VOLUME 10 ISSUE 2 2024

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 10, No. 2, 1089-1105 (2024)

International Journal of Zoological Investigations
Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

A Novel Probiotic Bacteria Isolated from Epidermal Mucus and Instestine of Marine Catfish (*Plotosus lineatus*)

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Received: 8th May, 2024; **Accepted:** 28th August, 2024; **Published online:** 7th November, 2024

https://doi.org/10.33745/ijzi.2024.v10i02.107

Abstract: Probiotic bacteria play a crucial role in promoting the health and well-being of various organisms, including fish. In this study, we isolated and characterized a novel probiotic bacterium from the intestinal tract and mucus of the catfish *Plotosus lineatus*. The isolation process involved the collection of intestinal samples and mucus samples, followed by culture and identification using molecular techniques. The newly isolated bacterium, *Bacillus* species, *Enterobacter* species and *Pseudomonas* species displayed promising probiotic properties. It exhibited tolerance to the harsh conditions of the gastrointestinal tract and outer membrane of the catfish, such as acidic pH and the presence of bile salts. Furthermore, *Bacillus* species, *Enterobacter* species and *Pseudomonas* species demonstrated antagonistic activity against several potential fish pathogens, suggesting their potential as a biological control agent in aquaculture. To assess its probiotic potential, *in vivo* experiments were conducted, and it was found that the administration of *Bacillus* species, *Enterobacter* species and *Pseudomonas* species to catfish significantly improved their growth performance and enhanced disease resistance. This finding underscores the potential of this novel bacterium to be used as a beneficial probiotic in the aquaculture industry, contributing to the overall health and productivity of catfish. In conclusion, the isolation and characterization of "Bacillus species, Enterobacter species and Pseudomonas species" provide valuable insights into the diversity of probiotic bacteria in the intestinal tract and outer membrane of catfish. This bacterium holds promise as a biocontrol agent in aquaculture and highlights the importance of exploring novel probiotics for the sustainable management of fish health. Further research is needed to identify the species of the bacteria and fully elucidate the mechanisms underlying its probiotic effects and to assess its safety and efficacy in larger-scale aquaculture applications.

Keywords: Probiotic bacteria, Catfish, *Plotosus lineatus*, *Bacillus, Pseudomona*, *Enterobacter*

Citation: Thaiyeba M.B., Sasi Harini S. and Ahmed Fauzia: A novel probiotic bacteria isolated from epidermal mucus and instestine of marine catfish (*Plotosus lineatus*). Intern. J. Zool. Invest. 10(2): 1089-1105, 2024.

https://doi.org/10.33745/ijzi.2024.v10i02.107

Introduction

Probiotics is derived from Greek word and means "prolife. It has been redefined throughout the years as more scientific knowledge and better understanding of its relationship between intestinal

health and general well-being has been gained. FAO/WHO (Food and Agriculture Organization and World Health Organization, 2001) and Reid *et al.* (2003) concentrated exclusively on its health purpose: Live microorganisms which when administered in adequate amounts confer a health benefit on the host. Many probiotics are naturally found in fermented foods, which are created through the process of fermentation. These foods can be a dietary source of beneficial micro-organisms (Marco *et al.*, 2010). Probiotics are widely recognized for their role in maintaining a balanced and healthy gut microbiome. They can help regulate digestion, prevent diarrhea, and alleviate symptoms of gastrointestinal disorders such as irritable bowel syndrome (IBS) (Quigley, 2012). Certain probiotic strains have been shown to enhance the body's immune response, making them beneficial for defending against infections and illnesses (Scott *et al.*, 2014). Probiotic supplements are designed to provide specific strains and concentrations of beneficial bacteria, making them a convenient and controlled source of probiotics (McFarland, 2019). Probiotics are widely used to maintain a balanced gut microbiome, which can help regulate digestion and alleviate symptoms of gastrointestinal disorders. (Quigley,2012). Certain probiotic strains are known to enhance the immune system's response, making them valuable in defending against infections and supporting overall immune health (Scott *et al.*, 2014).

The striped eel catfish *Plotosus lineatus* (Thunberg,1787) is a member of the small family Plotosidae, which has 10 genera and about 40 tropical species, mostly marine but also found in fresh and brackish waters. It is a member of the class Actinopterygii, the ray-finned fishes. They feature a tail like an eel and a head covered with Barbels, just like a catfish. Goblet cells, which are ubiquitously distributed in both the fins and the rest of the fish body, are responsible for producing large amounts of mucus, which appears to be necessary for osmotic protection (Duval, 1925). Typically, the striped eel catfish occupy the coral reefs, estuaries, intertidal areas, and open waters (Gomon, 1984).The gray eel catfish, *Plotosus canius* (Hamilton, 1822), is a close relative of *P. lineatus*, which has gained a threatened status in several locations including India, Bangladesh, and Malaysia (IUCN Bangladesh, 2000; Patra *et al.*, 2005; Usman *et al.*, 2013a) and this condition is potentially to occur in *Plotosus lineatus*. To conserve the striped eel catfish population, there is an immediate need for practical data on its reproductive biology to design effective strategies. Striped eel catfish, *Plotosus lineatus* of the family Plotosidae, known as 'lele laut' or 'sembilang' in Indonesia, is one of the target species by small-scale fishermen (Ball and Rao, 1984; Jumiati *et al.*, 2017; Muhajirah *et al.*, 2018). As an important fishery resource, the fish is traditionally and nationally traded across the Indonesian region and serves as an important source of protein for many coastal communities. Striped eel catfish contains highly essential amino acid nutrients for human health (Manikandarajan *et al.*, 2014) such as monounsaturated fatty acid (MUFA)- 1.37%; polyunsaturated fatty acids (PUFAsω-6)- 18.0%; PUFAω-3- 32.0% (Osman *et al.*, 2001; Sahena *et al.*, 2009) and other PUFAs - 34.0%. These essential amino acids have important roles and usages in pharmaceutical products, food additives, food supplements, and antioxidants(Ray *et al.*,2014).Eel catfish meat also contains carbonate 3.26 mg g^{-1},protein 14.69 mg g^{-1}, and fat 1.48 mg g^{-1} (Suganthi *et al.*, 2015). Like in other fish species, the microbial community in the gut of *Plotosus lineatus* (striped eel catfish) plays an important role in various aspects of the fish's health and digestion. Probiotic bacteria are beneficial microorganisms that can aid in the maintenance of gut health and overall well-being. The presence of certain probiotic bacteria in *Plotosus lineatus* and other fish species can help with digestion and disease resistance. The gut microbiota of *Plotosus lineatus* may contain beneficial bacteria that aid in the control of potential pathogen growth. These beneficial bacteria compete for resources and space in the gut with harmful microorganisms, lowering the risk of

infection. Mucus plays a protective role due to the presence of biologically active molecules (Fletcher,1978). Mucus is also engaged in the defense mechanisms used by microorganisms (Hildemann, 1962; Kitzan and Sweeny, 1968). This mucus layer also lessens friction as the fish swims through the water, increasing its agility and environmental efficiency.

