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Isolation and Screening of Probiotic Bacteria from the Gut of Polychaetes as a Probiotic Potential for Fish Aquaculture

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ABSTRACT

In the present study, a total of 17 morphologically different gut-associated bacteria were isolated from four species of estuarine polychaetes: polychaetes *Capitella capitata*, *Scalibregma inflatum*, *Dendronereis aesturiana,* and *Namalycastis abiuma*. The isolated strains were evaluated for their probiotic activities, such as digestive enzymes including protease, amylase, and lipase, and antimicrobial activities by the agar well diffusion method against fish pathogens. Based on their better enzymatic and antibacterial activities, two bacterial strains, CMST Poly1 and CMST Poly2, were selected for further probiotic studies. Based on the biochemical and morphological characterization, both probiotic strains were characterized as Gram-positive, rod-shaped, non-motile, non-spore-forming, homofermentative, absence of catalyzing enzymes and notable proteolytic activity, and susceptibility to various antibiotics. Further, these two strains were confirmed by 16S rRNA gene sequence analysis as *Bacillus subtilis* CMST Poly1 and *Priestia megaterium* CMST Poly2. Our results revealed that strains *Bacillus subtilis* CMST Poly1 and *Priestia megaterium* CMST Poly2 can potentially be used as probiotic strains in aquaculture applications.

INTRODUCTION

The aquaculture sector is a promising source of high-quality animal protein and food supply for the entire world. There is a growing need for seafood globally, and global aquaculture output has made a significant contribution to the world economy. Fish raised in modern intensive aquaculture systems experience a range of harmful environmental conditions, which weaken their immune systems and make them more susceptible to diseases (Lieke et al. 2019). One of the main factors contributing to significant economic losses in the production of freshwater fish is bacterial fish diseases (Silva et al. 2012). The infections caused by *Aeromonas* spp., *Pseudomonas fluorescens*, *Vibrio anguillarum*, *Flavobacterium columnare*, *Edwardsiella tarda*, *Streptococcus* spp., and *Enterococcus* sp. are the primary causes of mortality in fish (Plumb 1997). Gastrointestinal disease in humans can also be brought on by certain virulent microorganisms (Igbinosa et al. 2012). Commonly used synthetic drugs, antibiotics, and chemotherapeutics for managing bacterial infections in aquaculture sectors may have unfavorable impacts such as bacterial resistance, drug resistance, and environmental contamination (Nya & Austin 2011, Wang et al. 2015). As a result, alternative methods of disease control must be developed, including eco-friendly disease prevention measures, to preserve the sustainability of aquaculture (Nawaz et al. 2018). According to many studies, probiotics are a viable alternative to antibiotics for treating bacterial infections because of their nutritional content and their ability to counteract the negative effects of antibiotics and other medications (Jinendiran et al. 2019, Chen et al. 2019). In aquaculture, probiotics are live microorganisms that provide health advantages to the host and are beneficial in disease prevention and encouraging growth through improving immunological response (Son et al. 2009, Abumourad et al. 2013), supplying nutrients, and enzymatic activities (Yang et al. 2015).

Almost all benthic marine and estuarine sediments contain polychaetes, which are frequently the dominant species and

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individuals in the macrobenthos (Fauchald 1977, Grassle & Maciolek, 1992, Ward & Hutchings 1996). In captivity, polychaetes are frequently added to shrimp brooders in addition to squid and oysters since they are the natural prey of wild penaeid shrimps. For the spawning effectiveness and reproductive success of brooders, the well-balanced food content of polychaetes is essential (Palmer et al. 2014). On this basis, the goal of this study was to examine the polychaete gut and evaluate its potential as a source of probiotics through *in vitro* testing of probiotic enzyme synthesis.

MATERIALS AND METHODS

Collection and Identification of Polychaetes

The samples of marine polychaetes were collected using a scoop from the estuarine sediments of Manakkudy (Lat. 8.08°N and Long. 77.48°E), Kanyakumari District, South West coast of India. The collected samples were immediately transported to the lab in sterile and live conditions in sterilized bottles. The morphological identification of samples depends on morphometric and meristic traits such as color patterns, the structure of wings and legs, the arrangement of head and mouth parts, and genitalia (Fauvel 1953, Day 1967).

Isolation of Probiotic Microbes

Samples were immediately brought to the lab for microbiological investigation in sterile polythene bags with sterile saltwater. Live polychaetes' body surfaces were cleaned with sterile water before being swabbed with 60-70% ethanol for surface sterilization. Using sterile forceps, the polychaetes' gut samples were extracted aseptically and homogenized in phosphate-buffered saline (10 mM PBS, pH 7.2). Following this, 100 microliters of the serially diluted sample was plated on Zobell marine agar medium supplemented with 1% peptone and incubated for 48-72 hours at 37°C. By repeatedly sub-culturing at least three times, the morphologically distinct colonies were chosen, purified, and then stored for future use at 4°C.

