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Novel Probiotic Bacteria *Sutcliffiella cohnii* from Gut of Brownspotted Grouper Fish *Epinephelus chlorostigma* (Valenciennes, 1828)

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Abstract: Probiotic bacteria can help maintain a healthy aquatic environment by improving water quality. They can compete with harmful bacteria for resources, reducing the population of pathogens and preventing diseases. Additionally, probiotics can break down organic waste and excess nutrients, reducing the risk of water pollution. The present study aimed to isolate marine bacteria with promising probiotic potential from fish. In the present study, fish gut samples of brownspotted grouper fish (*Epinephelus chlorostigma*) were collected from the Mudasalodai landing center, and microbial density was 2.4×10^4 CFU/ml used for the screening and isolation of promising marine bacteria for the probiotic role. Twelve bacteria isolated from the Brownspotted grouper fish gut showed the highest probiotic activity and were identified as *Sutcliffiella cohnii*. The pH tolerance was 6.5, Bile salt tolerance was 0.35 observed. Antimicrobial activity against fish pathogens MM-07 showed the zone of inhibition in *Vibrio cholera* (14 mm), likewise *E. coli* (8 mm), *Staphylococcus aureus* (16 mm) and *Aeromonas hydrophila* (16 mm). The antibiotic sensitivity test tested 10 antibiotics; MM-07 was resistant to most of the antibiotics.

Keywords: Brownspotted grouper fish, *Epinephelus chlorostigma*, Antimicrobial activity, *Sutcliffiella cohnii*

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Introduction

Fish belongs to a paraphyletic group of organisms consisting of all gill-bearing aquatic vertebrate animals. Probiotic bacteria are live microorganisms that provide health benefits to the host when consumed in adequate amounts. These bacteria are often referred as "good" or "friendly" bacteria because they help maintain a healthy balance in the gut microbiota (Oyebamij *et al*., 2013). These ectothermic creatures are thought to be excellent providers of animal protein. Several studies have shown that fish is an excellent source of high-quality protein, vitamins, and minerals needed to maintain human health. Probiotics are generally understood to be living microorganisms that positively impact their health when given to a host in sufficient quantities (FAO/WHO, 2002). Probiotics have been extensively researched for their ability to improve various health outcomes, including lowering blood cholesterol, treating lactose intolerance, preventing allergies, and producing bioactive metabolites like vitamins (Bhat and Bajaj, 2019). Fermented foods and other probiotic-containing foods are good for your health. This sparked interest in the study of diverse fermented food types and the identification of new probiotic strains. The need for non-dairy probiotics to combat the rising incidence of lactose intolerance worldwide also motivates research on novel probiotic strains (Argyri *et al*., 2013). Most probiotics may live in the intestinal tract and keep their potency for several days while being stored since they are nonpathogenic and nontoxic (Ramakrishnan *et al*., 2008). According to Vijayaram *et al.* (2014), the most widely utilized probiotic is gram-positive, non-motile, non-sporulation bacteria that mostly create lactic acid due to fermentation. As pathogens become resistant to medications, the use of antibiotics to treat bacterial infections and minimize fish mortality in aquaculture is becoming more restricted (Gonzalez et al., 2000; Gomez-Gil et al., 2000). According to studies of Murry *et al.* (2004), Raja *et al*. (2009) and Moghaddam *et al*. (2006), probiotics such as *Lactobacillus* spp. exhibit inhibitory efficacy against common human infections. They can create antimicrobial compounds like bacteriocins, which have a great deal of promise for usage as medicines and as bio-preservatives in food (Mobarez *et al*., 2008). In particular, the stomach's acidic environment and the presence of bile in the small intestine are two fundamental characteristics that indicate the probiotic microorganism's capacity to survive passage through the upper gastrointestinal tract (Hyronimus *et al*., 2000; Erkkila and Petaja, 2000). Lactic acid bacteria are widely known to form a component of the natural microbiota of aquatic animals from temperate locations, even though they are not the dominant population in Fish (Ringo *et al*., 2004). However, dietary, environmental, and genetic factors may impact the isolated probiotics from the endogenous microbiota. Probiotics from aquatic sources may be endogenous or exogenous microbiota. Human probiotics derived from aquatic species have a significantly more substantial impact on health status because the ambient environment has a more significant impact on aquatic animals' health than terrestrial animals (De *et al*., 1999).