In this study, we isolated and characterized a novel probiotic bacterium from the intestinal tract and mucus of the catfish *Plotosus lineatus*. The isolation process involved the collection of intestinal samples and mucus samples, followed by culture and identification using molecular techniques.

Materials and Methods

Fish collection

The healthy *Plotosus lineatus* (Thunberg,1787) fishes were collected during the month of August 2023, at N4 beach (13.1307°N, 80.3032°E), Chennai, Tamil Nadu, India. Fishes were collected with the help of local fishermen, and the collected fishes were immediately transported to the laboratory in covered plastic containers.

Sample preparation (*mucus and intestine*)

The harvested fish underwent a meticulous procedure in which they were initially rinsed with sterile distilled water, followed by a thorough cleansing with a 75% alcohol solution to eradicate any potential external microbial contaminants. Subsequently, the fish were subjected to anesthesia, and their intestines were aseptically extracted. These removed intestines were meticulously subjected to a triple-washing process using a normal saline solution (0.85% NaCl w/v). After this purification, the intestines were finely diced and subsequently homogenized with a saline solution (Allameh *et al.*, 2012).

In mucus collection after a 2-day acclimation period, the fish were subjected to a 48 h fasting regimen. No chemicals or anesthesia were administered to the fish during the process of collecting skin mucus. Five specimens of the striped eel catfish, *Plotosus lineatus* (225g) were chosen. The mucus was meticulously obtained from the dorsal surface of the fish's body by gently scraping it using a sterile plastic spatula, moving from the head to the tail in an anterior-posterior direction. Multiple mucus samples were collected at regular intervals (five attempts) within a single day. To prevent contamination from the intestines and urinogenital area, mucus collection from the ventral region was avoided (Chong *et al.*, 2005). The collected mucus samples were promptly frozen at -20ºC to prevent both microbial growth and any potential degradation of proteins. Buffered saline and nutrient agar was used to prepare culture.

Isolation of intestine and mucus bacteria

Both processes begin with sample collection and homogenization. The homogenized samples are then centrifuged at 10,000 rpm, resulting in the separation of a supernatant. For intestinal bacteria isolation, the supernatant is adjusted to a final volume of 10 ml with sterile seawater and subjected to serial dilution to obtain a total of 106 dilutions. Similarly, for mucus-associated bacteria isolation, the supernatant is adjusted to 10 ml with sterile seawater and subjected to serial dilution. Subsequently, each dilution from both processes is evenly spread onto Petri plates containing autoclaved Nutrient Agar. These plates are then incubated at 37°C for 24 h or until bacterial colonies become visible. After the incubation period, pure bacterial colonies are isolated through repeated streaking to ensure the isolation of a single colony. For intestinal bacteria, isolated colonies are transferred to fresh LB agar medium and stored at -80°C in LB broth supplemented with 30% glycerol for future studies and analyses (Nakanishi and Nishijima, 1996). Meanwhile, for mucus-associated bacteria, various bacterial colonies with distinct morphologies are selected and further purified through successive

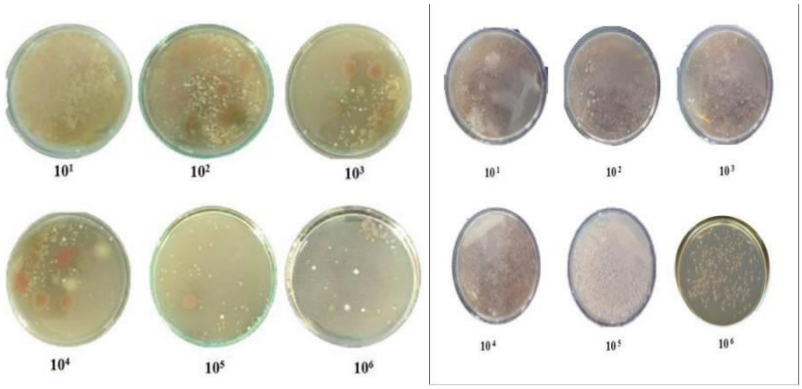

Fig. 1: Isolation of intestinal and mucus bacteria.

Fig. 2: Preparation of selected isolates (mucus and intestine).

re-streaking. These pure bacterial cultures are subsequently maintained on Nutrient Agar slants (Fig. 1).

Selected isolates (*mucus and intestine*)

The chosen isolates, consisting of two distinct colonies, underwent additional purification via a single colony isolation method. This process aimed to ensure the purity of the bacterial cultures by eliminating potential contaminants. The refined colonies were then carefully stored on Luria Agar at a temperature of 4°C, making them readily available for future applications. Similarly, two specific colonies were selected for further purification using the same technique. Once purified, these colonies were also maintained on Luria Agar at 4°C, awaiting future utilization (Fig. 2)(Asokan and Puttaswamy, 2007).

Cultural characterization of bacterial colony (*mucus and intestine*)

Following the incubation period of the inoculated Petri plates, the bacterial colonies were carefully examined for various characteristics (Table 1). These included assessing their size, which ranged from pinpoint to large colonies, and observing their coloration or pigmentation. Additionally, the form of the colonies was documented, categorizing them as circular (with an unbroken peripheral edge), irregular (featuring indented peripheries), or rhizoid (displaying root-like and spreading growth patterns) based on their growth patterns. The margin of each colony was also scrutinized, using terms such as entire (sharply defined and even), lobate (marked by noticeable indentations), undulate (showing wavy indentations), serrate (resembling a tooth-like appearance) or filamentous (exhibiting a thread-like spreading edge) to describe their appearance. Furthermore, the elevation of colony growth on the agar surface was assessed, distinguishing between"flat"

Table1: Culture characterization

S. No.	Cultural characterization	I-1	I-2	M-1	M-2
1	Size	Moderate	Pinpoint	Moderate	Large
2	Pigmentation	White	White yellow	White	White
3	Form	Irregular	Circular	Circular	Rhizoid
4	Margin	Filamentous	Entire	Entire	Serrate
5	Elevation	Umbonate	Flat	Flat	Raised

Table 2: Microscopical characterization

S. No.	Microscopical characterization	I-1	I-2	M-1	M-2
1	Motility	+	-	+	+
2	Gram's Stain	G+	G-	G+	G-
3	Shape	Rod	Rod	Rod	Rod

when the elevation was indiscernible, "raised" when slightly elevated, "convex" when displaying a dome-shaped elevation, or "umbonate" when raised with an elevated convex central region. This comprehensive characterization process enabled a thorough examination of the bacterial colonies' morphology and growth features, providing valuable insights into their identity and behavior.

