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Unraveling The Biochemical Strength of Gut Bacteria in *Monopterus Cuchia*

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Abstract

The swamp eel, Monopterus cuchia, is a commercially important farmed fish in northeast India due to its medicinal and nutritional properties. The gut microbiota dysbiosis and homeostasis have been associated with the pathogenesis of swamp eel disease and food digestion. The gut microbiome in fish contributes to its growth and health by producing bio-compounds with nutritional functions. The objective of the present study was to find out the bioactivity of these gut bacteria with the help of various biochemical and morphological parameters. This study indicates that bacteria associated with M. cuchia have strong biochemical properties consistent with disease resistance. It requires further investigation of these bacteria up to the species level.

Keywords: *Bacteria, Biochemical, Morphological, Bio compound, Species*

1. Introduction

Widely prevalent in India, Bangladesh, Nepal, Myanmar, and Pakistan, the swamp eel Monopterus cuchia is a common freshwater species belonging to the Actinopterygii class (Menon, 1999; Mirza & Alam, 2002; Zhou *et al*., 2002). They frequently hide under rocks and mud (Nasar, 1997). The eel moves serpentinely to a more favourable place if its waterbodies become unsuitable. *M. cuchia* can, therefore, be used to test the quality of the water and soil in the environment. By burrowing in damp earth, this Asian swamp eel may withstand extended droughts without food or water during dry seasons. *M. cuchia* is a tasty, nutrient-dense, and medicinally beneficial fish that has long been used to treat anaemia (Rahman *et al*. 1992).

Every creature has a complicated gastrointestinal tract home to various microorganisms, including aerobic, anaerobic, and required aerobic bacteria (Ringø *et al*., 1995). Since fish are aquatic creatures, their environment may impact their microbiota composition (Cahill, 1990). Despite its complexity, the gut bacterial population is crucial for fish nutrition, supporting host physiology, metabolism, and immunity (Cahill, 1990; Ring *et al*., 1995). Certain fish species from the gut flora obtain numerous intestinal enzymes. (Cahill, 1990; Hamid *et al*., 1979).

The gut of host fish contains probiotic bacteria that successfully colonize the gut and create antimicrobials to inhibit the growth of harmful bacteria. They also face competition from opportunistic diseases for resources and space. Although present in all fish species, the microflora exhibits variation based on various factors such as stress, age, geographic location, nutrition, intestinal microenvironment, and environmental conditions (Verschuere *et al*., 2004; Refstie *et al*., 2006; Skrodenyte-Arbaciauskiene *et al*., 2008; Yang *et al*., 2007; Kesarcodi-Watson *et al*., 2008). The traditional culture-based techniques are used to identify the bacteria found in the gut. The current study's objective was to characterize the gut microbiota of swamp eels by morphological and biochemical means.

2. Materials And Methods

Sample Collection

Samples of *M. cuchia* were collected from local fishermen in Guwahati, India (26.171616, 91.691278), in pre-sterilized plastic containers (Autoclaved). After collection, they were brought alive to the Cell and molecular biology laboratory, Department of Zoology, Gauhati University, Guwahati, Assam, India.

Isolation of Gut microbes

For the isolation of gut microbes, the entire body surface of the collected samples were sterilized with 70% alcohol. Then, under aseptic condition, all specimens were dissected lengthwise to remove the whole gut from the mouth to the anus. To isolate the microflora of the gut, it was homogenized with 0.89% NaCl solution (10:1; volume: weight) (Das and Tripathi, 1991). The homogenized mixture obtained was serially diluted upto 10^{-1}, 10^{-2},10^{-3},10^{-4} concentration. Bacteria were grown aerobically in Luria Bertani Agar medium plates from each diluted sample for 24-48 hours at 30^{0}C. From this primary culture, isolates with clear zone were selected for analysis,

Morphological Characterization

The different isolates obtained from primary culture were named as G1, G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, G14, G15, G16, G17, G18.Morphological characterization of these Bacterial isolates were done for examining their shape, size, colour, elevation, margin, opacity, consistency The results are depicted in table 1.