Agar Well Diffusion Assay

To verify the inhibitory activity, antagonistic strains were tested using an agar well diffusion assay against the aquaculture pathogen *Aeromonas hydrophila* (Cintas et al. 1995). ZMB broth was used to culture antagonistic microorganisms for 48-72 hours at 37°C. The bacterial suspension was separated by centrifugation after incubation, and cell-free culture supernatants were used for the antibacterial assay. A bacterial pathogen was cultured in a 15 mL glass test tube containing 5 mL of Muller Hinton (MH) broth and was then incubated aerobically at 37°C for 24 hours. Then, 50 mL of sterile-filtered supernatants were poured into wells (6 mm in diameter) that had been aseptically pierced using the base tip of a 200 mL sterilized pipette. The plates were then incubated at 37°C for 24 hours, and clear zones indicated the presence of antibacterial activity, and the zone was measured. The control was the same sterile broth (ZMB).

Screening of Probiotic Enzymatic Activities

Bacteria that produce extracellular enzymes were examined for amylase, lipase, and protease, respectively, on starch, tributyrin, and skim milk agar plates. Amylolytic activities of the isolates were screened on starch agar plates containing ($g.L^{-1}$): starch 10.0, peptone 5.0, glucose 5.0, agar 30.0, and NaCl 30.0, pH 7. By flooding the plates with iodine solution after 24 hours of incubation at 37˚C, the zone of the clearing was identified. By comparing the zone of clearance diameter to the colony diameter, promising amylase-producing strains were selected. Skim milk agar plates were used to test the isolated bacterial strains for protease activity. The isolate that showed a distinct zone forming surrounding the growing colony was identified as a protease producer. Tributyrin nutritional agar plates with 1% (v/v) of tributyrin were used to evaluate the isolate's capacity to produce lipase activity. Clear zones in bacterial colonies were considered to indicate the presence of bacteria that produce lipase.

Antibiotic Sensitivity and Hemolytic Test

By using a disc diffusion assay in nutrient agar, the isolates' antibiotic susceptibility was determined. The following antibiotic discs were used: Ampicillin (2 µg), Tetracycline (5 µg), Erythromycin-E (5 µg), Chloramphenicol (5 µg), and Gentamycin (10 µg) (Hi Media, Mumbai). Briefly, 0.1 mL of each antagonistic strain was inoculated into the ZMA, and then antibiotic discs were placed on the ZMA and incubated overnight at 37°C. The results of the clear zone were measured in diameters and denoted as Sensitive (S), Intermediate (I), and Resistance (R). On top of the blood agar medium, which contains 5% (v/v) human blood, strains were streaked. The plates were checked for the hemolytic reaction after 48 h of incubation at 37°C.

Identification of Probiotic Bacteria

Bergey's manual of determinative bacteriology (Garrity et al. 2001) was used to identify the potential probiotic strains based on their morphological, biochemical, and phenotypic properties. 16S rRNA gene sequencing was used to confirm the identification. The probiotic strains' genomic DNA was isolated using a modified method (Sharma & Singh 2005). Using universal primers

(FP: 5′-AGAGTTTGATCCTGGCTCAG-3′ and RP: 5′-CGTTACCTTGTTACGACTT-3′), the 16S rRNA gene was amplified. The following PCR conditions were used: initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Following purification, an automated DNA sequencer immediately began sequencing the PCR products. The CLUSTAL X program was used to examine the homology of gene sequences in GenBank. The neighbor-joining method (MEGA 6.0) was used to build the phylogenetic tree (Saitou & Nei 1987).

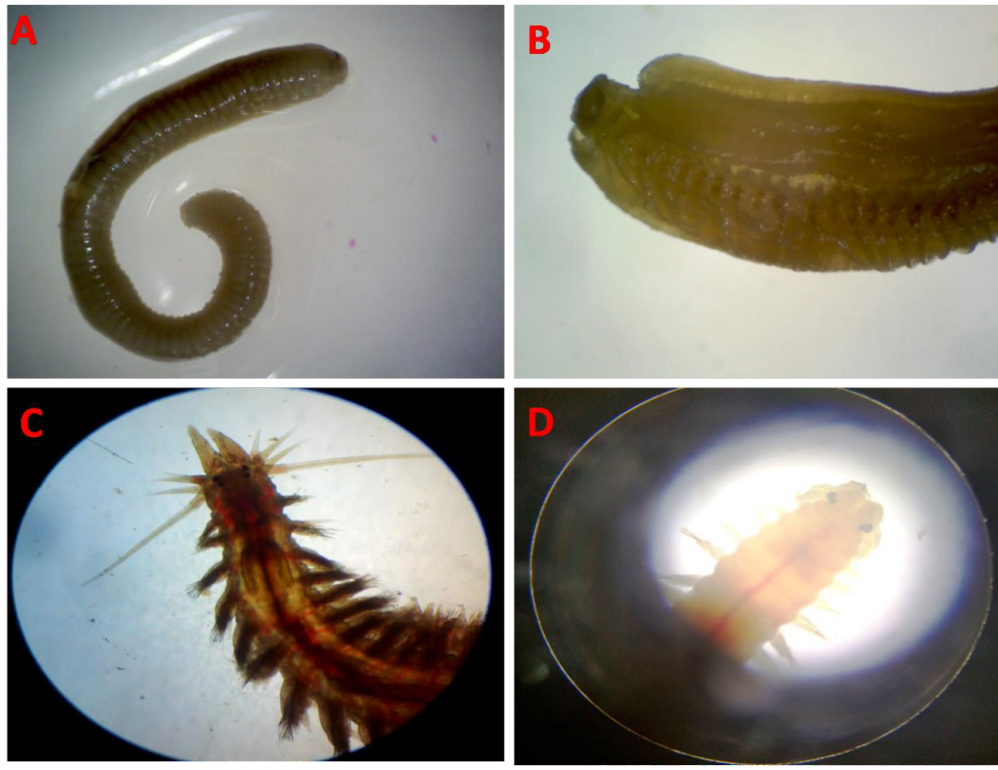

Fig. 1: Polychaetes collected from estuarine sediments of Manakkudy, Kanyakumari District, Southwest coast of India. A. *Capitella capitata*; B. *Scalibregma inflatum*; C. *Dendronereis aesturiana*; D. *Namalycastis abiuma*.