Microscopical characterization (*mucus and intestine*)
Motility test

Motility assessment was conducted using the hanging drop method. In this procedure, a ring of petroleum jelly was meticulously applied around the concave area of a depression slide using a cotton swab. A loopful of the bacterial culture was then carefully deposited at the center of a clean cover slip using aseptic techniques. The depression slide was positioned with its concave side facing downward, enclosing the culture drop on the cover slip. After gently pressing to seal the slide to the cover slip, it was swiftly inverted to ensure the culture drop adhered to the inner surface of the cover slip. The examination of the culture drop commenced with the low-power objective (10x) to bring it into focus, followed by observations under the high-power objective (40x). This method provided a detailed examination of bacterial motility under the microscope, offering valuable insights into the microorganisms' movement patterns (Table 2).

Gram staining

The initial screening process commenced with Gram staining (Table 2). This involved carefully applying a small quantity of overnight bacterial culture onto a clear glass slide to create a uniform smear. The slide was then gently heated to secure the bacterial cells in place. Next, a liberal application of crystal violet solution was administered to the slide, and it was allowed to stand for one minute. Subsequently, the slide was rinsed with water and generously coated with Gram's iodine solution. After another minute, the slide underwent a decolorization phase using 95% ethyl alcohol, followed by another round of rinsing. To conclude the staining procedure, the slide was

Table 3: Biochemical assays

S. No.	Tests	Intestine		Mucus	
		Positive	Negative	Positive	Negative
1.	Casein hydrolysis test				
	A) Fine white	+ve			-ve
	B) White striated	+ve		+ve	
2.	Catalase test				
	A) Fine white	+ve		+ve	
	B) White striated	+ve		+ve	
3.	Potassium hydroxide test				
	A) Fine white	+ve		+ve	
	B) White striated	+ve		+ve	
4.	Starch hydrolysis test				
	A) Fine white	+ve		+ve	
	B) White striated	+ve			-ve
5.	Gelatin liquefication test				
	A) Fine white	+ve			-ve
	B) White striated	+ve		+ve	

counter-stained with safranine and observed under a light microscope. During the microscopic examination, Gram-positive organisms manifested a violet color, while Gram-negative organisms exhibit a pink hue (Fig. 3).

Shape

Following Gram's staining procedure, a microscopic examination was carried out at 40x magnification to determine the shape of the bacteria present (Table 2). The observed bacterial shapes were categorized as short rods, straight rods, slightly curved rods, ovoid(oval-shaped), rod chains, or cocci(spherical).

Biochemical characterization (mucus and intestine)

Biochemical characterizations (Table 3) such as catalase, potassium hydroxide test, casein hydrolysis, starch hydrolysis and gelatin hydrolysis were performed as follow:

Catalase test

To identify potential probiotic strains, a pure colony of each presumptive isolate was positioned on a slide. Subsequently, a drop of hydrogen peroxide (H_2O_2) with a concentration of 3% was introduced to the colony. The observation was focused on the immediate formation of bubbles, which indicated a positive result. Those isolates exhibiting this prompt bubble formation, suggestive of catalase activity, were considered potential candidates for probiotics (Fig. 4)(Lelliott and Stead, 1987).

Potassium hydroxide test

To identify potential probiotic strains, a pure colony of each presumptive isolate was positioned on a slide. Subsequently, a drop of potassium hydroxide with a concentration of 3% was introduced to the colony. Mix the bacterial sample and hydrogen peroxide solution thoroughly by spreading the mixture on the slide using the sterile needle. If the loop is not seen, then the bacterium is positive suggestive of potassium hydroxide activity (Fig. 5) (Ryu, 1940).

Casein hydrolysis

To identify potential probiotic strains, prepare casein hydrolysis medium by using skim milk powder 50 g, peptone 2.5 g, agar 7.5 g in distilled water whereas maintained at pH 7.2 and the solution was autoclaved. It was Poured on plates and the medium allowed to solidify. The cultured bacterium was streaked on the medium and incubated for 48 h in an inverted position and checked for clear zone along the line around the growth of bacterium. The observation was focused on the plate around or beneath colony growth (hydrolysis) it indicates the positive result. Suggestive of casein activity and are considered potential candidates for probiotics (Fig. 8)(Verniere *et al.*, 1998).

Starch hydrolysis

To identify potential probiotic strains, starch hydrolysis test (also known as amylase test which is a differentiate nutritive medium), the test organisms are inoculated onto a starch plate and incubated at 30°C until growth is seen (i.e., up to 48 h). The petri plate is then flooded with iodine solution if there is no enzyme present and therefore no hydrolysis amylase and iodine react together to form a blue color depending on the concentration of the iodine used, iodine turns into blue, purple, black in the presence of starch. The observation was focused on purple black color appearing in the medium however, clear halo will appear around the colonies of amylase positive species. Suggestive of starch activity, were considered potential candidates for probiotics (Fig. 7)(Fahy and Persley, 1983).

Gelatin liquefication

To identify potential probiotic strains, a media solution comprising 1.5 g beef extract, 2.5 g peptone, and 60 g gelatin dissolved in 500 ml of distilled water was dispensed into 5 ml test tubes, sealed, and autoclaved for sterilization. The test tubes were inoculated with fresh bacterium culture of 24 h old and incubated at 27°C. After 72 h the tube were placed at 4°C for 25 to 30 min. After incubation, the tubes are examined for changes in the gelatin. The liquefication can be observed as a liquid or semi liquid streak within the otherwise solid gelatin column. That indicates a positive result. Suggestive of gelatin liquefication activity, this was considered as potential candidates for probiotics (Fig. 6)(Cowen, 1974).