The present study aimed to isolate marine bacteria with promising probiotic potential from fish. In the present study, fish gut samples of brownspotted grouper fish (*Epinephelus chlorostigma*) were collected from the Mudasalodai landing center, and microbial density was 2.4×10^4 CFU/ml used for the screening and isolation of promising marine bacteria for the probiotic role.

Materials and Methods

Sample collection and Isolation of Bacteria:

The fish sample Brownspotted grouper fish (*Epinephelus chlorostigma*) were collected from Mudasalodai Landing Centre, Cuddalore district of Tamil Nadu, India (Fig. 1). The fish samples were collected in a sterile polythene bag in an aseptic condition. The samples were stored at low temperature to protect the samples from debasement and contamination. The fish samples were washed with distilled water. The digestive tracts were taken out from the fish sample in an aseptic condition. The digestive tracts removed from the fish sample were homogenized using sterile distilled water. The homogenized sample was serially diluted. 1 ml of the homogenized sample was inoculated on Nutrient Broth for 24 h

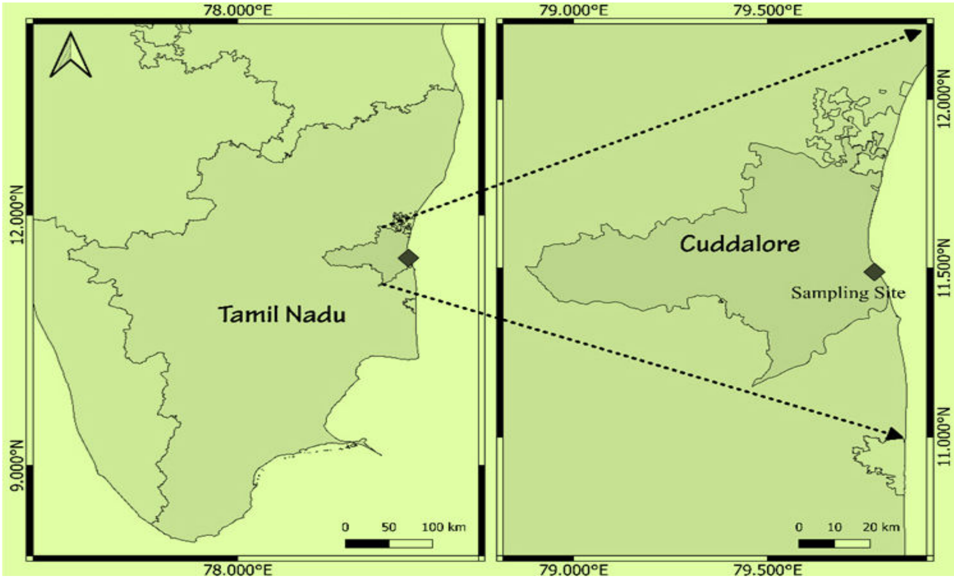

Fig. 1: Sample site.

at room temperature. After incubation, the Nutrient Broth culture was plated by the spread plate method (0.1 ml) in Nutrient agar plates. The bacterial colonies were isolated from each plate and subculture in a Nutrient agar plate to acquire the pure culture in single colonies.

pH tolerance:

At different pH, the acid tolerance of the selected bacterium was investigated. Nutrient broths with distinct pH, including 3, 4, 5, 6, 7, 8 and 9 were prepared using 1% of hydrochloric acid and 1N of sodium hydroxide (Hi-media) and separated in Universal containers (Samelis *et al.*, 1994). The broth media and control containers were autoclaved at 121°C for 15 min. Then it was inoculated with an overnight culture of the selected strain in Nutrient broth and incubated at 37°C. The growth rate of bacteria was measured by spectrophotometer at 600 nm.