Table 1: The important key characters of the collected polychaetes.

S. No.	Family	Species Name	Key character	Reference
1.	Capitellidae	*Capitella capitata*	• Abdominal segments without branchiae • Peristomium and the next six segments with winged capillaries • Prostomium conical with a pair of ventro-lateral eyes.	Fauvel, (1953); Day (1967)
2.	Scalibregmatidae	*Scalibregma inflatum*	• Body rusty brown, swollen anteriorly but narrowed posteriorly. • Length up to 60 mm, with 60 segments. Skin tessellated. • Prostomium pale with divergent processes forming a stout T.	Fauvel, (1953); Day (1967)
3.	Nereidae	*Dendronereis aesturiana*	• Body about 50 mm in length and only 1 mm wide, belongs to the genus *Dendronereis* this species and other mud-dwelling polychaetes. • Most members of this family have four pairs of tendril-like appendages. • In *Dendronereis* the gills are the most developed and resemble a well-branched tree.	Fauvel, (1953); Day (1967
4.	Nereidae	*Namalycastis abiuma*	• Brown epidermal pigment dorsally and on pygidium; prostomium with shallowly cleft anteriorly, antennae extending short of tip of palpophore. • *N. abiuma* species group except body uniform in width anteriorly, tapering gradually posteriorly. Eyes, 2 pairs, black.	Fauvel, (1953); Day (1967)

RESULTS AND DISCUSSION

Totaling four species of polychaetes *Capitella capitata*, *Scalibregma inflatum*, *Dendronereis aesturiana* and *Namalycastis abiuma* (Fig. 1) were identified and their key important characters were presented in Table 1.

Over the past few decades, aquaculture production has significantly increased around the world. One of the most dynamic and promising economic sectors for global food supply is sustainable shrimp and fish production (Jinendiran et al. 2019). Probiotics have positive impacts on aquaculture, including increased growth performance (Merrifield et al. 2010), pathogen reduction, and the prevention of infectious illnesses by enhancing innate and acquired immunity (Qi et al. 2009, Nayak 2010). The greatest sources to screen beneficial bacteria based on their effective combative impact against infectious disease in aquaculture are the symbiotic associations of microbes within the host or with the aquatic environment (inter-intra-specific interactions) (Verschuere et al. 2000). *Bacillus subtilis*, *B. licheniformis*, *B. pumilus*, *B. megaterium*, and *B. halotolerans* were among the additional gut-associated bacteria that were identified and described from several polychaetes (Priscilla et al. 2022). In this work, a total of 17 types of distinguishable bacterial strains were isolated from four different polychaetes species - *Capitella capitata*, *Scalibregma inflatum*, *Dendronereis aesturiana*, and *Namalycastis abiuma* - and evaluated for probiotic activity.

The five isolates were given the names Poly1, Poly2, Poly3, Poly4, and Poly5. Of the five isolates, three produced only lipase and protease, but only two isolates (Poly1 and Ploy2) had activity for all three enzymes, such as protease, lipase and amylase (Fig. 2 and Table 2). Of the five isolates, two isolates, Poly1 and Poly2, produce antimicrobial activity against the fish pathogen *Aeromonas hydrophila* (Table 3) and produce a clear zone greater than 10 mm.

Probiotic microorganisms reportedly produce digestive enzymes to enhance the host's digestibility, according to Mohapatra et al. (2012). Additionally, the protease enzyme enhances functional qualities by creating antimicrobial peptide (AMP)-mediated defensive mechanisms against encroaching pathogens by cleaving their receptors in the intestinal epithelial cell wall (Hosoi & Kiuchi 2003). In this study, it was discovered that the probiotic bacteria *Priestia megaterium* CMST Poly2 and *Bacillus subtilis* CMST Poly1 have protease, amylase, and lipase activities. These enzymes may help the host's GI tract to digest

