

Original Research Article

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Bioprospecting Fish Gut-Associated Microbes for Potential Probiotic Applications in Aquaculture

P. Gokul¹ and S. Priya Grace^{id}2*

Department of Microbiology, Hindustan College of Arts & Science, Chennai, Tamil Nadu, India.

*Corresponding author

ABSTRACT

The increasing demand for sustainable aquaculture practices has driven the exploration of probiotics as alternatives to antibiotics, which are associated with antimicrobial resistance and environmental concerns. This study focused on the bioprospecting of fish gut-associated microbes from Barramundi (*Koduva*, *Lates calcarifer*) collected from the coastal waters of Chennai, India, to isolate and characterize potential probiotic strains. The gut microbiota was homogenized, serially diluted, and cultured on selective media (MRS, Starch Casein Agar, and Nutrient Agar) supplemented with seawater. Twenty bacterial strains were isolated and screened for probiotic properties, including antagonistic activity against fish pathogens (*Staphylococcus aureus*, *Aeromonas salmonicida*, *Aeromonas caviae*, *Vibrio harveyi*, *Vibrio vulnificus*, and *Edwardsiella tarda*), extracellular enzyme production (amylase, protease, lipase, cellulase), siderophore synthesis, and tolerance to acidic pH and bile salts. Two promising strains, K5 and K8, exhibited strong antagonistic activity, enzyme production, and siderophore synthesis. Strain K8 demonstrated superior pH tolerance and bile salt resistance, surviving at pH 2 and 3% ox bile, while K5 showed higher hydrophobicity, indicating better gut adhesion potential. Both strains were Gram-negative and displayed auto-aggregation and antioxidant activity, further supporting their probiotic candidacy. The findings highlight the potential of native fish gut microbes as eco-friendly probiotics to enhance aquaculture productivity by improving nutrient absorption, pathogen inhibition, and gut health. Future studies should validate these results through in vivo trials to assess their efficacy in aquaculture systems. This research contributes to the development of sustainable alternatives to antibiotics, aligning with global efforts to promote environmentally responsible fish farming practices.

Keywords

Probiotics, aquaculture, *Lates calcarifer*, gut microbiota, antagonistic activity, enzyme production.

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Introduction

India is one of the largest producers of aquaculture products, contributing significantly to global fish production. The country ranks second in aquaculture production after China and plays a vital role in ensuring food security, livelihood generation, and economic growth. The sector has witnessed rapid expansion due to favorable climatic conditions, a vast network of inland water bodies, and government initiatives promoting sustainable fish farming (FAO, 2022).

Major species cultivated in India include freshwater fish like *Labeo rohita* (rohu), *Catla catla* (catla), and *Pangasius* species, along with shrimp varieties such as *Penaeus vannamei* and *Macrobrachium rosenbergii* (Kumar *et al.*, 2020). With increasing global demand for seafood, India's aquaculture sector is poised for further growth, supported by policies like the Pradhan Mantri Matsya Sampada Yojana (PMMSY) and advancements in biotechnology-based solutions for disease management and nutrition enhancement (GoI, 2023). Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host by modulating the gut microbiota, improving digestion, and stimulating the immune system (Hai, 2015).

The fish gastrointestinal tract harbors a diverse community of microbes that play a crucial role in digestion, nutrient absorption, immune modulation, and overall fish health. These gut-associated microorganisms include bacteria, archaea, fungi, and viruses, with bacteria being the most dominant group (Ringø *et al.*, 2016).

The gut microbiome plays a crucial role in fish health, digestion, and disease resistance, making it a valuable resource for isolating microbes with aquaculture applications (Egerton *et al.*, 2018). *Koduva* fish (Lates calcarifer), commonly known as Asian sea bass, is a commercially important species in aquaculture due to its high market demand and adaptability to diverse environmental conditions.

The present study was carried out to isolate, characterize, and evaluate potential probiotic bacterial strains from the gut microbiota of fish, with a focus on their beneficial properties such as antimicrobial activity, digestive enzyme production, and gut health enhancement for aquaculture applications."