Biochemical characterization of the isolates

The following biochemical tests are performed in order to identify the biochemical characterization of the isolates obtained from gut of *M. cuchia.*

Gram Staining

To Gram stain the bacterial cells, a HiMedia gram staining kit was utilized. Smears of bacteria were carefully made on sterile microscope slides and then heated to fix them. The HiMedia kit's instructions were followed precisely for the application of crystal violet, iodine solution, ethanol decolorization, and safranin counterstaining, as well as for the duration of each stage. Growing the bacteria on specific media for Gram-positive bacteria, such as mannitol salt agar, further validated the Gram-positive result obtained by Gram staining.

Starch hydrolysis

In bacterial cultures, starch hydrolysis was considered a crucial enzymatic activity. The methods mainly adhered to the defined protocol with minor adjustments. In summary, starch agar plates were streaked with bacterial isolates and then incubated under predetermined parameters. The plates were then saturated with iodine solution, which, when combined with starch, caused a blue-black coloring. The creation of amylase enzymes was suggested by the clear zones surrounding bacterial colonies, which showed signs of starch hydrolysis.

Lipid hydrolysis

With a few minor modifications, the experimental protocol adhered to accepted practices. On tributyrin agar plates, bacterial cultures were infected and then allowed to grow under carefully monitored circumstances. Clear zones surrounding bacterial colonies on the agar surface demonstrated tributyrin hydrolysis, which is an indication of lipase enzyme activity. Lipid hydrolysis is an important metabolic activity that affects the physiology of microorganisms.

Gelatin hydrolysis

After being streaked onto gelatin agar plates, bacterial cultures were cultured under carefully monitored circumstances. The plates were checked for the existence of clear zones surrounding the bacterial colonies after the incubation period, which is a sign of gelatin breakdown and the generation of gelatinase enzymes. Understanding the proteolytic capabilities of microbes is particularly interested in gelatin hydrolysis.

Sugar utilization

This study aimed to investigate the use of sugar to comprehend the metabolic capacities of bacterial isolates. Different sugar substrates, such as glucose, lactose, and sucrose, were added to bacterial colonies in specific growth media. The use of sugars was evaluated by keeping an eye on growth patterns, pH variations, or other particular signs. The presence of metabolic byproducts or alterations in the properties of the media provided unambiguous evidence of the use of sugar. Understanding the dietary preferences and metabolic adaptability of the microorganisms under study requires knowledge of this information.

Lactose fermentation

This study mainly focused on lactose fermentation to clarify the fermentative potential of bacterial isolates of lactose as a carbon source. Lactose-containing medium was added to bacterial cultures and then incubated under carefully monitored circumstances. Changes in pH or the development of gas bubbles in Durham tubes were indicators of fermentation, which produced acid and gas. Understanding the metabolic preferences of the microorganisms under study depends critically on their capacity to ferment lactose.

Citrate utilization test

Bacterial cultures were streaked onto Simmons citrate agar plates and incubated under controlled circumstances in order to assess the viability of the isolated bacterial colonies. The medium's hue changed to reflect the alkalization brought on by citrate metabolism, indicating the use of citrate. This test is important because it reveals how adaptable the bacteria's metabolism is and how well they can use citrate as a carbon source.

Catalase test

In this experiment, hydrogen peroxide was added to bacterial cultures and the instantaneous emission of oxygen bubbles was seen. The presence of catalase enzymes, which are essential for shielding bacteria from oxidative stress, was suggested by this effervescence. The catalase test is essential for comprehending the physiology of microorganisms and their capacity to adapt to various environmental circumstances.

Urease production

The urease production assay was used in this investigation to assess the bacterial isolates' capacity to hydrolyze urea, an enzyme activity important to a number of microbial functions. After being injected into urea agar, bacterial colonies were cultured under carefully monitored circumstances. When ammonia was released, urease activity was visible in the medium's colour shift.

