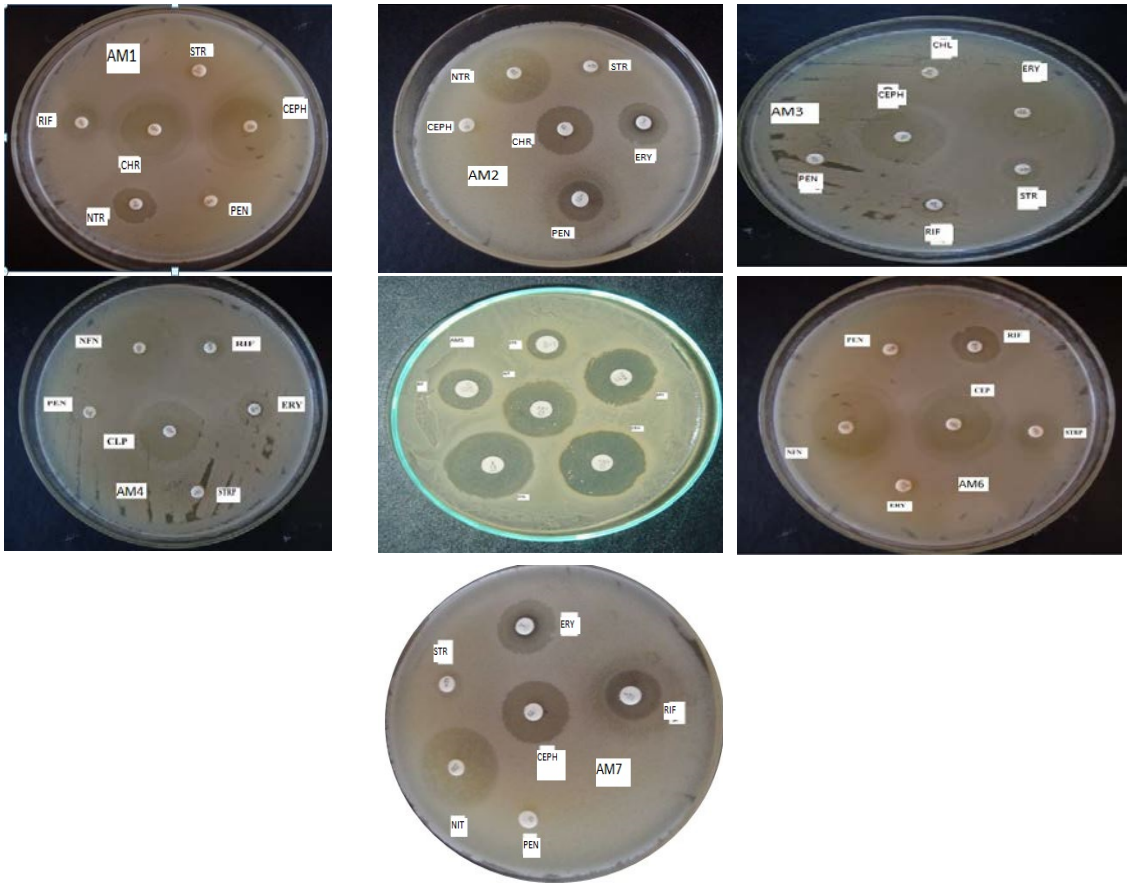

Figure 2. Antibiotic Susceptibility of isolates AM1 to AM7

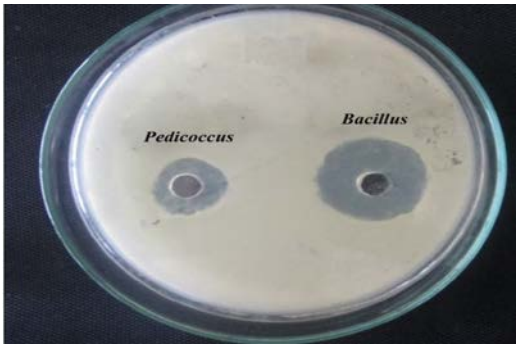

Figure 3. Probiotic efficacy of *Bacillus* and *Pedicococcus* against isolate AM2

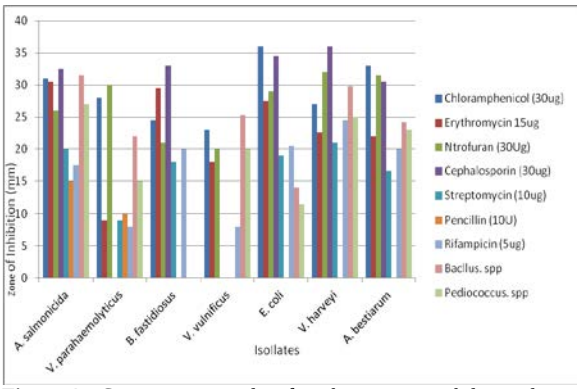

Figure 4. Comparative results of antibiotic susceptibility and probiotic efficacy against the identified pathogens**.**