Materials and Methods

Sample collection and isolation of bacterial strains

Marine fish, *Barramundi* (Koduva) was collected from the coastal area of Chennai, Tamil Nadu. The fish were dissected aseptically, and the intestine gut was removed and washed with sterile distilled water (Pathak & Joseph, 2021). The intestine samples were homogenized in a surface sterilized mortar and pestle and with sterilized distilled water for centrifugation. After centrifugation the supernatant was taken and serially diluted in sterile distilled water in the test tubes to 10^{-3} , 10^{-4} and 10^{-5} dilution and plated in three different media like Morgan R Shape agar (MRS), Starch-casein agar (SCA) and Nutrient Agar (NA) medium. The dilutions of 100 μ L is spread evenly with the help of glass L-rod and plates were incubated at 37°C for 24 hours. All the media used in this study were properly sterilized by autoclaving at 121°C for 20 mins. Therefore about 27 strains of bacteria were isolated based on the morphological differences and sub-cultured in Nutrient agar. Pure bacterial cultures were maintained as a slant stock on Nutrient agar. All the media used in this study were prepared in 50% filtered sea water unless otherwise stated.

Screening of Fish Gut Bacterial Cultures for Antagonistic Activity

Fish Pathogens

Fish pathogens used in this study are *Staphylococcus aureus*, *Aeromonas salmonicida*, *Aeromonas caviae*, *Vibrio vulnificus*, *Vibrio harveyi* and *Edwardsiella tarda* common fish pathogens

Inoculum preparation

A single colony of fish pathogen was inoculated into 2ml of nutrient broth and incubated at 37°C for 30mins. Nutrient broth was used to prepare for *Staphylococcus aureus*, *Aeromonas salmonicida*, *Aeromonas caviae*, *Vibrio vulnificus*, *Vibrio harveyi* and *Edwardsiella tarda*.

Testing of antagonistic activity

Antagonistic activity of the isolates was done according to (Prakash *et al.*, 2024) with modifications. MHB were

inoculated with the gut associated bacteria and kept in shaker overnight for incubation. The next day the MHB inoculated with bacteria were centrifuged for about 13000rpm at 4°C for around 20 mins to collect supernant. The MHA plates were marked with fish pathogens (*Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio vulnificus*, *Aeromonas caviae*, *Staphylococcus aureus*, *Edwardsiella tarda*) Using a sterile cotton swab the bacteria samples are inoculated in the marked plates. Then using a well cutter wells were created in the MHA plates. The created well is filled with supernant of the gut bacteria and the plates are marked as 9 in each plate. Then the plates were incubated for the observation of Antagonistic activity.

Enzymatic Activity

Amylase Test

Bacterial isolates were inoculated on agar medium containing starch 1%, glucose, peptone and yeast extract are incubated at 37°C for 48 hours. After incubation, plates were flooded with iodine solution. Positive culture exhibited clear zone around the colony against the blue-black background due to hydrolysis of starch.

Protease Test

Bacterial strains were inoculated on the protease media containing 1% Skimmed milk, glucose, peptone and yeast extract are incubated at 37°C for 48 hours. After incubation, the positive strains showed clear zone around the colony due to protein hydrolysis

Lipases Test

Bacterial strains were inoculated in the lipase media which contains TBA (Tributyryn agar, tributyrin oil 1%) The prepared media is sonicated for about 5 mins in order to break down the oil. Then the plates are incubated at 37°C for around 48 hours. After incubation the positive strains showed clear zone due to triglycerides (fats and oils) into glycerol and free fatty acids.

Cellulase Test

Bacterial isolates were inoculated on CMC (Carboxyl-methyl cellulose) medium containing carboxyl methyl cellulose 1%, glucose, peptone and yeast extract are incubated at 37°C for 48 hours. After incubation, plates

were flooded with Gram's iodine solution. Positive culture exhibited clear zone around the colony against the blue-black background due to breakdown of a complex polysaccharide into simpler sugars like glucose.

Siderophore Assay

The strains were assayed for the production on the Chrome Azurol S agar according to the method of Alexander and Zuberer (1991). CAS dye added on NA (Nutrient agar) plates were prepared and spot inoculated with bacterial strains and incubated at 37°C for 48 hours. The colonies producing yellow to orange colour were considered positive for siderophore production.