Bile salt tolerance:

Bile salt tolerance is tested in Nutrient broth consisting of 0.1, 0.2, 0.3, 0.4 and 0.5% (w/v) Bile salt. Identical containers of Nutrient broth containing purified different concentrations of bile salt were inoculated by the cultured strain of 30 µl, and it was incubated at room temperature. The optical density (OD) was measured at 600 nm after 0, 2, 4 and 8 h incubation (Balcazar *et al.*, 2008; Kim and Austin, 2008; Allamesh *et al.*, 2012)

Screening for antibacterial activity of autochthonous isolates against bacterial fish pathogens:

Twelve different autochthonous bacterial isolates (MM01 to MM12) isolated from the healthy Brownspotted Fish (*Epinephelus chlorostigma*) gut was screened for antimicrobial activity against different fish bacterial pathogens such as *Vibrio cholera, S. aureus, E. Coli* and *Aeromonas hydrophila* using agar well diffusion method on Muller Hinton agar plates (Millette *et al.*, 2007). The cell-free culture supernatant from each ABI inoculated in Muller Hinton broth was obtained by centrifugation and loaded into the wells made on each bacterial pathogen spread plate and incubated at 37ºC for 18-24 h. Based on the diameter of the zone of inhibition, the most potential autochthonous bacterial isolate with antimicrobial activity was selected for further study.

Antibiotic sensitivity test:

The antibiotic sensitivity test was performed on the most frequently used antibiotics in

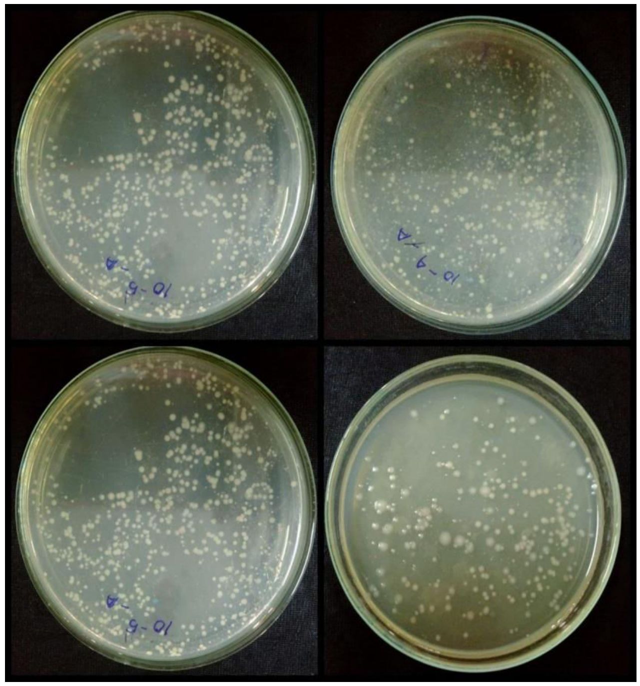

Fig. 2: Isolation of Microbes.

aquaculture for selected strains by disc diffusion method of Akinjogunla *et al*. (2010).

Identification of the potential strain:

Bergey's manual of Determinative Bacteriology describing morphological, cultural, biochemical, and physiological properties was used to determine the strain with the highest potential (Buchanan *et al.*, 1974). Test like biochemical identification like indole production test, methyl red, Voges-Proskauer test, citrate utilization test, catalase test, oxidase test, carbohydrate fermentation, and nitrate reduction test were performed and also 16S rRNA sequencing was done using standard procedures as given below.

Molecular identification of potential strains using 16S rRNA sequencing:

Molecular identification of the potential strain was made based on 16S rRNA sequencing using the bacterial primers 16SRS-F (5'-CAGGCCTAACACATGCAAGTC-3') and 16SRS-R (5'-GGGCGGWGT GTACAAGGC-3'). PCR amplification was done in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA). The PCR product was purified using a Qiagen PCR kit and sequenced in ABI 3730 DNA Analyzer (Applied Biosystems, USA). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems, USA). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 (Drummond *et al.*, 2012). The phylogenetic tree was generated using partial 16S rRNA gene sequences from the isolates compared to other NCBI database sequences closely related to the reference strain. For multiple sequence alignment of sequences, Clustal Omega was used. A neighbour joining tree was created using the MEGA 6.0 programme and bootstrapping at 10,000 bootstrap attempts using the Kimura-2 parameter. (Das and Tiwary, 2013).