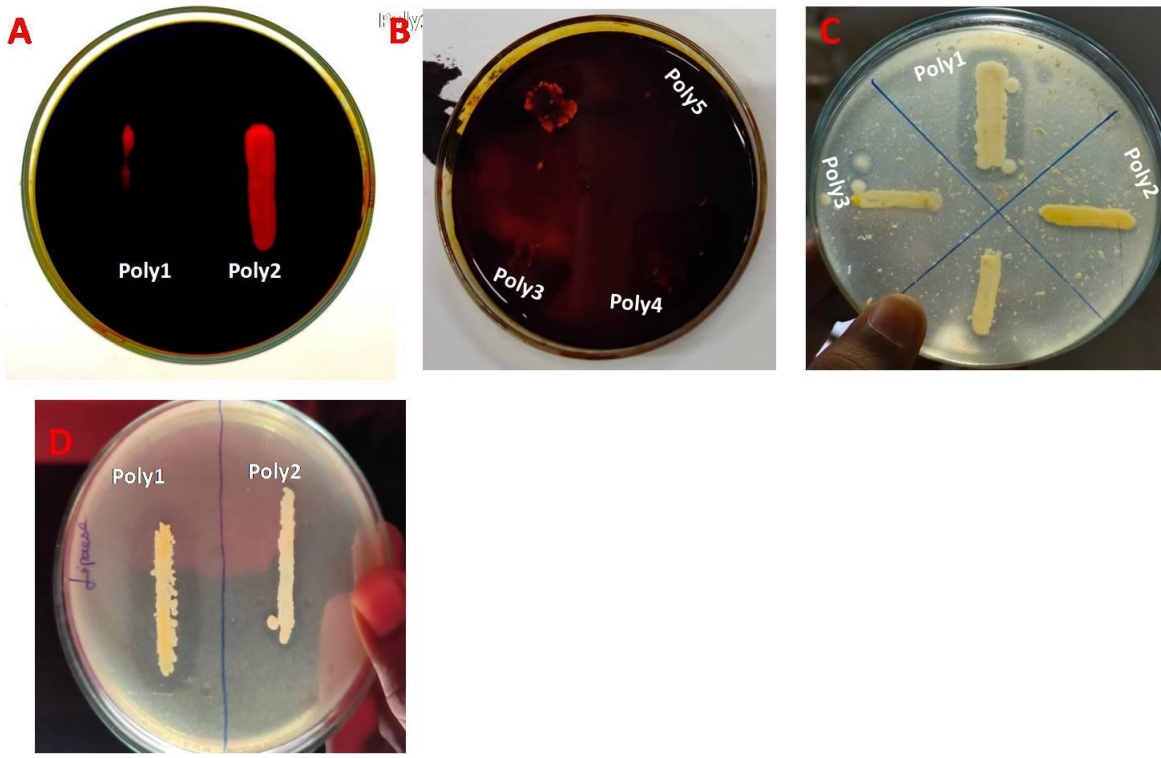

Fig. 2: Screening of extracellular enzyme activities. A. Amylase activity, B. Protease activity, C. Lipolytic activity (clear zone indicating positive results; Absence of zone indicating negative results for the enzyme activities).

Table 2: Screening of enzymatic activities for the probiotics bacteria.

S. No.	Strains Name	Extracellular enzymes activity		
		Protease	Amylase	Lipase
1.	Poly1	+	+	+
2.	Poly2	+	+	+
3.	Poly3	-	-	+
4.	Poly4	+	-	+
5.	Poly5	+	-	+

'+': Positive Activity; '-': Negative activity

Table 3: The screening of antimicrobial activity against fish pathogen *Aeromonas hydrophila* using the agar well diffusion assay.

Sl. No	Strains Name	Fish pathogen *Aeromonas hydrophila*
		Zone of inhibition (mm)
1.	Poly1	+
2.	Poly2	+
3.	Poly3	-
4.	Poly4	-
5.	Poly5	-

'+': Positive Activity; '-': Negative activity

proteins, lipids, and carbohydrates. Similar findings were made by Dawood et al. (2016) and Priscilla et al. (2022) on the production of hydrolytic enzymes amylase, lipase, and proteinase by Gram-positive *Bacillus* spp., isolated from the intestines of polychaetes. The hydrolytic enzymes enhance the host's enzyme activity and cause the creation of digestive enzymes, which improve feed utilization, survival, and immune response in the host. The ability of *Bacillus* spp. to create extracellular enzymes, such as amylolytic, proteolytic, cellulolytic, and lipolytic in the GI tracts of tropical freshwater fish, was also the subject of numerous research studies (Bairagi et al. 2002, Kar & Ghosh, 2008, Mondal et al. 2010, Ray et al. 2012, Banerjee et al. 2013).

The probiotic strains Poly1 and Ploy 2 produce no holozone on the blood agar, which indicates that they did not produce any hemolytic activity and are harmless to cultured animals. The results of the antimicrobial disc diffusion susceptibility tests on two strains for five antibiotics are summarized in Table 4. The probiotic bacterial strains Poly1 and Ploy2 showed no resistance to the majority of tested antibiotics such as ampicillin, penicillin, tetracycline, and kanamycin (Fig. 3). Further screenings for the probiotic candidates can be done by examining the antibiotic susceptibility test results of isolates. The present results clearly showed that candidate bacterial strains had no antagonistic effect against tested commercial antibiotics. Similar to Thankappan et al. (2015), they reported that *Bacillus* spp. was susceptible to the various antibiotics amoxicillin, cephalexin, streptomycin, penicillin-G, gentamycin, and erythromycin. Likewise, Sorokulova et al. (2008) have described the commercial probiotics *B. subtilis* and *B. licheniformis* as being susceptible to common antibiotics.