Resistance to temperature

Bacterial isolates were initially cultured in MRS broth overnight. Each of the overnight cultures was then separately introduced and incubated at various temperatures:15°C, 25°C, 37°C, and 45°C for a duration of 48 h (Table 4). The growth of these isolates was assessed by measuring turbidity at a wavelength of 610 nm using a spectrophotometer. Subsequently, 1 ml of the bacterial inoculum was transferred to MRS plates via the pour plate method and incubated (Figs. 13, 15)

Resistance to pH

Bacterial cultures that had been grown overnight at 37°C were collected by centrifugation at 10000 rpm for 5 min at 4ºC.The resulting cell pellets were washed twice with sterile phosphate-buffered saline (PBS) at pH 7.3 and then resuspended in 1 ml of PBS. Each bacterial isolate was subsequently diluted 1:100 in PBS adjusted to different pH levels, specifically pH 3, 4, 5, 6 and 7. The pH levels were adjusted using 1N HCl. The bacterial cells in these various pH-buffered solutions were then incubated at 37ºC, and the viable bacterial cells were assessed

Table 4: Resistance to temperate for intestine sample

S. No.	Isolate	Growth at temperature (℃)			
		15	25	37	45
1	I-1	+	++	++	+
2	I-2	+	++	++	-

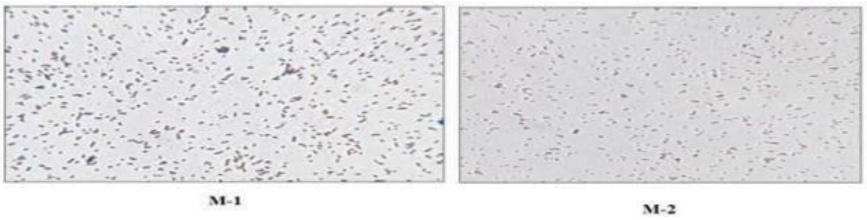

Fig. 3: Gram's staining.

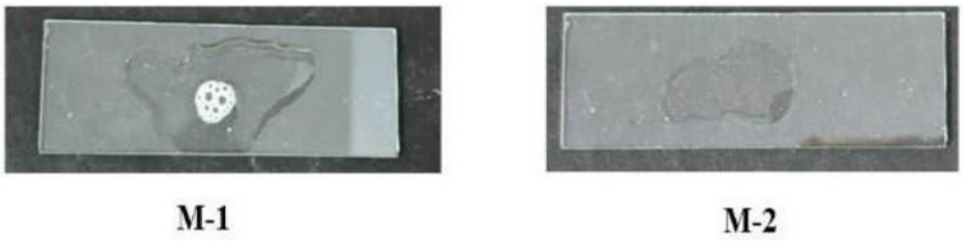

Fig. 4: Catalase Test.

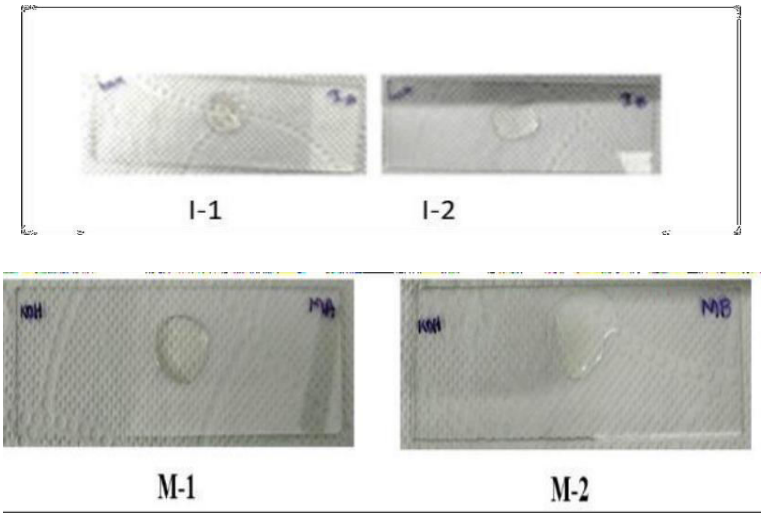

Fig. 5: Potassium hydroxide test.

at 0, 1, 2, and 3 h intervals by plating 100 µl from each tube onto nutrient agar plates. These plates were incubated at 37 °C for 24 h, and bacterial growth was quantified in terms of colony-forming units (CFU) per milliliter. Additionally, the percentage of bacterial cultures that survived exposure to different pH values was calculated (Figs. 11, 12, 16) (Ahire *et al.*, 2011).

$$\text{Survival (\%)} = \frac{\text{Number of viable cell survived (CFU/ml)}}{\text{Number of initial cell inoculated (CFU/ml)}} \times 100$$

Bile salt tolerance

Growth of bacteria in 0.3%(w/v) ox bile was tested (Trivedi *et al.*, 2013). Initially, bacterial cultures, grown overnight at 37°C, were subjected to centrifugation at 10,000 rpm for 5 min at 4°C. The pellet obtained from this step was then collected and reconstituted in PBS (pH 7.3) to the same volume. Following this, a bacterial cell suspension containing 0.5 ml with a concentration of 108 CFU ml^{-1} was introduced into 10 ml of nutrient broth, which was supplemented with 0.3% (w/v) ox-bile. In parallel, a control group was set up using nutrient broth without ox-bile. All these bacterial cultures were incubated at 37°C for varying durations, including 0, 2, 4, and 6 h, during which cell counts were determined by culturing 0.1 ml from each culture onto nutrient agar plates. The agar plates were then incubated at 37°C for 24 h to facilitate bacterial growth, quantified in terms of colony-forming units (CFU) per milliliter. Finally, the percentage of surviving bacterial cultures was computed based on established calculations (Figs. 14, 17).

$$\text{Survival (\%)} = \frac{\text{Number of viable cell survived (CFU/ml)}}{\text{Number of initial cell inoculated (CFU/ml)}} \times 100$$

Collection of bacterial pathogens

A probiotic should have a desirable antagonistic activity towards pathogenic microorganisms. To screen the antibacterial activity of the isolates, the bacterial fish pathogens such as *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio angullarium*, *Aeromonas hydrophilia* were obtained from the lab culture and the cultures were maintained on tryptone soy agar (TSA) slants at 4ºC.

Preparation of cell-free supernatant

The bacterial CFS was prepared as follows. The bacterial cultures were grown in nutrient broth for 24 h at 37°C under aerobic conditions. The broth cultures were centrifuged at 10,000 rpm at 4°C for 10 min. The collected culture-free supernatants were stored at -20°C (Aslim *et al.*, 2005).

Antibacterial activity

Briefly, 100 µl of the bacterial pathogens (Adjusted to 0.5 McFarland standards) were spread plated on Mueller-Hinton agar. The agar surface was bored using a sterile corn borer to create wells of 6mm diameter and filled with 100 µl of CFS of the isolates (Manhar *et al.*, 2016). The plates were incubated at 37ºC for 24 h and zones of clearance around the wells were measured and recorded (Figs. 9, 10).