Results and Discussion

Microbial density:

In the present study, the gut of the Brown spotted grouper collected from the Mudasalodai landing centre was taken for bacterial analysis of the gut. Plated on Zobell marine agar and the observed microbial density was 2.4×10^4 CFU/ml. A total of 12 strains were collected from the guts of the *Epinephelus chlorostigma* animal (Fig. 2).

pH Tolerance Test:

MM01 to MM12 bacterial species were selected from these microbes to test against the probiotic properties, and 6 strains show potential. It resembles the findings of Dhanasekaran *et al.* (2008, 2010) who reported that a maximum population of *Lactobacillus* in freshwater fishes, The 6 isolates were culturally, morphologically and biochemically characterized in five groups; Isolated strains MM03, MM05, MM06, MM07, MM10 & MM12). All the isolates were Gram-positive rods with entire margins and able to grow at 25°C to 35°C. Isolates showed the same sugar fermentation pattern. Among the biochemical test, Indole, VP, Citrate utilization, and Catalase are positive, and Methyl Red reactions are adverse. The ideal growth rate (w/v) ranged between 1% and 9%. However, different species were used for the current investigation. This might be the consequence of various hosts, environments, or completely distinct identification and further probiotic characteristic techniques. Probiotic selection criteria used *in vitro*, such as pH and bile salt tolerance tests, showed encouraging findings for MM07, which had a pH tolerance of 6.5. The same outcomes were achieved using *L. mesenteroides*, which was isolated from the gut of *Channa striatus* under neutral conditions. The maximum activity was seen at pH 7 (Allamesh *et al.*, 2012). Potential viability at low pH is one of the crucial requirements for probiotic organisms, according to this study. Probiotic Cyanobacterial strains that were isolated from rainbow trout intestines grew at pH levels between 5 and 10 (Kim and Austin, 2008). This isolate can pass through the stomach, as shown in this research by its survival and growth at low pH.

Bile Salt Tolerance Test:

Tolerance to detrimental actions of bile salts (0.0, 0.15 and 0.3% at 2 h incubation period) was recorded for 12 isolates. The results show activity and growth in all three concentrations for 2 h incubation period. From the isolates MM03, MM05, MM06, MM07, MM10 and MM12, bile salt concentration increased, and the growth rate of isolates decreased significantly ($p<0.5$). In this study, the bile salt affected the growth rate of isolates MM03 and MM06, limiting their ability, but the bile salt induced the growth rate of isolate MM07 to maximum ability. Furthermore, these strains show the different ability to survive and grow in bile salt. Bile salt tolerance is required for probiotic bacteria to grow and survive in the fish intestines (Salminen *et al.*, 2004). Cebeci and Gurakan (2003) determined that *L. plantarum*, as a probiotic, could survive in 0.3% of bile salt. The probiotics that can tolerate low pH and bile salt means they not only can transit through the stomach and be active in the intestine but also are able to be alive and survive in stressful conditions (Cebeci and Gurakan, 2003). In the present study, MM07 isolates had bile and acid-tolerant and may appear to have high potential probiotics (Fig. 3).

Antibacterial Activity:

Gram-negative intestinal bacteria, especially *Salmonella*, *Shigella*, and *E. coli*, are the most important causes of diarrhea in developing countries (Adesokan *et al.*, 2008). The study examined the antibacterial activities of the isolated strains against the test bacteria *Vibrio cholera, E.coli, Staphylococcus aureus* and *Aeromonas hydrophile*. Isolate MM-07 shows the zone of inhibition in *Vibrio cholera* (14 mm). Likewise, *E. coli* (8 mm), *Staphylococcus aureus* 16 mm) and *Aeromonas hydrophila* (15 mm) (Table 1, Fig. 4). This finding demonstrates the antibacterial activities of probiotic strains against pathogenic organisms, whose safety was taken into consideration. Kaynar and Beyatli (2012) noted the same pattern. The antagonistic actions of *B. subtilis* (5.80 mm), *B. pasteurii* (4.95 mm), and *B. licheniformis* (5 mm) produce a zone of inhibition against *P. fluorescens*. Aly *et al*. (2008) showed that three species of *Bacillus* bacteria used as probiotics suppressed the growth of *A. hydrophila*, and Rengpipat *et al*. (2008) verified this growth suppression using a cell-free cultured broth of five LAB. Kim and Austin (2008) tested two probiotic strains obtained from the intestines of rainbow trout for their antibacterial efficacy against *A.*