Bacillus, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Shewanella*, *Aeromonas*, *Clostridium*, and *Saccharomyces* species are among the probiotic bacteria frequently utilized in aquaculture (Nayak 2010). The immune systems and health advantages of Japanese flounder, black tiger prawns, white leg prawns, and western king prawns can all be improved by probiotics (Rengpipat et al. 1998; Chiu et al. 2007). In Nile tilapia, *Bacillus pumilus* may improve health and reduce illness (Aly et al. 2008). Infection with *Aeromonas hydrophila* was prevented by the probiotic bacteria *Bacillus*

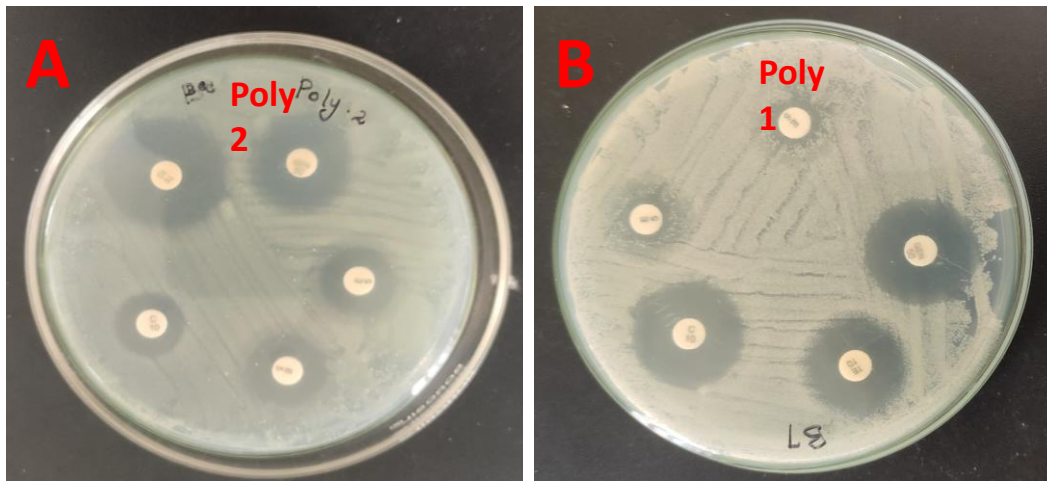

Fig. 3: Antibiotic susceptibility profile of the probiotics strains. A: *Priestia megaterium* CMST Poly2, B: *Bacillus subtilis* CMST Poly1.

Table 4: Morphological and biochemical characters of probiotic bacteria.

Characters	Probiotic bacterial strains	
	CMST Poly1	CMST Poly2
Gram's staining	+	+
Shape	Rod	Rod
Capsule staining	–	–
Spore formation	+	+
Motility	+	+
Glucose	+	+
Mannitol	-	+
Sucrose	-	+
Indole	–	–
Methyl red	+	+
Voges Proskauer	+	+
Citrate	+	+
Urease	–	–
Casein hydrolysis	+	+
Oxidase	+	+

licheniformis and *Bacillus pumilus*, as previously described by Ramesh et al. (2015). The isolated probiotic strains Poly 1 and Poly 2 were identified using biochemical traits (Table 4) and 16S rRNA gene sequencing, and the accession number was then submitted to GenBank. Both isolates are gram-positive, rod-shaped microorganisms that can use glucose, galactose, methyl red, Voges Proskaevuer, and catalase negatively. It was determined to be from the genus *Bacillus* spp. Our morphological and biochemical findings were found to be consistent with those of previous reports (Chu et al. 2010, Rajashekhar et al. 2017, Lee et al. 2017). The isolated strain belongs to the Firmicutes, Bacillaceae, according to phylogenetic analyses. The phylogenetic tree produced using the neighbor-joining approach explains the strain's evolutionary relationship to other genera and species (Fig. 4). The isolate's 16S rRNA gene sequence was uploaded to GenBank as *Bacillus subtilis* CMST Poly1 and *Priestia megaterium* CMST Poly2, respectively (Accession number: OP435726). Probiotic bacterial strains *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus amyloliquefaciens* were discovered using morphological and biochemical testing,