4. Discussion

The maximum bacterial load was found to exist in the soil sample of the mixing water zone of Kolatali, Cox's Bazarand Sonapara, Cox's Bazar. The waste water of the hatchery, discharged with poor or no treatment, is supposed to be responsible for making the raw seawater contaminated. Wang and his co-workers published their studies on the total bacterial counts of new and three-year-old grow-out ponds for the cultivation of *Liptopenaeus vannamei*. Their findings revealed that the total bacterial count of a recently-constructed pond was 1.11×10^6 CFU/mL, while it was 6.25×10^6 CFU/mL for a three-year-old pound. Most of the Hatcheries' total bacterial count and total vibrio load count were found similar. The *Vibrio* count of treated water was found slightly lower for some hatcheries. The *Vibrio* species were *V. vulnificus*, *V. harveyi* and *V. parahaemolyticus* which are commonly termed as the pathogenic bacteria for shrimp larvae. The other identified bacteria have also detrimental effects for shrimp hatchery management. The root causes of these bacterial infections include the improper treatment of raw water and the insufficient storage conditions of storage tanks to maintain them as contamination-free. Moreover, in hatcheries, the algal culture tank constitutes another vital source of potential bacterial contamination where both the total bacterial count and total vibrio count were high. It is evident that *V. harveyi* is the most dominant pathogenic *Vibrio* species that has a greater effect on shrimp PL during the rearing period. According to Lavilla-Pitogo *et al*., (1998) and Karunasagar *et al*., (1994), Luminous bacteria, particularly *V. harveyi*, and occasionally other luminous species, have become recognized as a devastating pathogen of Penaeid shrimp larvae and adults throughout Southeast Asia. The salinity of this area facilitates the pathogenic *Vibrio* growth. This environment proved congenial for harmful bacterial species like *Vibrio harveyi, V. fisheri, V. spplendidus* and *V. vulnificus* for their survival and multiplication. To prevent diseases' outburst in shrimp hatcheries, the temperature of the rearing water tanks, in particular, needs to be maintained at optimum levels, and least fluctuations in temperature would lead to luminous vibriosis. Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose part of the normal intestinal microflora of healthy fish. Therefore, the presence of these bacteria, by themselves, is not indicative of a disease, and consequently, stress is often considered to be a contributing factor in the outbreaks of disease caused by these bacteria. In the present study, the bacterial genus *Aeromonas* was identified as the second most dominant bacteria in the shrimp culture system. The prevalence of this bacterium is an indication of its relation to pathogenic infections of cultured shrimp. Two of the other bacteria identified, namely *B. fastidiosus* and *E. coli* were also reported to be present in the shrimp culture system of which *Bacillus* spp. is used as the probiotic treatment in shrimp hatcheries to control other bacterial growth. Although *E. coli* is not so much reported in shrimp culture systems, the presence of *E. coli* is not unexpected due to the widespread availability of this organism which is also regarded as the pathogenic microbes affecting shrimp growth. All penaeid shrimp hatcheries encounter bacterial problems that impact the production. Antibiotic treatments to control pathogenic bacteria problems yield varying results. However, in the current research work, some of the antibiotics showed effective results in controlling bacterial growth in aquaculture. At present, the introduction of Probiotics, as 'bio-friendly agents' such as lactic acid bacteria and *Bacillus* spp. into the culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms is gaining popularity. The present study has used the following antibiotics: Chloramphenicol, Erythromycin, Penicillin, Rifampicin, Nitrofuran, Cephalosporin, Streptomycin and some commercially available probiotics including *Bacillus.* spp, and *Pediococcus*. spp.. Both probiotics showed good results against all pathogens except *B. fastidiosus* because *B. fastidiosus* itself is a genus of the applied probiotic bacteria. Two common pathogens of shrimp hatcheries, namely *V. parahaemolyticus* and *V. vulnificus* exhibited resistance against the antibiotic cephalosporin and streptomycin, but showed significant zones of inhibition (22 mm against *Bacillus* spp., and 15 mm against *Pediococcus* spp. for *V. parahaemolyticus*, and 25.3 mm against *Bacillus* spp, and 20 mm against *Pediococcus* spp for *V. vulnificus*) against probiotics. This indicates that the presence of probiotic bacteria within the shrimp aquaculture can cause a significant decrease of pathogenic microorganisms through their antimicrobial action against a wide range of shrimp pathogens. With the use of antibiotics or disinfectants to kill bacteria, some bacteria survive (either strains of the pathogen or others) because they carry genes for resistance (Moriarty 1998). These will then grow rapidly because their competitors are removed.. Antibiotic-resistant bacterial strains develop and flourish over a short period of time. In contrast, Probiotic bacteria produce substances with bactericidal or bacteriostatic effects on other microbial populations (Servin 2004) such as bacteriocins, hydrogen peroxide, siderophores, lysozymes, proteases, among many others (Panigrahi 2007 and Tinh 2007). Besides, some bacteria produce organic acids and volatile fatty acids (e.g., lactic, acetic, butyric and propionic acids), that can result into the reduction of pH in the gastrointestinal lumen, thus, preventing the growth of opportunistic pathogenic microorganisms (Tinh 2007).

5. Conclusion

Waste water discharged from shrimp hatcheries and aquaculture without any or proper treatment is a potential source for microbial contamination within the shrimp culture. The untreated waste water gets mixed with seawater which is further used for hatchery operation. The representative microbial population within a shrimp culture includes the *Vibrio* spp., *Aeromonas* spp., *Bacillus fastidiosus* and *E. coli* among which *Vibrio* and *Aeromonas* are the pathogenic microorganisms which cause diseases to shrimp. The antibiotic effects against shrimp pathogens are strong enough to prevent any microbial growth; however, therapeutic regimen antibiotics used leave some negative impacts such as their residual toxicity, an emerging drug resistance, immunosuppression, and the reduction of consumers' preferences for drug-treated aquatic products in the market. Accordingly, the demand for non-antibiotic-based, and environmentally friendly agents is highly desired for health management in aquaculture. The use of probiotics is an effective alternative sustainable source of beneficial microbes with bactericidal or bacteriostatic effects against pathogenic bacteria, and with anti-bacterial, anti-viral, and anti-fungal activities. Further studies on the probiotic efficacy are still required to determine the appropriate dosage per unit of the aquaculture water system before commercial use.

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