Hydrophobicity

Potential strains K5 and K8 were inoculated, centrifuged and wash with PBS for 3 times and centrifuged to get the pellet. OD value is taken 0.1 and the tubes is flooded with toluene and the sample is incubated for 20 minutes. 0.6ml of toluene is added in 3 ml of sample. Then using a formula, the percentage of the bacteria present is calculated and resulted.

$$\text{Hydrophobicity (\%)} = (A^0 - A^1 / A^0) \times 100$$

pH Tolerance

Potential bacterial culture was incubated overnight in Nutrient broth at 37°C. The sterile broth media adjusted to different pH levels ranging from pH 2, 3 and 7 as control. Using 1N NaOH. pH 2 and 3 is to mimic acidic stomach conditions. pH7 is neutral for the intestine.

$$\text{pH TOLERANCE (\%)} = (\text{OD test pH} / \text{OD control pH}) \times 100$$

Bile Salt Tolerance

Bile tolerance is an important property for probiotic bacteria in fish gut because it helps them colonize the gut. The isolated bacteria were grown overnight in a Nutrient broth. Various concentration of ox-bile (0, 1%, 2%, and 3%) were added to 10 ml w/w to Nutrient broth. Then the OD value is taken for 3 times and the value is calculated.

$$\text{Bile tolerance (\%)} = (\text{OD Bile} / \text{OD Control}) \times 100$$

>80% = highly tolerance strain

50-80% = moderately tolerance strain

<50% = Low tolerance strain

Autoaggregation

The potential strain K8 were inoculated overnight in nutrient broth. Then the sample were centrifuged at 3900rpm at 4°C for 20 minutes. Again, the sample were washed with PBS solution for thrice and the initial OD value was taken using microplate reader. Then sample were incubated for 1hr, 2hr and for 24hrs each time the sample OD value was taken.

$$AA = (OD_{\text{initial}} - OD_{\text{final}}) / OD_{\text{initial}} \times 100$$

Anti-Oxidant Assay

In DPPH assay 0.1mM solution of DPPH was prepared in methanol and 500ml of DPPH solution was added to 2500ml of the BS in different concentrations i.e.20%, 40%, 60%, 8%, and 100% Mg/ml respectively.

Then, absorbance was measured at 517 nm using spectrophotometer. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min (Zhou *et al.*, 2023). The percent DPPH scavenging effect was calculated by using following equation:

$$\% \text{ Inhibition} = (A^0 - A^1 / A^0) \times 100$$

Where, A^0 was the absorbance of control reaction
 A^1 -was the absorbance in presence of test or standard sample.

Result and Discussion

Sample Collection

Marine fish, *Barramundi* (Koduvu) was collected from the coastal area of Chennai, and gut was removed from the fish and homogenized in a surface sterilized mortar and pestle. (Figure 1) The gut of *Barramundi* collected from Chennai (Lat: 12.9494° N, Long: 80.2617° E) coastal area, Tamil Nadu.

It was processed under sterile conditions to ensure isolation of native gut microbes. This method is standard for studying gut associated bacteria with potential roles in digestion and aquaculture applications (Ghanbari *et al.*, 2015).

Enumeration

The enumeration of isolated bacteria was performed using the plate count method. The enumeration plate yield of 10^{-3} , 10^{-4} and 10^{-5} . These plates were then isolated and 20 strains were sub cultured. In this procedure, serial dilutions (10^{-3} , 10^{-4} , and 10^{-5}) were prepared to obtain countable colonies. These dilution levels suggest the sample had a high microbial load. From the dilution plates, 32 bacterial strains were successfully isolated and subcultured, indicating a diverse microbial community

Isolation

The bacteria isolated from dilution plates were cultured in NA plates as mother culture. The isolation process involved transferring bacteria from dilution plates to nutrient agar (NA) plates to establish mother cultures. These mother cultures serve as the primary source for further subculturing. Subculturing is a common practice to maintain pure bacterial strains and ensure their availability for future experiments, especially in case of contamination or depletion of the original culture. This step is essential in 23 microbiology to preserve the viability and genetic stability of isolated strains over time (Cappuccino & Welsh, 2017)

Characterization of bacteria

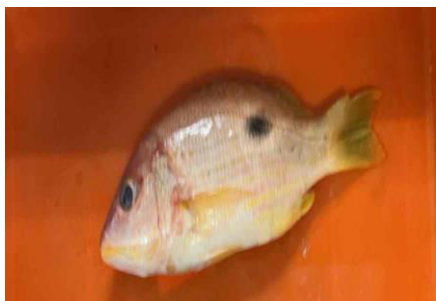
Antagonistic Activity

In this study, the bacterial strains recovered from the fish gut were screened for antagonistic activity against fish pathogens. The activity was carried on by well diffusion method. The strains showed zone of inhibition in some fish pathogens. (Figure 4)

In this study, bacterial strains isolated from the fish gut were evaluated for antagonistic activity against known fish pathogens using the well diffusion method. This method is widely used to test the antimicrobial potential of microorganisms. The presence of a zone of inhibition around the wells indicates that some of the strains produced antimicrobial compounds capable of suppressing the growth of certain fish pathogens.