Nitrate reduction test

Determining the degree of nitrate reduction is critical to understanding the metabolic pathways of microbes, providing information about denitrification mechanisms and the possible ecological functions of the bacteria under investigation. Within the scope of this study, the nitrate reduction test was carefully carried out to examine the ability of bacterial isolates to reduce nitrate enzymatically. Bacterial cultures were incubated under exacting conditions in a medium containing nitrate, according to established techniques with subtle modifications. After the incubation period, chemicals such as sulfanilic acid and alpha-naphthylamine were added one after the other to aid in detecting nitrite and determining the amount of nitrate reduction.

Oxidative fermentation test

Within this study, the oxidative fermentation (OF) test was carefully conducted to determine the metabolic inclinations of bacterial isolates concerning the utilization of carbohydrates. The experimental modality followed published protocols to the letter, making wise adjustments. OF essential medium supplemented with a particular carbohydrate and an indicator (usually bromothymol blue) was used to cultivate the bacterial cultures. Observable colour changes were used to distinguish between the oxidative and fermentative metabolism outcomes: an acidic shift that indicated fermentative activity and an alkaline shift that indicated oxidative metabolism. The detailed evaluation of oxidative and fermentative potentials has significant consequences for understanding the complexities of microbial metabolism.

Indole production

The evaluation of indole synthesis was a crucial component in determining the enzymatic potential of the bacterial isolates. Bacteria cultures were grown in a medium enriched with tryptophan and incubated under carefully regulated circumstances. Following the incubation period, Kovac's reagent was added to ascertain indole's presence by promoting a distinctly red complex formation. The measurement of indole synthesis is a crucial step in deciphering the complex mechanisms of microbial metabolism, namely the activity of tryptophanase.

H_2S production test

The Hydrogen Sulfide (H2S) generation test was carefully carried out within this study's parameters to determine bacterial isolates' capacity to metabolize sulfur-containing substances. Bacterial colonies

were incubated under carefully monitored circumstances after being grown on media containing sulfide indicators such as sodium thiosulfate and ferrous ammonium sulfate. H2S generation was indicated by the appearance of a distinctive black precipitate representing ferrous sulfide development. Analyzing H2S generation is crucial for understanding microbial sulfur metabolism and environmental adaption.

Methyl red and Voges Prousker test

The Methyl Red and Voges-Proskauer (MR-VP) test was used in conjunction with other tests to determine which metabolic pathways were most common in the bacterial isolates for glucose. In order to perform the Methyl Red test, bacterial cultures were grown in media containing glucose, and then Methyl Red reagent was added. The red hue that was seen to develop indicated an acidic pH, which was consistent with mixed-acid fermentation. Alpha-naphthol and potassium hydroxide were added after bacterial cultures were grown in the glucose-rich medium to conduct the Voges-Proskauer test concurrently. The emergence of a distinctive red hue highlighted the existence of acetoin, indicating a fermentation pathway for 2, 3-butanediol that is not acidic. The results of the biochemical analysis tests are summarized in table 2.

3. Results and Discussion

Morphological characterization

Table1. Morphological characterization of isolated bacterial colony

Bacterial isolates	Colour of colony	State	Margin of colony	Shape
G1	White	Mucoid	Uneven	Rod
G2	White	Mucoid	Uneven	Rod
G3	Off white	Mucoid	Uneven	Rod
G4	Off white	Mucoid	Uneven	Rod
G5	Off white	Mucoid	Uneven	Rod
G6	Off white	Mucoid	Uneven	Rod
G7	Off white	Mucoid	Even	Cocci
G8	White	Mucoid	Uneven	Rod
G9	Off white	Mucoid	Uneven	Rod
G10	Off white	Mucoid	Uneven	Rod
G11	Off white	Mucoid	Uneven	Rod
G12	Off white	Mucoid	Uneven	Rod
G13	Off white	Mucoid	Uneven	Rod
G14	Off white	Mucoid	Uneven	Cocci
G15	Off white	Mucoid	Even	Cocci
G16	White	Mucoid	Even	Rod
G17	White	Mucoid	Uneven	Rod
G18	White	Dry	Even	Rod

Gram staining

All the isolates except for G9 isolates were found to be Gram-positive. Moreover, all bacterial isolates, except G11, did not grow on MacConkey agar and Eosin Methylene Blue agar. These are selective culture media only for Gram-negative bacteria (Table2).