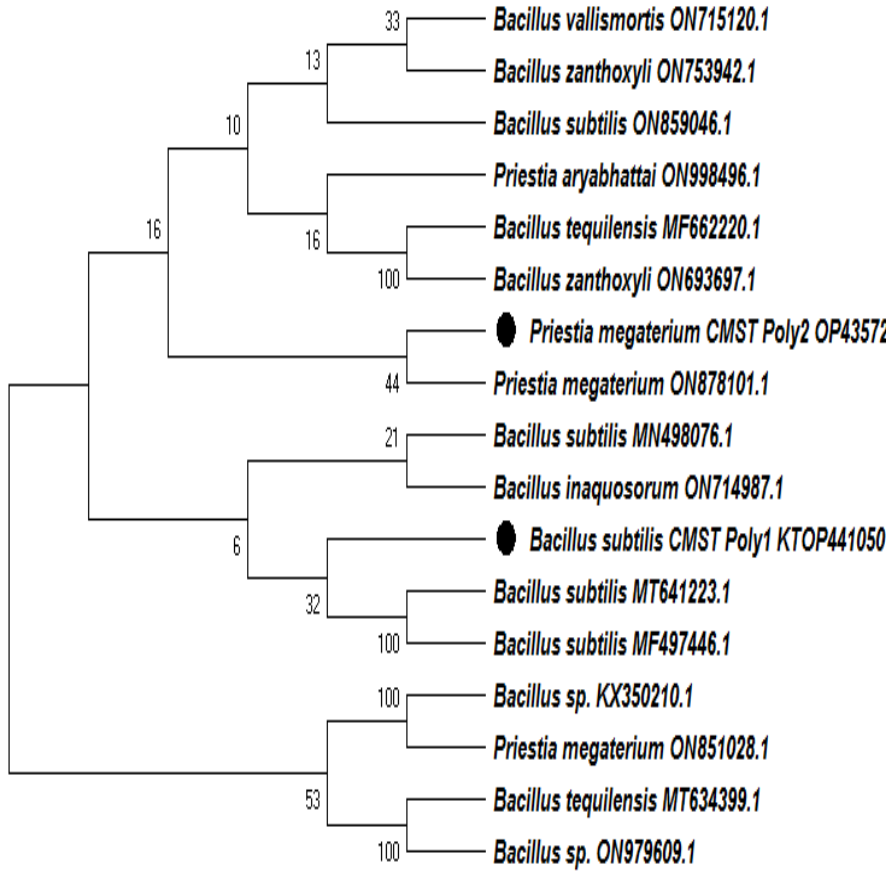

Fig. 4: Neighbor-joining phylogram based on comparative analysis of 16S ribosomal RNA gene sequences of two probiotic bacterial strains CMST Poly1 and CMST Poly2 and the closest related type strains. Percentage bootstrap values are shown at the branching points of 1000 replicates.

and their identification was further confirmed using 16S rRNA gene sequencing. Similar discoveries have also been published by Kavitha et al. (2018) and Shah et al. (2010). Several authors have reported that *B. subtilis*, *Bacillus* sp., and *B. amyloliquefaciens* are safe to use as probiotics for aquaculture use based on current investigations (Banerjee et al. 2017, Nandi et al. 2017a, & Nandi et al. 2017b).

CONCLUSION

Our findings concluded that two bacteria, *Bacillus subtilis* CMST Poly1 and *Priestia megaterium* CMST Poly2, were isolated and identified as potential probiotic candidates from polychaetes. These bacteria demonstrated good hydrolytic enzyme activity, were susceptible to common antibiotics, and exhibited no hemolytic activity *in vitro*. Additionally, the antimicrobial assay screening revealed that two prospective probiotic bacteria have inhibitory effects against the fish disease *Aeromonas hydrophila*, although more research is required to identify the antimicrobial metabolites. To screen and develop the prospective use of probiotics in fish aquaculture, this baseline study should be used as a reference.

REFERENCES

Abumourad, I. M.K., Abbas, W.T., Awaad, E.S., Authman, M. M.N., El-Shafei, K. and Sharaf, O. M. 2013. Evaluation of *Lactobacillus plantarum* as a probiotic in aquaculture: emphasis on growth performance and innate immunity. J. Appl. Sci. Res., 9: 572-582.

Aly, S.M., Ahmed, Y.A., Ghareeb, A.A. and Mohamed, M.F., 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish Shellfish Immunol., 25: 128-136. https://doi.org/10.1016/j.fsi.2008.03.013

Bairagi, A., Ghosh, K.S., Sen, S.K. and Ray, A.K. 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacul. Int., 10: 109-121. https://doi.org/10.1023/A:1021355406412

Banerjee, G., Ray, A.K., Askarian, F. and Ringø, E., 2013. Characterization and identification of enzyme-producing autochthonous bacteria from the gastrointestinal tract of two Indian air-breathing fish. Benef. Microbes., 4: 277-284. https://doi.org/10.3920/bm2012.0051

Banerjee, G., Nandi, A. and Ray, A.K. 2017. Assessment of hemolytic activity, enzyme production and bacteriocin characterization of *Bacillus subtilis* LR1 isolated from the gastrointestinal tract of fish. Arch. Microbiol., 199: 115-124. https://doi.org/10.1007/s00203-016-1283-8

Cintas, L. M., Rodriguez, J. M., Fernandez, M.F., Sletten, K., Nes, I. F., Hernandez, P.E. and Holo, H. 1995. Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. Appl. Environ. Microbiol., 61(7): 2643-2648. https://doi.org/10.1128%2Faem.61.7.2643-2648.1995

Chen, N. Z. and Wang, W. 2019. Effects of Lactobacillus pentosus on the growth performance, digestive enzyme and disease resistance of white shrimp, Litopenaeus vannamei (Boone,1931). Aquacult. Res., 48:2767-2777. https://doi.org/10.1111/are.13110

Chiu, C. H., Guu, Y. K., Liu, C. H., Pan, T. M. and Cheng, W. 2007. Immune responses and gene expression in white shrimp, Litopenaeus vannamei, induced by Lactobacillus plantarum. Fish & Shellfish Immunology., 23(2): 364-377. https://doi.org/10.1016/j.fsi.2006.11.010

Chu, W., Lu, F., Zhu, W. and Kang, C. 2010. Isolation and characterization of new Potential probiotic bacteria based on quorum-sensing system. J. Appl. Microbiol., 110: 202-208. https://doi.org/10.1111/j.1365-2672.2010.04872.x

Day, J. H. 1967. A monograph on the polychaeta of Southern Africa. Trustees of the British Museum (Natural History). Publication No., 656.