This suggests potential for developing probiotic or biocontrol agents in aquaculture to reduce reliance on antibiotics (Balcázar *et al.*, 2006)

Fig.1 Sample collection



Barramundi (Koduva)



Fish gut

Fig.2 Enumeration

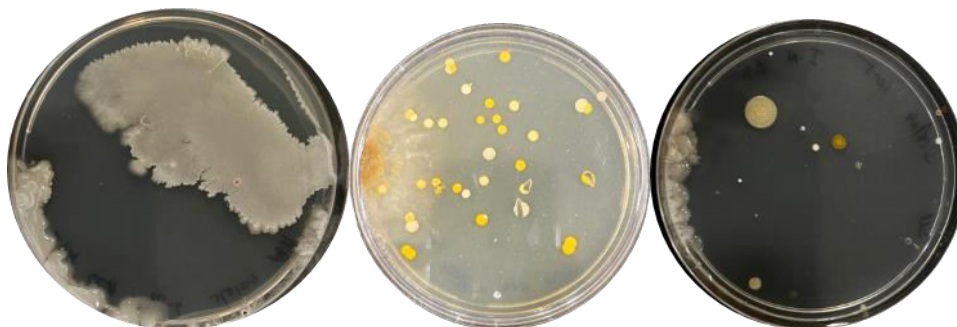
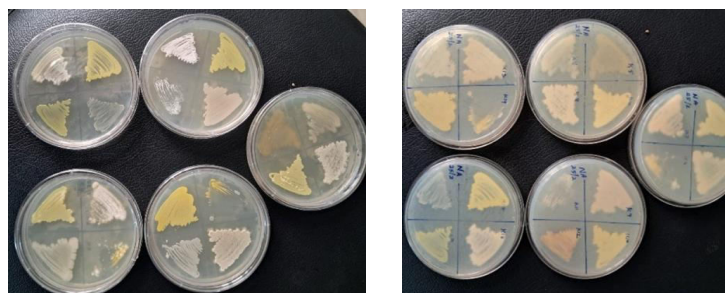
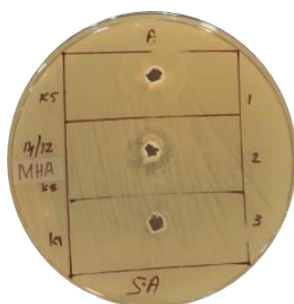