Strach Hydrolysis

In all the bacterial isolates, G1, G5, G6, G7, G9, G10, G11, G13, G14, G15, G16, G17, G18 showed signs of starch hydrolysis (Table2).

Lipid hydrolysis

All the bacterial isolates have not shown any lipid hydrolysis property (Table2).

Gelatin hydrolysis

No isolates are found to be gelatin hydrolysis positive (Table2).

Sugar utilization

It was found that isolates G1 G2 G6 G7 G13 G14 G15 G17 showed positive results for sugar utilization test (Table2).

Lactose fermentation test

No isolates are found to cause lactose fermentation.

Citrate utilization test

Only G1 showed citrate-positive results among the bacterial isolates, which was inferred by observing the blue-coloured slant of Simmon's citrate agar medium (Table2). It is a characteristic property of the bacteria in the family Enterobacteriaceae.

Catalase test

All the bacterial isolates except G9 are found to be catalase negative (Table2).

Urease test, **Nitrate reduction test, Oxidative fermentation test**

All the isolates except G5 G7 G11 G13 G18 showed positive urease test. G3 G4 G8 G9 G10 G14 G15 G16 showed no nitrate utilization (Table2). while all the isolates showed negative oxidative fermentation results.

Indole and H_2S production

All the isolates showed negative indole production while only G18 G19 showed positive H_2S production (Table2).

Methyl red and Voges Prousker test

All the isolates except G7 G11 G13 showed positive **Voges Prousker test**

Indicating production of acetoin. While G1 G2 G3 G4 G5 G6 G9 G10 G13 G15 G16 showed acid production thereby dropping the pH that leads to positive MR test (Table2).

Bacterial isolates → Biochemical test ↓	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**	**G9**	**G10**	**G11**	**G12**	**G13**	**G14**	**G15**	**G16**	**G17**	**G18**
Gram staining	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Mannitol salt agar	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
EMB agar	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
MacConkey agar	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Tributyrin agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose fermentation	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+	-	+	-
Maltose fermentation	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+
Mannitol fermentation	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Gas production	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dextrose utilization	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-
Lactose utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+
Urease test	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-
Gelatin utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidation fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+

Citrate utilization	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
Voges proskaur	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	-	-

The microbiota in the fish gut differs between species depending on the surroundings. The autochthonous gut bacteria of fish significantly influence the host species' growth, physiology, and immunity. Most fish gut microbiota support the host's immunity by taking up as much space as possible and secreting bacteriocins to fend off harmful bacteria. This study found distinct gut bacteria, each with unique biochemical properties. The blue-coloured slope of Simmon's citrate agar medium indicated that only G1 of the bacterial isolates had citrate-positive results. It is an attribute exclusive to members of the Enterobacteriaceae family of bacteria. The differential colour changes in the culture medium caused by pH variations allowed researchers to study the sugar fermentation processes of various isolates. Bacteria secreting enzymes was the cause of this. The bacterial isolates fermented numerous types of carbohydrates. Tolerance to bile salts is an essential characteristic of the probiotics under investigation. The entire gut microbiota of the mud eel produced positive findings for the bile salt utilization test. Probiotic bacteria are becoming more and more necessary to help fish resist disease growth as aquaculture techniques advance.

Bacilli were one of the bacterial strains that Sivasubramanian *et al.* (2012) found and described from the freshwater fish *Oreochromis mossambicus*, *Oreochromis leucostictus*, and *Oreochromis suratensis*. These bacteria are hostile toward powerful human infections such as *Vibrio cholera* and *Klebsiella pneumonia*.