Results and Discussion

When developing microorganisms for use as probiotics in aquaculture, it is important they should be regarded as safe, not only for the aquatic hosts, but also for their surrounding environments and humans (Muñoz–Atienza *et al.*, 2013). A list of characteristics for potential probiotic bacteria to be used in aquaculture species was reported by Vine *et al.* (2006) and extended by Merrifield *et al.* (2010). First, the candidate microorganism should be naturally occurring and nonpathogenic in the host organisms native habitat. The microorganism should also be easily cultured with advantageous growth characteristics, such as a short lag period and generation time, as well as strong growth at the host organisms rearing temperature. The candidate probiotic should have positive traits to culture the host gut, such as resistance to bile salts and low pH values, as well as the adherence within intestinal mucus, and the ability to colonize the intestinal epithelial surface. It is also essential that there are no health risks; therefore, it is important that the

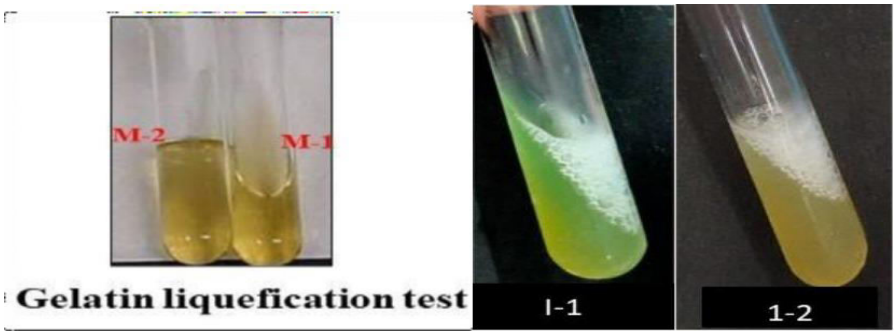

Fig. 6: Gelatin liquefication test.

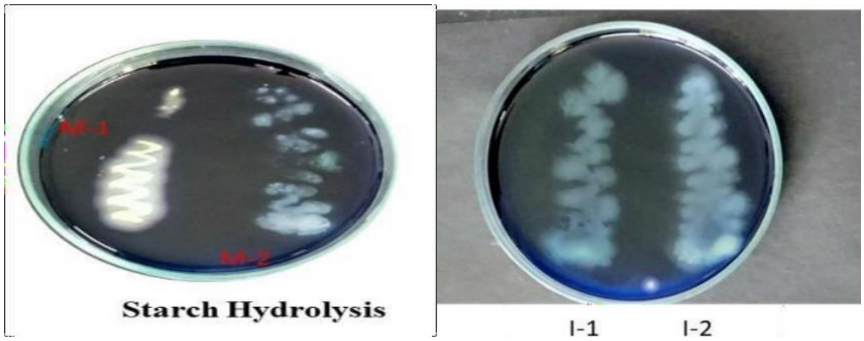

Fig. 7: Starch Hydrolysis test

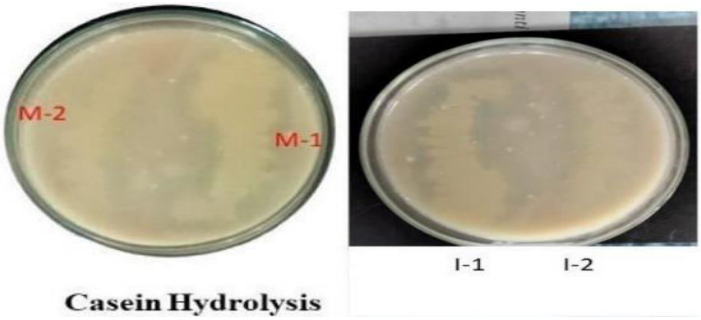

Fig. 8: Casein Hydrolysis test.

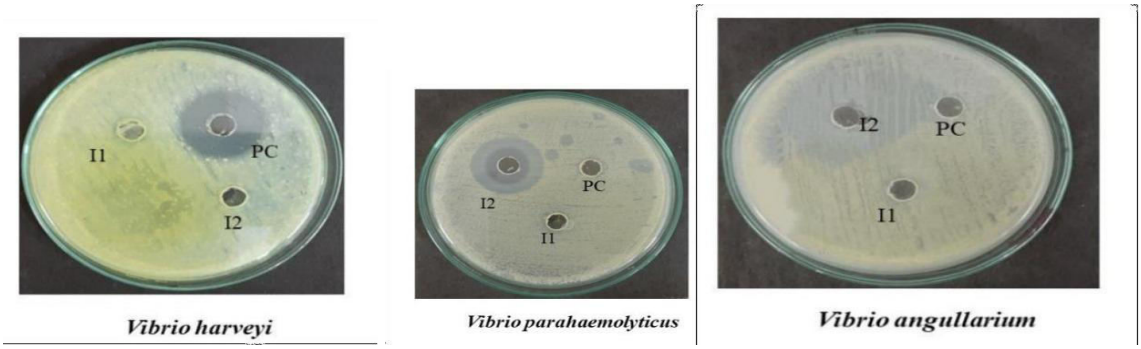

Fig. 9: Antibacterial activity of the selected mucus isolates against *Vibrio parahaemolyticus*, *Vibrio angullarium*, and *Vibrio harveyi.*

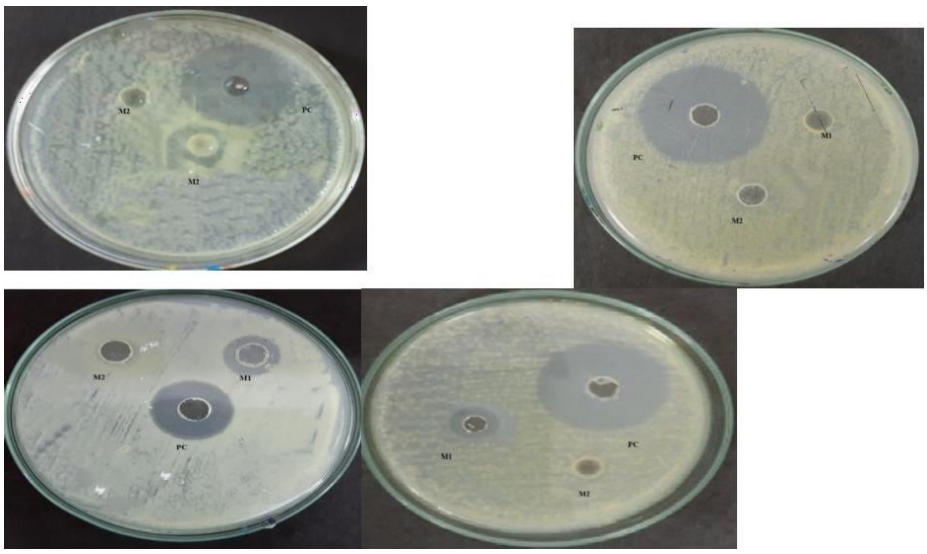

Fig. 10: Antibacterial activity of the selected (M1 and M2) mucus isolates against *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio angullarium*, and *Aeromonas hydrophilla.*