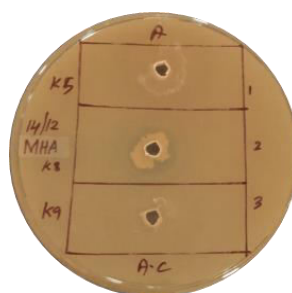
Fig.3 Isolation of bacterial strains



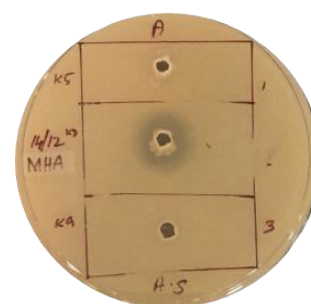
Antagonistic activity



Staphylococcus aureus



Aeromonas caviae



Aeromonas salmonicida

Enzymatic Activity

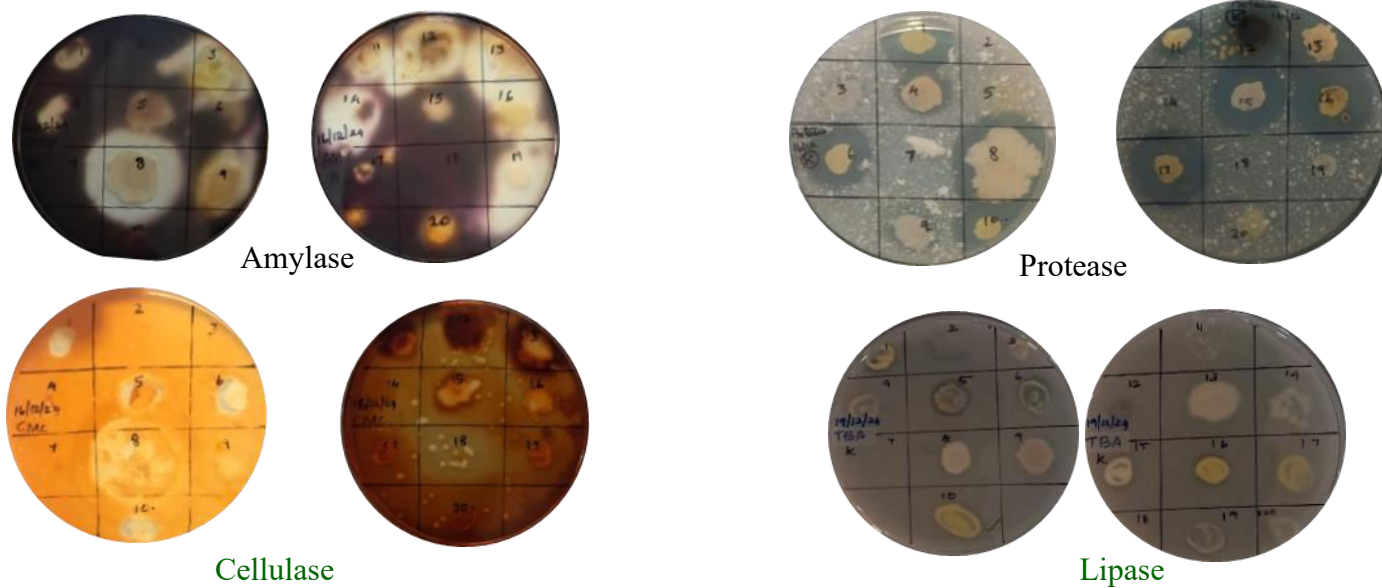


Fig.6 Siderophore assay

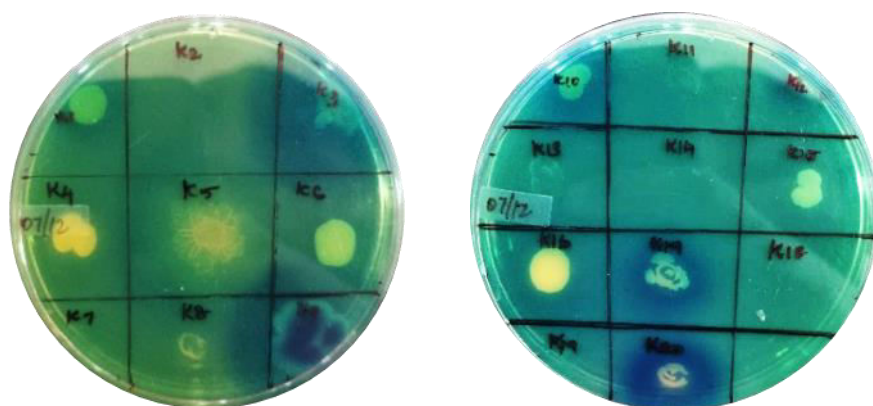


Fig.7 Hydrophobicity

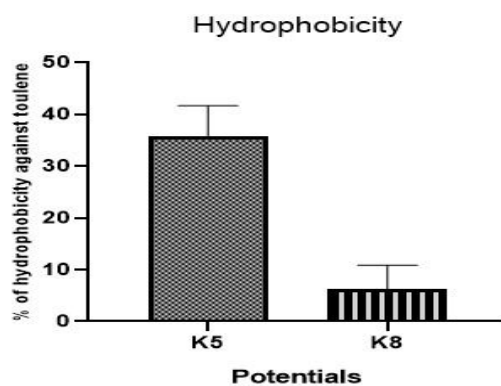


Figure.8 pH-Tolerance

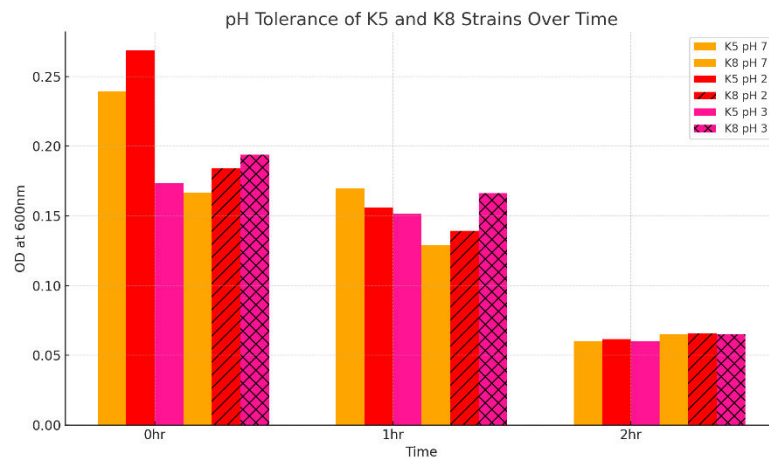


Fig.9 Bile graph

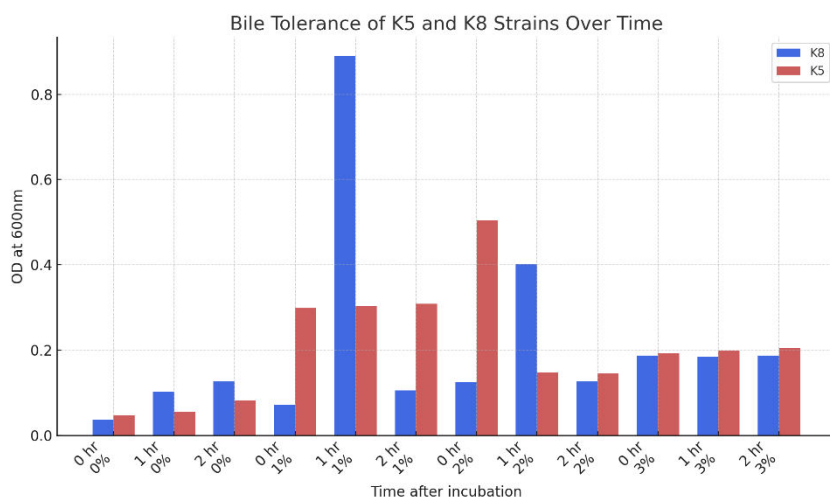


Fig.10 Bile salt



Fig.11 Auto aggregation