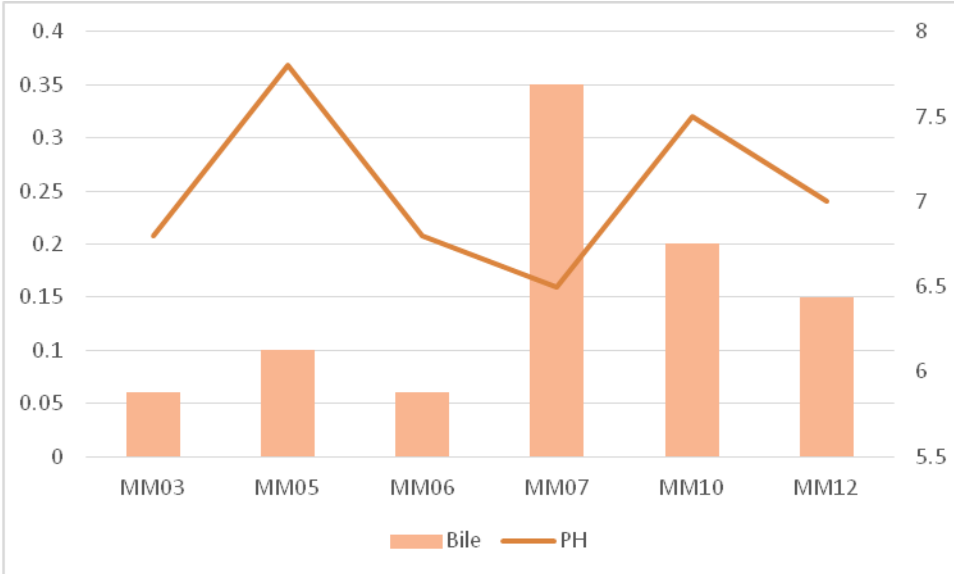

Fig. 3: pH and Bile salt tolerance value.

Table 1: Antibacterial activity:

Pathogens	Zone Of Inhibition(mm)					
	MM-03	**MM-V**	**MM-06**	**MM-VII**	**MM-X**	**MM-XII**
Vibrio cholera	**05**	**-**	**07**	**14**	**02**	**02**
E. coli	**0**	**07**	**-**	**08**	**04**	**-**
S. aureus	**02**	**-**	**02**	**16**	**0**	**01**
Aeromonas sp.	**05**	**03**	**09**	**15**	**05**	**-**