microorganisms do not possess any plasmid encoded antibiotic resistance genes. Finally, the candidate probiotic should have positive effects on fish health and/or nutrition. An example would be the exhibition of antagonistic properties towards one or more key pathogens (e.g., *Aeromonas salmonicida* and *Yersinia ruckeri*). The probiotic may also have relevant nutritional benefits, such as production of extracellular digestive enzymes. For example, production of chitinase or cellulase in cases where fish are being fed chitin-rich or plant-based diets, or production of vitamins. The aim of this work was to isolate lactic acid bacteria (LAB) from the intestinal content of catfish (*Plotosus lineatus*). In the present investigation, bacterial isolates showed high inhibitory activity against fish pathogens, *Vibrio parahaemolyticus, Vibrio angullarium* and *Vibrio harveyi*. Similar observation was recorded by Mukherjee and Ghosh (2014) where strains of Bacilli isolated from the gut of *Catla catla* inhibited growth of 7 fish pathogens that includes species of *Aeromonas* and *Pseudomonas* by double agar layer method. In another study (Muthukumar and Kandeepan, 2015), gut adherent probiotic strains isolated from four Indian fish species showed antagonism to *A. hydrophila. Aeromonas* are known to be the commonest disease-causing bacteria for freshwater fish (Austin and Austin, 2007), whereas *Pseudomonas sp*. is also described as an opportunistic pathogen of various fish species (Bøgwald and Dalmo, 2014). Hence, antagonism to potential fish pathogens is considered as an essential criterion for probiotic selection in aquaculture (Verschuere *et al.*, 2000). Del'Duca *et al.* (2013) have demonstrated capacity of LAB isolated from fish in inhibiting pathogen such as *Bacillus sp*. isolated from gut of Nile tilapia against *Aeromonus hydrophila*. *Edwardsiella tarda*, *Pseudomonas fluorescens* and *Pseudomonas pestida*. Sergio Alonso *et al.* (2018) have worked on isolation of *Lactobacillus spp*. in African catfish *Clarias gariepinus* as probable probiotics in aquaculture and isolated probiotic bacteria from cultured African catfish *Clarias gariepinus* that could be used to fight bacterial fish pathogens. *Lactobacillus spp*. was successfully isolated and identified from the intestine of *C. gariepinus* using biochemical characteristics. Pathogenic bacteria were also isolated from the environment. Similar species include *E. coli*, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila*. The solitary *Lactobacillus spp*. was tested for antagonistic activity against

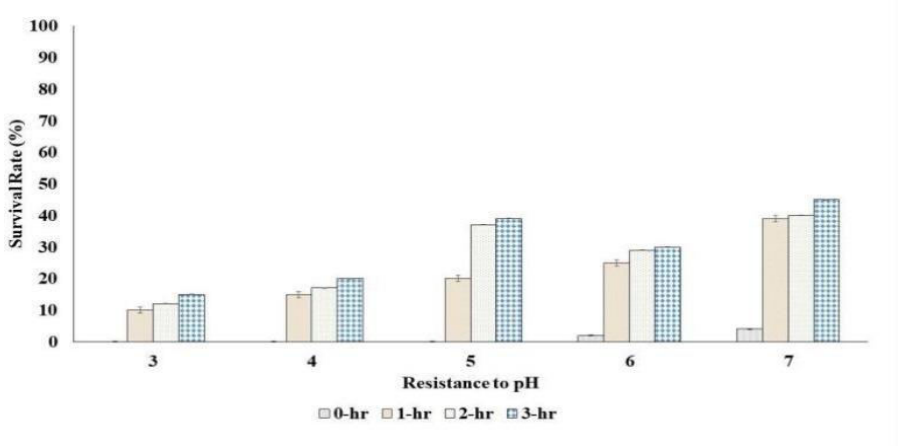

Fig. 11: Isolates of bacteria survival rate against the pH.

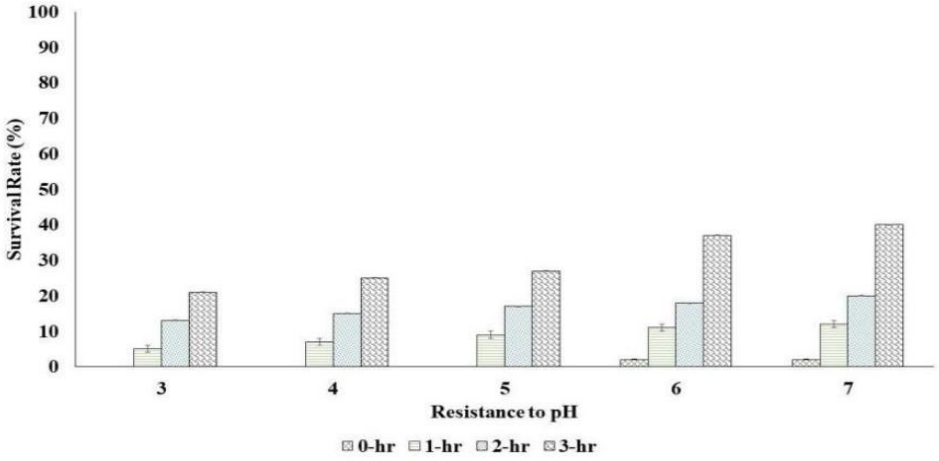

Fig. 12: Isolates of bacteria survival rate against the pH.

isolated bacterial pathogens *in vitro*. Disc diffusion method was used. Two *Lactobacillus spp*. each plate of pathogenic bacteria cultured. *Lactobacillus spp*. was found to be comparable to the antibiotic (Amoxicillin) in terms of antagonistic activity, as measured by measuring the zone of inhibition against *E. coli*, *V. parahaemolyticus*, and *A. hydrophila. Lactobacillus spp.* had the greatest zone of inhibition against *A. hydrophilia* (8.5 mm) and the lowest zone (7.25 mm) for *E. coli*. Antibiotic (Amoxicillin) antagonistic activity was demonstrated. The zone of inhibition was 14 mm for *E. coli* and 7 mm for *V. parahaemolyticus. Lactobacillus spp.* was successfully isolated from the intestine of catfish. The present study revealed the antibacterial impregnated paper discs and one antibiotic (Amoxicillin) and one blank disc were placed in effect of skin mucus extract of *Plotosus lineatus* against *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio angullarium*, *Aeromones hydrophila*. The skin mucus of *Clarias batrachus* exhibited strong inhibitory activity against five pathogenic bacteria, namely *Escherichia coli*, *A. hydrophila*, *Pseudomonas aeruginosa*, *Vibrio fischeri* and *Vibrio anguillarum,* on agar plates (Loganathan *et al.*, 2011). In the present study *Plotosus lineatus* exhibited the antibacterial activity which is against the pathogen such as *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio angullarium* and *Aermones hydrophil* of agar well diffusion assay on agar plates. The acidic