The biochemical characteristics of bacterial isolate G19 revealed a striking resemblance to those of *Bacillus cereus* during the current experiment. According to earlier research, numerous Bacillus species show antibacterial qualities against Gram-positive and Gram-negative harmful bacteria. It has been shown by Kavitha *et al*. (2018) that *Bacillus cereus* is tolerant of varying bile salt concentrations. This suggests that this particular kind of bacteria plays a significant role as a probiotic. Additionally, their research has demonstrated that *Bacillus cereus* is resistant to ciprofloxacin, ampicillin, and amoxicillin. The research revealed that the antibiotic activity of *Bacillus cereus*, isolated and identified from *Labeo calbasu*, was effective against *Aeromonas hydrophila*. Ridgeon (2012) and KozinŅska *et al*. (2002) state that this important fish pathogen causes haemorrhagic septicaemia, fin rot, tail rot, and epizootic ulcerative syndrome in major Indian carps. The *Bacillus cereus* JAQ04 strain was examined by Bernard *et al.* (2013) about its possible probiotic activity in red tilapia (*Oreochromis species*). This study also demonstrated the safety of this bacterial strain for tilapia farming. According to our current research, the mud eel's various behavioural adaptabilities may also be significantly influenced by the G19 bacterial isolate. This has to be looked at more. Strains of Bacillus isolated from the intestines of channel catfishes or soil were hostile to *Aeromonas hydrophila* and *Edwardsiella ictaluri*. According to Ran *et al*. (2013), these strains cause motile *aeromonad septicemia* and enteric septicemia in catfish. Several Bacillus species have been discovered using biochemical characterisation in the current investigation.

Numerous bacteria might also be essential for the host *M. cuchia*'s immunity and disease resistance. The shape and biochemical characteristics of the colony of *Bacillus subtilis* and the bacterial isolate G1 are similar. According to Zokaeifar *et al*. (2014), adding *Bacillus subtilis* to a shrimp (*Litopenaeus vannamei*) pond for two months at a concentration of 108 CFU/mL led to more significant growth, enhanced immunological response, and increased resistance to disease. Furthermore, *Bacillus subtilis* can be added as a probiotic at a rate of 3% to the experimental food given to the *Macrobrachium rosenbergii* culture of freshwater shrimp, according to research conducted by Seenivasan *et al*. (2012). Improved nutritional indicators, growth, and survival were all benefits of the diet.

4. Conclusion

Furthermore, it has been demonstrated that *Bacillus subtilis* boosts antioxidant activity and immunity. Thus, determining the species of these bacterial isolates would aid in our comprehension of the mud eel *M. cuchi*a's growth, physiology, immunology, and distinctive behavioural traits. From the present work, we now better understand the biochemical characteristics of the microbiota in the gut of healthy mud eels. Given that *M. cuchia* is a mud-dwelling fish able to feel environmental changes, a study of the gut microbiota up to the species level will aid in our assessment of whether the gut microbiota plays a role in the eel's ability to perceive environmental changes. Furthermore, research on the autochthonous gut microbiota can be used to assess a fish's ability to survive in a nutrient-deficient, drought-prone environment. Autochthonous and probiotic bacteria linked to the mucosal membrane of *M. cuchia* may play a critical role in maintaining the host's disease resistance in drought environments, helping the mud

eel withstand environmental pressures on its physiology and immunity. These probiotic and autochthonous bacteria are only connected to the host's growth. Therefore, molecular study is needed to resolve and explain these unanswered questions regarding *M. cuchia*, a medicinally valuable mud eel.

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Declarations

Banasri Mech contributed to the study conception and design. Material preparation, data collection and analysis were performed by Banasri Mech. The first draft of the manuscript was written by Banasri Mech, Rahul Sarma, Jayshree Deka, Priyam Sarmah. All the authors approved the final manuscript.

Conflict of Interest: Authors declare there is no conflict of interest.

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