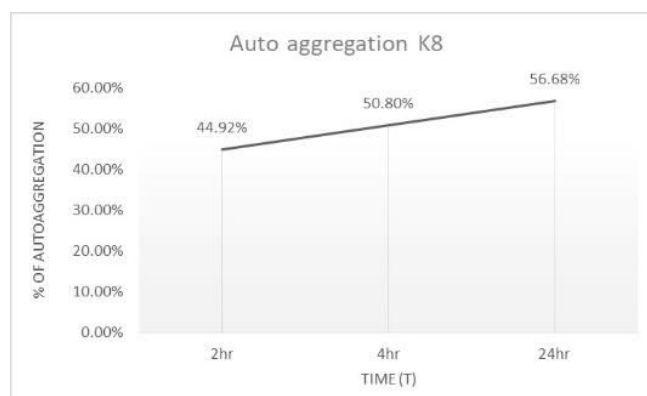


Fig.12 Anti-oxidant assay

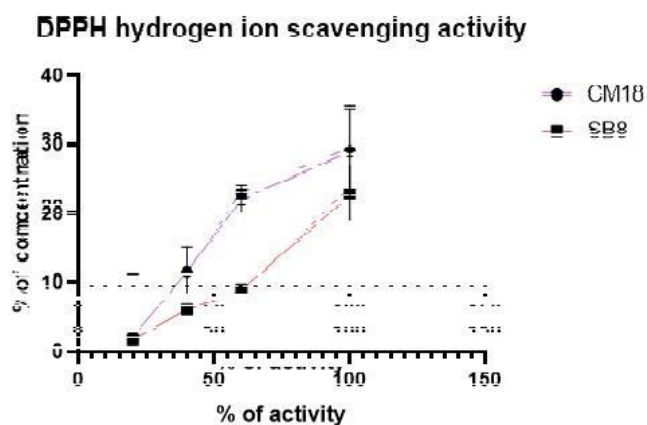


Table.1 Characterization of bacteria

CHARACTERIZATION OF BACTERIA			
S. NO	STRAIN NAME	COLOUR	CONSISTENCY
1	K1	Yellow	Slimy
2	K2	Dark-yellow	Slimy
3	K3	White	Slimy
4	K4	Colourless	Dry
5	K5	Orange	Slimy
6	K6	Yellow	Slimy
7	K7	Colourless	Dry
8	K8	White	Slimy
9	K9	White	Slimy
10	K10	Yellow	Slimy
11	K11	Colourless	Slimy
12	K12	White	Powdery
13	K13	White	Slimy
14	K14	Colourless	Slimy
15	K15	White	Slimy
16	K16	Yellow	Slimy
17	K17	Yellow	Slimy
18	K18	Colourless	Dry
19	K19	Colourless	Dry
20	K20	Yellow	Slimy