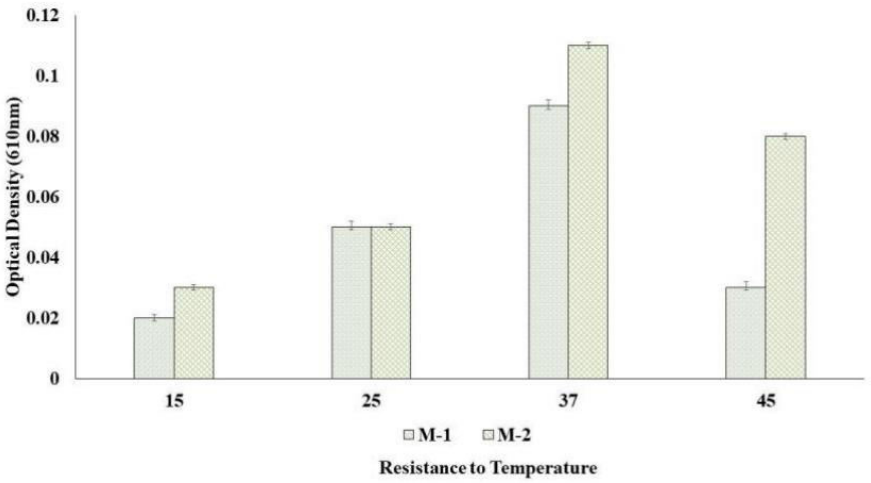

Fig.13: Isolates of bacteria optical density against the temperature.

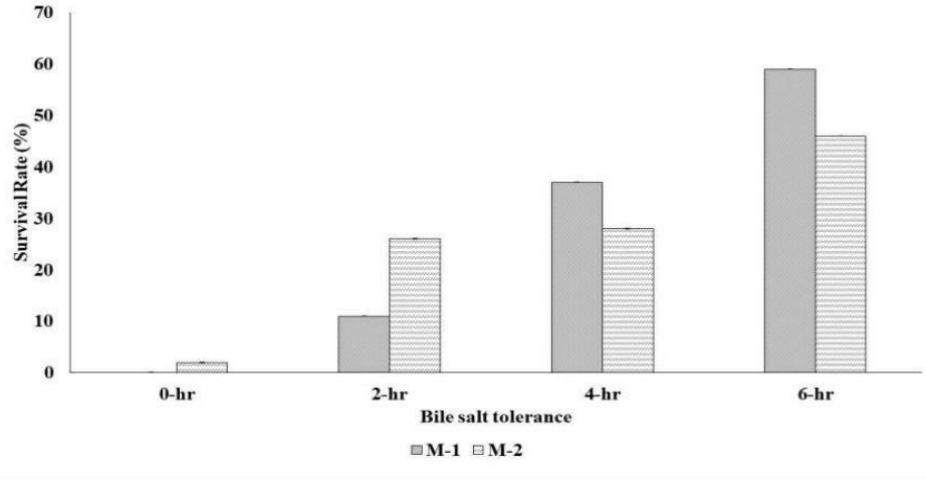

Fig.14: Isolation of bacteria survival rate against bile salt tolerance.

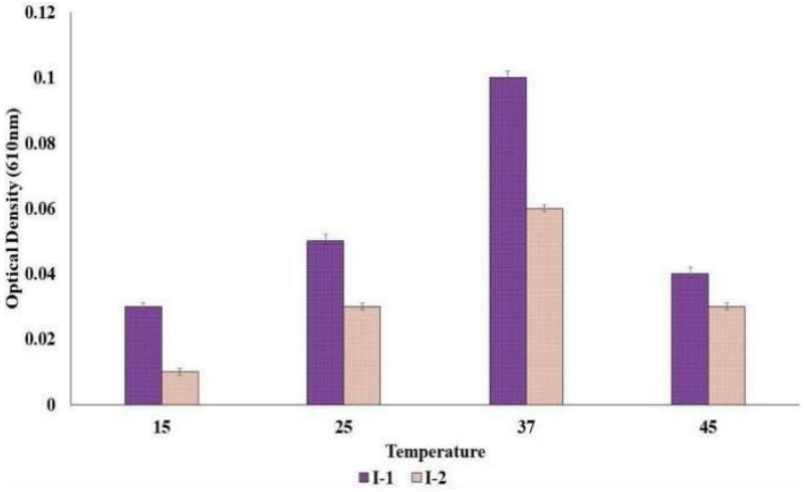

Fig.:15 Isolates of bacteria optical density against the temperature.

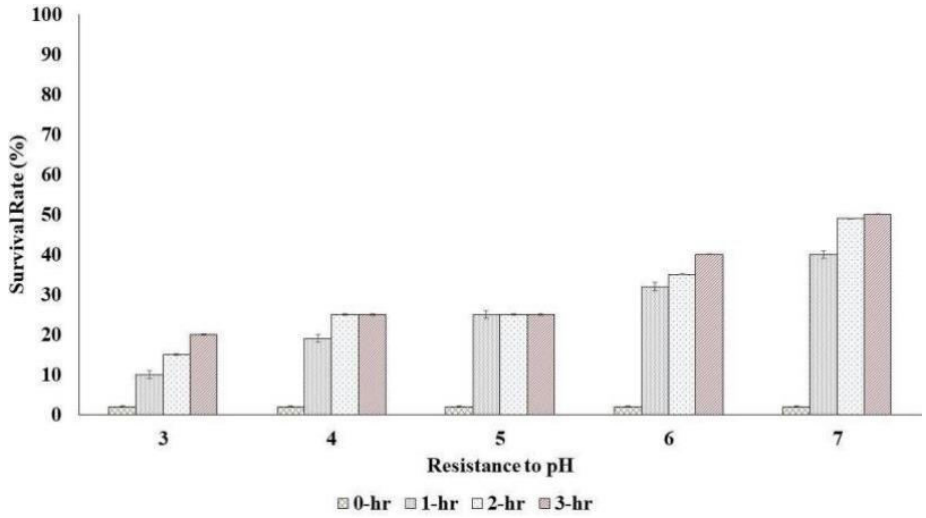

Fig.16: Isolation of bacteria survival rate against the pH.