Dawood, M.A.O., Koshio, S. Ishikawa, M. and Yokoyama, S. 2016. Effects of dietary inactivated *Pediococcus pentosaceuson* growth performance, feed utilization and blood characteristics of red sea bream, Pagrus major juvenile. Aquac. Nutr., 22: 923-932. https://doi.org/10.1111/anu.12314

Fauchald, K. 1977. The polychaete worms, definitions and keys to the orders, families and genera. Nat. Hist. Mus. LA County Mus. Sci. Ser., 28: 1-190.

Fauvel, P. 1953. Annelida Polychaeta. The Fauna of India Including Pakistan, Ceylon, Burma and Malaya. 507.

Grassle, J.F. and Maciolek, N.J. 1992. Deep-sea species richness; regional and local diversity estimates from quantitative bottom-sampling. Am. Nat., 139: 313-41.

Garrity, G. M, Boone, D. R. and Castenholz, R. W. 2001. Bergey's Manual of Systematic Bacteriology, 2nd ed., Springer, New York.

Hosoi, T. and Kiuchi, K. 2003. Handbook of Fermented Functional Foods. CRC Press, pp. 227-245.

Igbinosa, I.H., Igumbor, E.U., Aghdasi, F., Tom, M. and Okoh. A. I. 2012. Emerging *Aeromonas* species infections and their significance in public health, Sci. World J.,625023: 1-13. https://doi.org/10.1100/2012/625023

Jinendiran S., Boopathi, S., Sivakumar, N. and Selvakumar, G. 2019. Functional characterization of probiotic potential of novel pigmented bacterial strains for aquaculture applications. Probiotics Antimicrob Proteins., 11: 186-197. https://doi.org/10.1007/s12602-017-9353-z

Kar, N., Roy, R.N., Sen, S.K. and Ghosh, K. 2008. Isolation and characterization of extra-cellular enzyme-producing bacilli in the digestive tracts of rohu, Labeo rohita (Hamilton) and murrel, Channa punctatus (Bloch). Asian Fish. Sci., 21 (4): 421-434. http://dx.doi.org/10.33997/j.afs.2008.21.4.006

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

Lieke, T., Meinelt, T., Hoseinifar, S.H., Pan, B., Straus, D. L. and Steinberg, C.E.W. 2019. Sustainable aquaculture requires environmental-friendly treatment strategies for fish diseases. Rev. Aquac., 12(2): 943-965. https://doi.org/10.1111/raq.12365

Lee, A., Cheng, K.C. and Liu, J.R. 2017. Isolation and characterization of a Bacillus amyloliquefaciens strain with zearalenone removal ability and its probiotic potential. PLoS One, 12(8): e0182220. https://doi.org/10.1371/journal.pone.0182220

Mohapatra, S., Chakraborty, T., Prusty, A. K., Das, P., Paniprasad, K. and Mohanta K.N. 2012. Use different microbial probiotic in the diet of rohu, Labeo rohita fingerlings: effect on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora. Aquacult Nutr., 18(1): 1-11. https://doi.org/10.1111/j.1365-2095.2011.00866.x

Mondal, S., Roy, T. and Ray, A.K. 2010. Characterization and identification of enzyme‐producing bacteria isolated from the digestive tract of Labeo bata. J. World Aquacult. Soc., 41: 369-377. https://doi.org/10.1111/j.1749-7345.2010.00378.x

Merrifield, D. L., Harper, G. M., Dimitroglou, A., Ringø, E. and Davies, S. J. 2020. Possible influence of probiotic adhesion to intestinal mucosa on the activity and morphology of rainbow trout (Oncorhynchus mykiss) enterocytes. Aquacult. Res., 41: 1268-1272. https://doi.org/10.1111/j.1365-2109.2009.02397.x

Nya, E.J. and Austin, B. 2011. Development of immunity in rainbow trout (Oncorhynchusmykiss, Walbaum) to Aeromonas hydrophila after the

dietary application of garlic. Fish Shellfish Immunol., 30: 845-850. https://doi.org/10.1016/j.fsi.2011.01.008

Nawaz, A., Javaid, A. B., Irshad, S., Hoseinifar, S. H. and Xiong. H. G. 2018. The functionality of prebiotics as immunostimulant: Evidences from trials on terrestrial and aquatic animals., Fish Shellfish Immunol., 76: 272-278. https://doi.org/10.1016/j.fsi.2018.03.004

Nayak, S. K. 2010. Probiotics and immunity: a fish perspective. Fish Shellfish Immunol., 29: 2-14. https://doi.org/10.1016/j.fsi.2010.02.017

Nandi, A., Dan, S.K., Banerjee, G., Ghosh, P., Ghosh, K. Ringø, E. and Ray, A.K. 2017a. Probiotic potential of Autochthonous bacteria isolated from the gastrointestinal tract of four freshwater teleost's. Probiotics Antimicrob. Proteins., 9: 12-21. https://doi.org/10.1007/s12602-016-9228-8

Nandi, A., Banerjee, G., Dan, S.K., Ghosh, P., Ghosh, K. and Ray, A.K. 2017b. Screening of autochthonous intestinal microbiota as candidate probiotics isolated from four freshwater teleosts. Curr. Sci., 113(4): 25.