Table.2 Antagonistic activity

Strain No	<i>S. aureus</i>	<i>Aeromonas ceviae</i>	<i>Aeromonas salmonicida</i>	<i>Vibrio harveyii</i>	<i>Edwardsiella tarda</i>
K5	-	+	+	-	+
K8	17 mm	18 mm	15 mm	-	-
K9	-	-	-	+	-

Table.3 Enzymatic Activity

ENZYME ACTIVITY				
STRAIN NO	PROTEASE	AMYLASE	LIPASE	CELLULASE
K1	+++	+	+	+
K2	-	-	-	-
K3	+	++	-	-
K4	+	+	-	-
K5	-	-	+	-
K6	++	-	+	+
K7	-	-	-	-
K8	+++	+++	+	+++
K9	+	+	+	-
K10	+++	-	+	+
K11	+	+	-	+
K12	+++	++	-	-
K13	+++	++	-	+
K14	-	+++	-	-
K15	+++	-	+	+
K16	+++	+	+	+
K17	+++	-	-	-
K18	-	-	-	++
K19	-	++	+	-
K20	-	+	-	-

(+) Positive

(++) Strong Positive

Table.4 Siderophore Assay

SIDEROPHORE ACTIVITY		
STRAIN NO	SIDEROPHORE	COLOUR
K1	+	YELLOW
K2	-	-
K3	-	-
K4	+	YELLOW
K5	+++	YELLOW
K6	+	YELLOW
K7	-	-
K8	+	YELLOW
K9	-	-
K10	+	YELLOW
K11	-	-
K12	-	-
K13	-	-
K14	-	-
K15	+	YELLOW
K16	+	YELLOW
K17	+	YELLOWISH BLUE
K18	-	-
K19	-	-
K20	+	YELLOW

(-) Negative

(++) Moderate production

(+) Positive

(+++) Strong production

Table.5 pH-Tolerance

0 th Hour	7pH			2pH			3pH		
K5	0.333	0.545	0.129	0.122	0.105	0.264	0.263	0.156	0.127
K8	0.209	0.215	0.27	0.144	0.14	0.12	0.147	0.197	0.192
1st Hour									
K5	0.173	0.221	0.071	0.121	0.105	0.258	0.215	0.142	0.125
K8	0.116	0.123	0.199	0.142	0.13	0.124	0.13	0.165	0.176
2nd Hour									
k5	0.055	0.057	0.057	0.066	0.068	0.06	0.059	0.06	0.064
k8	0.053	0.059	0.059	0.062	0.072	0.076	0.081	0.066	0.061

Table.6 Bile salt

% of bile concentration	K5			K8		
	0hr	1hr	2hr	0hr	1hr	2hr
0%	0.046	0.054	0.081	0.036	0.102	0.126
1%	0.298	0.303	0.308	0.071	0.89	0.105
2%	0.505	0.147	0.145	0.124	0.402	0.126
3%	0.193	0.199	0.205	0.187	0.184	.0.187

Table.7 Auto aggregation

Hours	K8
2hr	44.92%
4hr	50.80%
24hr	56.68%

Enzymatic Assay

The media required for amylase, protease, lipase and cellulase was prepared and poured in petri dishes then bacterial culture was spotted in each plate. Then plates were incubated for 24hrs at 37°C. Then plates were flooded with iodine for amylase and with Congo red for cellulase. After 15 min appearance of zone is observed. Strains K8, K5 and K1 showed activity for amylase, lipase, protease and cellulose. (Govindaraj *et al.*, 2021). The formation of clear zones around colonies indicated positive enzyme activity. Strains K8, K5 and K1 demonstrated the ability to produce all four enzymes, highlighting their potential for industrial or biotechnological applications.

Enzymatic activity

Siderophore Assay

A clear yellow halo around the bacterial colonies of strains K5 and K8 indicates positive siderophore production, typically observed using Chrome Azurol S (CAS) agar medium. Siderophore-producing bacteria can play an important role in biocontrol, plant growth promotion, and pathogen inhibition by limiting iron availability to competing microbes (Neilands, 1995).

Hydrophobicity

The hydrophobicity assay was used to evaluate the cell surface properties of bacterial strains, which can influence adhesion to host tissues and colonization. In

this study, strain K5 showed higher hydrophobicity values compared to K8, suggesting a stronger ability to adhere to surfaces or host cells. This trait is particularly important for potential probiotic candidates, as higher hydrophobicity is often associated with better colonization in the gastrointestinal tract (Rosenberg *et al.*, 1980).

pH-Tolerance

K8 appears to have better pH tolerance overall, showing a more stable response over time. K8 struggles significantly in acidic conditions, especially at pH 2, but shows slight adaptation at pH 3. This implies that K5 may have a higher potential for gastrointestinal survival.