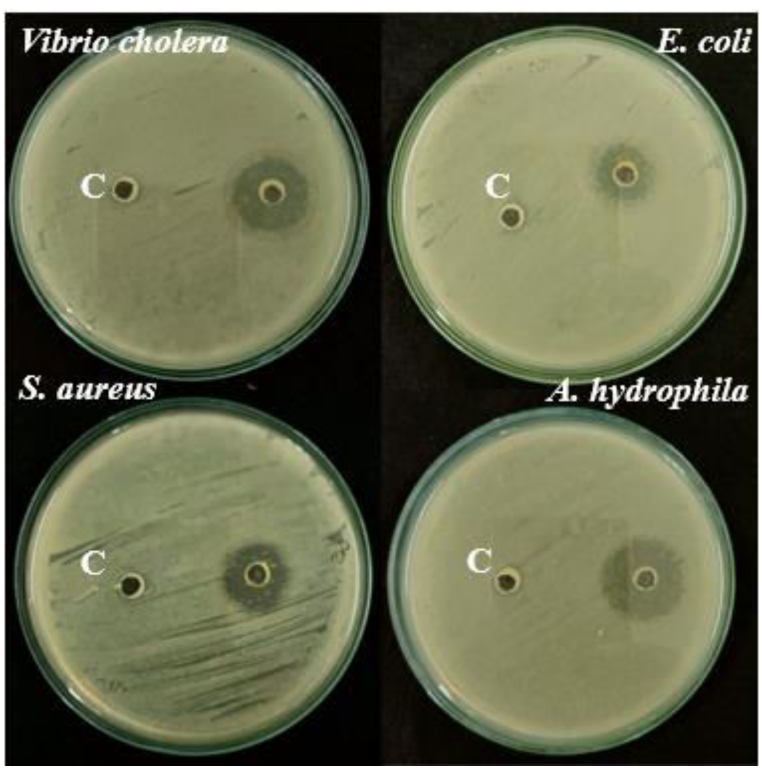

Fig. 4: Antimicrobial activity against fish pathogens.

Table 2: Antibiotic sensitivity test

Antibiotic	MM-03	MM-05	MM-06	MM-07	MM-10	MM-12
Neomycin	**Sensitive**	**Sensitive**	**Sensitive**	**Resistant**	**Resistant**	**Sensitive**
Gentamycin	**Sensitive**	**Sensitive**	**Sensitive**	**Resistant**	**Sensitive**	**Sensitive**
Vancomycin	**Sensitive**	**Sensitive**	**Resistant**	**Sensitive**	**Sensitive**	**Resistant**
Ampicillin	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Resistant**	**Sensitive**
Bacitracin	**Sensitive**	**Sensitive**	**Sensitive**	**Resistant**	**Sensitive**	**Sensitive**
Streptomycin	**Sensitive**	**Sensitive**	**Sensitive**	**Resistant**	**Sensitive**	**Sensitive**
Erythromycin	**Resistant**	**Sensitive**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
Penicillin G	**Sensitive**	**Sensitive**	**Resistant**	**Sensitive**	**Sensitive**	**Sensitive**
Chloramphenicol	**Sensitive**	**Sensitive**	**Sensitive**	**Resistant**	**Resistant**	**Sensitive**