Plumb, J. A., Infectious diseases of tilapias, In: Costa-Pierce, B. A. and Rakocy, J.E. (Eds.).1997. Tilapia aquaculture in the Americas. J. World Aquac Soc., 1: 212-228.

Palmer, P.J., Wang, S., Houlihan, A. and Brock, I., 2014. Nutritional status of a nereidid polychaete cultured in sand filters of mariculture wastewater. Aquac. Nutr., 20: 675-691. https://doi.org/10.1111/anu.12129

Priscilla, L., Rajeev, M. and Pandian. S. K. 2022. Gut associated culturable bacterial community in intertidal polychaete worms (Annelida: Polychaeta), their characterization and implications in captive shrimp aquaculture. Regional Studies in Marine Science, 52: 102274. https://doi.org/10.1016/j.rsma.2022.102274

Qi, Z., Zhang, X.H., Boon, N. and Bossier, P. 2009. Probiotics in aquaculture of China current state, problems and prospect. Aquaculture, 290: 15-21. https://doi.org/10.1016/j.aquaculture.2009.02.012

Ray, A.K., Ghosh, K. and Ringø, E. 2012. Enzyme-producing bacteria isolated from fish gut: a review. Aquacult. Nutr., 18: 465-492. https://doi.org/10.1111/j.1365-2095.2012.00943.x

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S. and Menasveta, P. 1998. Probiotics in aquaculture: a case study of probiotics for larvae of the black tiger shrimp (*Penaeus monodon*). In: Flegel, T.W. (Ed.), Advances in Shrimp Biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Ramesh, D., Vinothkanna, A., Rai, A.K. and Venkada Subramanian, V. 2015. Isolation of potential probiotic Bacillus spp. and assessment of their sub cellular components to induce immune responses in Labeo rohita against Aeromonas hydrophila. Fish Shellfish Immunol., 45: 268-276. https://doi.org/10.1016/j.fsi.2015.04.018

Rajashekhar, M. Shahanaz, E. and Vinay, K. 2017. Biochemical and molecular characterization of Bacillus spp. isolated from insects. J. Entomol. Zool. Stud., 5(5): 581-588.

Silva, B.C., Mouriño, J.L.P., Vieira, F.N., Jatoba, A., Seifert, W. Q. and Martins. M. L. 2012. *Haemorrhagic septicaemia* in the hybrid surubim (*Pseudoplatystom acorruscans* and *Pseudoplatystom afasciatum*) caused by *Aeromonas hydrophila*. Aquacult. Res., 43: 908-916. https://doi.org/10.1111/j.1365-2109.2011.02905.x

Son, V.M., Changa, C. C., Wu, M. C., Guu, Y. K., Chiu, C. H. and Cheng, W. 2009. Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the grouper *Epinephelus coioides*. Fish Shellfish Immunol., 26: 691-698. https://doi.org/10.1016/j.fsi.2009.02.018

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Sorokulova, B., Pinchuk, V., Denayrolles, M., Osipova, G., Huang, J. M., Cutting, S. M. and Urdaci, M. C. 2008. The safety of two *Bacillus* probiotic strains for human use. Dig Dis Sci., 53(4): 954. https://doi.org/10.1007/s10620-007-9959-1

Sharma, A. D. and Singh, J. 2005. A nonenzymatic method to isolate genomic DNA from bacteria and Actinomycete. Anal. Biochem., 337: 354-356. https://doi.org/10.1016/j.ab.2004.11.029

Shah, K., Mody, K., Keshri, J. and Jha, B. 2010. Purification and characterization of a solvent, detergent and oxidizing agent tolerant protease from Bacillus cereus isolated from the Gulf of Khambhat. J. Mol. Catal. B: Enzym., 67: 85-91. https://doi.org/10.1016/j.molcatb.2010.07.010

Thankappan, B., Ramesh, D., Ramkumar, S., Natarajaseenivasan, K. and Anbarasu, K. 2015. Characterization of *Bacillus* spp. from the gastrointestinal tract of *Labeo rohita* towards to identify novel probiotics against fish pathogens. Applied biochemistry and biotechnology., 175(1): 340-353. https://doi.org/10.1007/s12010-014-1270-y

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete W. 2000. Probiotic bacteria as biological agents in aquaculture. Microbiol. Mol. Biol. Rev., 64(4): 655-671. https://doi.org/10.1128%2Fmmbr.64.4.655-671.2000

Wang, J.L., Meng, X. L. Lu, R.H. Wu, C. Luo, Y. T. and Yan, X. 2015. Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio* L.). Aquaculture. 435: 293-300. https://doi.org/10.1016/j.aquaculture.2014.10.004

Ward, T. and Hutchings, P. A. 1996. Effects of trace metals on in faunal species composition in polluted intertidal and subtidal marine sediments near a lead smelter, Spencer Gulf, South Australia. Mar. Ecol. Progr. Ser., 135: 123-35.

Yang, F., Wang, A., Zeng, X., Hou, C., Liu, H. and Qiao, S. 2015. *Lactobacillus reuteri* I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. BMC Microbiology., 15(1): 1-11. https://doi.org/10.1186/s12866-015-0372-1