Bile Salt

K8 is more bile-tolerant than K5 as shown by its consistently higher values at all bile concentrations. K8 shows stress at bile concentrations above 1%, with minimal recovery. K5 adapts better even at higher bile levels, making it a stronger candidate for survival in high bile environments, such as the human digestive system. Bile tolerance is a crucial trait for probiotic bacteria, as it reflects their ability to survive and function in the host gastrointestinal tract, where bile is naturally present in accordance with previous findings

Therefore, K8's enhanced adaptability suggests it is a promising probiotic candidate for gut health applications (Gilliland *et al.*, 1984).

Auto Aggregation

K8 has a higher initial aggregation ability than K5, but it declines more quickly over time. K8's rapid loss of aggregation may impact its ability to form stable biofilms, which could be relevant for probiotic adhesion and gut colonization studies. The aggregation ability of a bacterial strain is important for its role in biofilm formation and gut colonization.

In this case, K8 demonstrated a higher initial aggregation, indicating strong cell-to-cell interactions early on. However, the rapid decline over time suggests that may struggle to maintain stable aggregation, which could reduce its effectiveness in forming durable biofilms. This trait is significant in probiotic studies, as persistent aggregation supports better adhesion to intestinal surfaces and enhances colonization potential (Kos *et al.*, 2003).

Anti-Oxidant Assay

Higher concentration correlates with stronger antioxidant activity. The increase is not linear—there is a slight plateau at 40%-60%, followed by a sharp rise at 80%-100%.

The present study demonstrated that the gut of *Lates calcarifer* (barramundi) is a promising source of bacteria with probiotic-associated properties. Twenty morphologically distinct bacterial isolates were recovered and screened for antagonistic activity, extracellular enzyme production, siderophore production, hydrophobicity, acid and bile tolerance, auto-aggregation and antioxidant activity.

Among them, K5 and K8 showed the most promising overall characteristics. K5 exhibited antagonistic activity against *Aeromonas caviae*, *A. salmonicida* and *Edwardsiella tarda*, whereas K8 inhibited *Staphylococcus aureus*, *A. caviae* and *A. salmonicida*. Such pathogen inhibition is an important probiotic trait and may involve antimicrobial compounds, bacteriocins, organic acids and competition for nutrients or attachment sites (Balcázar *et al.*, 2006). K8 showed strong protease, amylase, cellulase and lipase activities, while K5 exhibited lipase activity. These extracellular enzymes may contribute to improved feed digestion and nutrient utilization (Hai, 2015), consistent with previous findings in Asian seabass (Adorian *et al.*, 2019). K5 showed

pronounced siderophore production and higher cell-surface hydrophobicity, suggesting potential roles in iron competition and bacterial adhesion, although these properties require further confirmation (Neilands, 1995; Rosenberg *et al.*, 1980). K8 demonstrated good acid and bile tolerance, while its auto-aggregation increased from 44.92% at 2 h to 56.68% at 24 h, indicating potential for persistence and interaction with intestinal surfaces, although auto-aggregation does not directly confirm colonization (Kos *et al.*, 2003). Overall, K5 and K8 exhibited complementary probiotic-associated characteristics, supporting their potential as candidates for aquaculture probiotic development. These findings are consistent with previous reports of beneficial properties in indigenous bacteria from *L. calcarifer* (Adorian *et al.*, 2019). However, the present findings are based on in-vitro screening, and further molecular identification, safety assessment, antimicrobial-resistance and virulence screening, adhesion studies, and in-vivo feeding and pathogen-challenge trials are required before their practical application.

In conclusion, the current study successfully isolated and preliminarily characterized two fish gut-derived bacterial strains with strong probiotic potential. These findings support the viability of using native microbial strains from fish as eco-friendly and sustainable alternatives to conventional antibiotics in aquaculture. While the initial characterization has shown encouraging results, further investigations such as antioxidant assays and biofilm formation studies are needed to confirm their full probiotic profile and ensure their effectiveness and safety in in vivo aquaculture systems. This work lays the foundation for developing natural microbial-based solutions to promote healthier and more sustainable fish farming practices.

Author Contributions

P. Gokul: Conceived the original idea and designed the model and wrote the manuscript. Dr. S. Priya Grace: Designed the model and the computational framework and analysed the data.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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