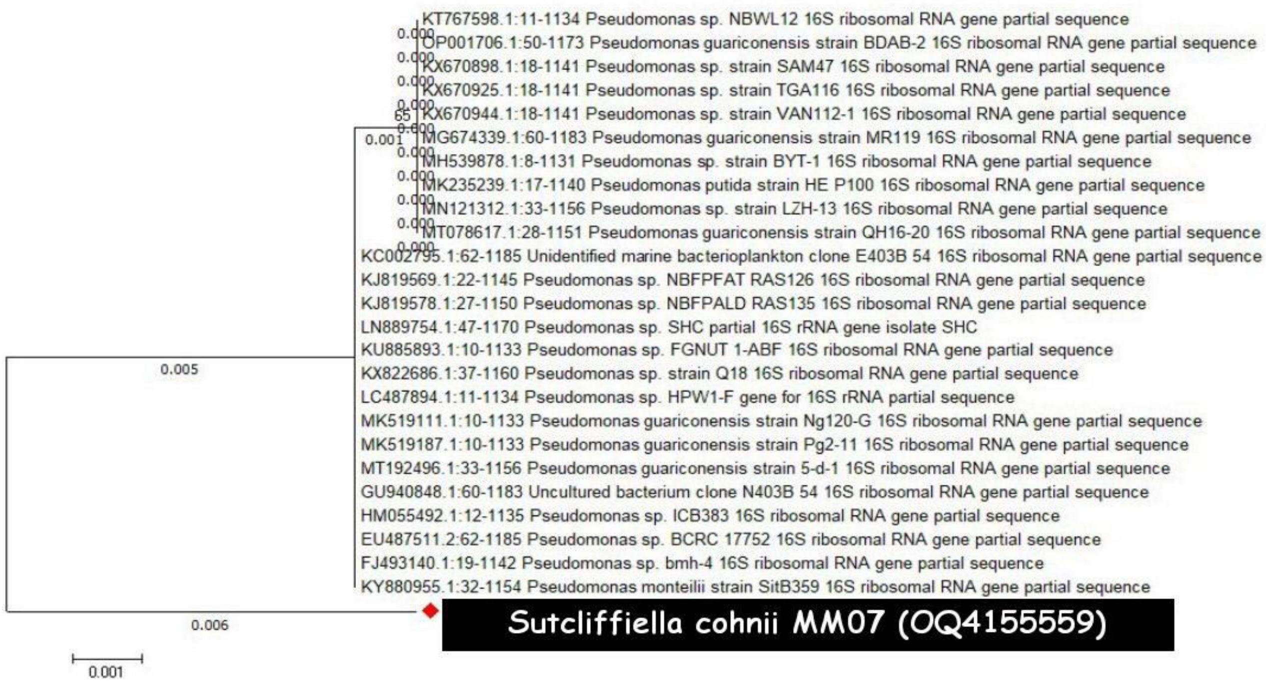

Fig. 5: Phylogenetic tree of *Sutcliffiella cohnii.*

Table 3: Bio-Chemical Test

S. No.	Gram staining	Indole	Methyl Red	VP	Citrate	Catalase	H_2s
MM-03	–	+	+	–	–	+	–
MM-05	+	–	+	+	–	+	–
MM-06	–	–	+	–	–	+	+
MM-07	+	+	–	+	+	+	+
MM-10	+	–	+	+	–	+	+
MM-12	–	–	–	–	+	+	–

hydrophila and *A. salmonicida.* These strains prevented both *A. hydrophila* and *A. salmonicida* from growing. Additionally, Allamesh *et al*. (2012) observed comparable outcomes for *Leuconostoc mesenteroides*. Different probiotic bacterial species may prevent the development of harmful bacteria in various ways. The results of this research therefore support the hypothesis that isolated bacteria may have strong probiotic and anti-adhesion effects against infections.

Antibiotic sensitivity test:

The antibiotic sensitivity and resistance patterns against the nine antibiotics discovered using the six isolated species are shown in Table 2. Six isolated species were found to be mostly antibiotic-susceptible, and there was little evidence of resistance, which may be seen as a good characteristic for bacteria used in probiotics. According to the zone size of the chart of Kirby-Bauer test findings (Bauer *et al*., 1966), inhibitory zone interpretations were made. When certain antibiotics are necessary for therapy, the probiotic may be used concurrently. According to Cebeci and Gurakan (2003) and Kim and Austin (2008), the microflora of the gut may recover more rapidly. Kim and Austin (2008) determined the antibiotic susceptibility of Corynebacterium strains According to their findings, the bacteria were sensitive to chloramphenicol, tetracycline, and cotrimoxazole but resistant to ampicillin, gentamycin, kanamycin, streptomycin, and penicillin. Additionally, they think that using probiotics that are resistant to antibiotics may help when giving fish antibiotics and preserve healthy bacteria in the gut for a long time.

Biochemical and Molecular Identification of Potential Probiotic Isolate:

The isolates were screened for their pH Tolerance, Bile salt Tolerance, Antibacterial activity against fish bacterial pathogens and Antibiotic activity. The results showed, among the Brownspotted fish (*Epinephelus chlorostigma*) gut bacterial isolates, the MM07 isolate showed the highest activity against the wide range of fish pathogens. Hence, it was further identified Biochemically and using 16S rRNA-based molecular identification. The strain was identified as *Sutcliffiella cohnii* NCBI Accession number OQ415559 (Table 3, Fig. 5).

Conclusion

Probiotic bacteria can be used in the rearing of larval stages in aquaculture. Providing a suitable microbial environment during early life stages is crucial for the development and survival of larvae. Probiotics can help establish a beneficial microbial community, outcompeting potentially harmful bacteria and improving the survival and growth rates of larvae. The results of the present investigation suggest that the bacteria *Sutcliffiella cohnii* isolated from the fish gut sample Brown spotted grouper (*Epinephelus chlorostigma*) from the Mudasalodai landing center seems to be a promising candidate for probation.

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