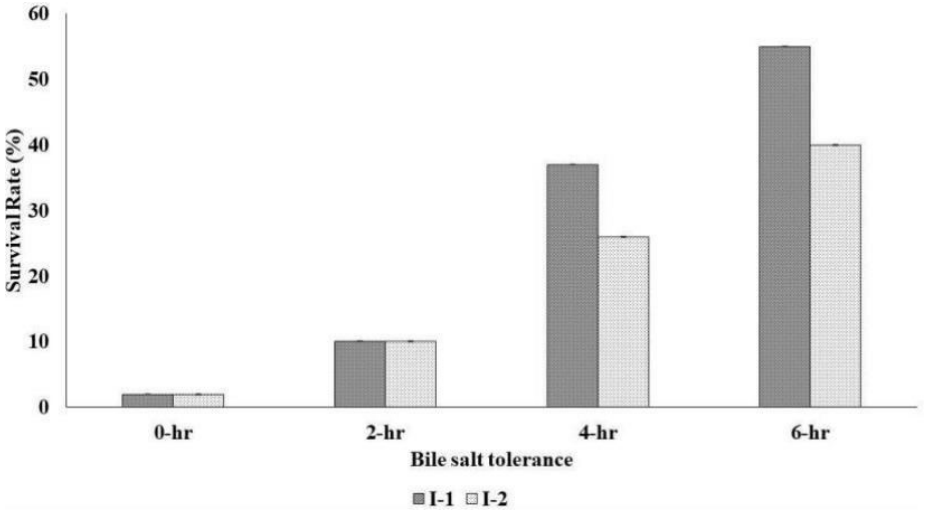

Fig.17: Isolation of bacteria survival rate against the Bile salt tolerance.

mucus extracts from koi carp and striped bass revealed no detectable levels of antibacterial activity (Subramanian *et al.*, 2008). In the present study the mucus extract from *Plotosus lineatus* revealed a detectable levels of antibacterial activity of pathogens such as *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio angullarium* and *Aeromanes hydrophilia* which is against the selected bacterial strains. Kuppulakshmi *et al.* (2008) reported that in the aqueous skin mucus extracts of *Cirrhinus mrigala*, the inhibition zones against the same bacterial strains were in the range 7– 30 mm. The variation in the inhibition zone, reported by different workers in the same fish species could be due to the differences in the ecological condition of the habitat or could be due to the different methods used for antibacterial assay. The acidic mucus extract of *Channa striatus* inhibited the growth of three human pathogens, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and not inhibited fish pathogen *Aeromonas hydropila*. The acidic mucus extracts of brook trout, haddock and hagfish showed bactericidal activity against a wide range of fish and human pathogens (Subramanian and Mackinnon, 2008). In the present study, the skin mucus extract of *Plotosus lineatus* exhibited

antibacterial activity of pathogens such as *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio angullarium* and *Aeromones hydrophilia* against the selected strains. Hellio *et al.* (2002) had investigated the antibacterial effects of the aqueous mucus extracts of 13 fish species and did not observe any antibacterial activity; however, these authors did not study the properties of crude mucus extract. Ebran *et al.* (2000) stated that only the hydrophobic components of crude epidermal mucus of fresh water and sea water fish exhibited strong pore-forming properties, which were well correlated with antibacterial activity. Bragadeeswaran and Thangaraj (2011) had revealed the strong antimicrobial effect of crude mucus extract of *Angullia angullia* against *E. coli*, *P. aeruginosa* and *S. aureus* and no activity was seen against *K. pneumoniae*. Furthermore, they did not notice bactericidal effect in aqueous mucus extract against *P. aeruginosa* pathogens. The current study shows that the isolated candidate could be a promising probiotic for use in aquaculture, feeding, due to its high potential in vitro activity against aquatic pathogenic bacteria. In the present study, the intestine's weight was determined and an equal proportion of PBS by volume was added. The content was then homogenized in ice using 15 ml borosilicate glass tissue homogenized under sterile conditions. Following that, 0.5 ml of the gut homogenate was diluted in 4.5 ml PBS. PBS was used to serially dilute the mixture and 0.1 ml of the aliquot was spread on Luria-Bertani agar plates. The plates were incubated at 30°C for 24 h. Under the same culture conditions, single colonies were chosen at random and inoculated into LB media for mass culture. To obtain very pure colonies, the isolates were streaked repeatedly. To characterize bacterial cultures thoroughly, this study used rigorous microscopic techniques. The combination of motility testing, Gram staining, and shape analysis not only aided in bacterial species identification but also provided detailed insights into their motility and morphological characteristics. The biochemical properties of the bacterium were investigated by subjecting bacterial isolates to various biochemical tests, including KOH, starch hydrolysis, gelatin liquefication, casein hydrolysis and catalyze tests. The goal of this screening process was to find probiotic isolates that were resistant to different environmental conditions, tolerated bile salts, and had antagonistic activity against fish pathogens. The researchers were able to isolate a unique probiotic bacterium from the catfish's intestinal microbiota. The isolated strain exhibited several beneficial properties, including the ability to improve gut health and overall performance in catfish.

Conclusion

Novel probiotic bacteria isolated from epidermal mucus and intestine of marine catfish (*Plotosus lineatus*) have yielded promising results and insights. This study has revealed the presence of bacteria with probiotic properties in a previously underexplored ecological niche. The findings indicate that these mucus-associated bacteria have the potential to contribute to the health and well-being of marine catfish, potentially enhancing their resistance to pathogens and promoting growth.

The research underscores the significance of the epidermal mucus and intestinal bacteria as a reservoir of beneficial microorganisms, which can be harnessed for aquaculture and environmental management. Whereas the identified bacterial species were *Bacillus species* and *Pseudomonas species, Enterobacter species* (mucus sample). This newly discovered strain holds great promise for enhancing the health and growth of catfish in aquaculture systems. Our findings demonstrate its ability to positively influence gut health and overall performance, making it a valuable candidate for sustainable aquaculture practices. The discovery of beneficial microorganisms in previously underexplored areas like epidermal mucus underscores the importance of diversifying sources for probiotics in aquaculture. The findings suggest that these bacteria could fortify catfish against pathogens and improve overall well-being, thereby

contributing to sustainable aquaculture methods. The isolation and identification of specific strains offer valuable candidates for further studies and potential applications in sustainable aquaculture practices. To fully unlock the practical utility of these probiotic candidates, additional research is required to elucidate their mechanisms, optimize their production, and conduct field trials.

As we move forward, further research is essential to unravel the precise mechanisms through which this probiotic bacterium benefits catfish. Additionally, field trials and practical applications are required to fully harness its potential. The isolation of this probiotic strain from the intestinal microbiota of catfish highlights the importance of exploring unique ecological niches for the discovery of beneficial microorganisms, which can play a pivotal role in advancing the field of aquaculture and ensuring the sustainability of this vital industry. Future work can be carried out to assess the species of the two bacterial genera that have been isolated from the intestine of catfish and this would serve as a breakthrough in the probiotic industry and improve the wellbeing of the commercially important fishes.

Acknowledgements

The authors are thankful to the P.G and Research Department of Zoology at JBAS College Teynampet in Chennai and University of Madras in Chennai for providing the facilities for this research.

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