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Original Paper

Isolation and characterization of novel bacteria *Macrococcus canis* from the gut of the freshwater fish *Macrognathus* spp.

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Abstract

Macrococcus canis was isolated from the gut of the omnivorous freshwater fish *Macrognathus* spp. In the 16S rRNA gene sequence, the bacterial isolate M2 showed a 99.43% similarity with *Macrococcus canis* (accession NR_156154.1). *M. canis* was found to be morphologically a Gram-positive and non-motile cocci-shaped bacterium that formed smooth, yellowish-orange, convex colonies approximately 2 mm in diameter. According to biochemical characterization, *M. canis* had positive reactions for Voges-Proskauer, oxidase, catalase, and nitrate reduction and negative reactions for urease, indole, citrate, methyl red, and urease. Furthermore, the isolate *M. canis* had positive lipase and protease activity, suggesting that it may play a role in lipid and protein metabolism within the fish gut microbiota. The results provide evidence that *M. canis* colonizes a new habitat – the aquatic environment, as previous studies already pointed to the existence of *Macrococcus* species within the fish gut microbiota. Additional studies, such as whole gene sequence and functional tests, would be required to understand the ecological and physiological roles of *M. canis* in water-living organisms.

Key words: fish gut bacteria, *Macrococcus canis, Macrognathus* spp., *Staphylococcus.*

Introduction

The fish gastrointestinal microbiota is important for immunity, nutrition, and general health. A diverse community of bacteria with functional significance has been discovered through in-depth investigation of the fish gut environment made possible by recent developments in microbial ecology and molecular tools (Morshed, Lee 2023). With variation based on host species, diet, habitat, and environmental conditions, the most prevalent phyla among these bacteria are Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes.

Nevertheless, little is known about the gut microbiota of numerous freshwater species that have received less attention, like *Macrognathus aral* and *Macrognathus pancalus*. *Macrognathus* species, native to freshwater bodies of South Asia, are benthic feeders that inhabit nutrient-rich environments, making them a potential reservoir for diverse gut-associated microbes (Sarmah, Kardong 2024). Understanding the gut microbiota of such species not only contributes to microbial ecology but may also identify novel bacterial strains with functional and probiotic potentials.

Macrococcus is most closely related to the genus *Staphylococcus* (Kloos et al. 1998; Baba et al. 2009; Mazhar et al. 2019). *Macrococcus*, a Gram-positive coccoid and catalase-positive bacterium, might be mistaken for a coagulase-negative *Staphylococcus* species; therefore, further separation is necessary. Before being allocated to its own *Macrococcus* genus in 1998, it was categorized under the *Micrococcus* genus, followed by the *Staphylococcus* genus (Kloss et al. 1998). Currently, eight species, including *Macrococcus canis*, which is found in dogs, are known to exist in the genus (*Macrococcus caseolyticus, Macrococcus carouselicus, Macrococcus equipercicus, Macrococcus bovicus, Macrococcus brunensis, Macrococcus hajekii,* and *Macrococcus lamae*) (Kloss et al. 1998; Mannerov et al. 2003; Gobeli et al. 2017).

In 2017, a novel species called *Macrococcus canis* was isolated from a dog's infection site (Gobeli et al. 2017). Both genera contain common animal commensals, some of which are opportunistic pathogens that need to be treated with antibiotics (Cotting et al. 2017; Natsis, Cohen 2018; Li et al. 2018; Heilmann et al. 2019). The majority of *Macrococcus* are found in the microbiota of mammals, while *M. caseolyticus* has also been detected in meat and milk.

The objective of this study was to examine the gut microbiota of freshwater fishes belonging to the *Macrognathus* species, which have not received much attention in studies on microbial ecology. In this study, we aimed to isolate, identify, and characterise gut bacteria from *Macrognathus aral* and *Macrognathus pancalus* collected from the waters in the beels of Dibrugarh, Assam, India.

Materials and methods

Isolation of fish gut bacteria

Macrognathus aral and *Macrognathus pancalus* were collected from the local market, fished from the beels of Dibrugarh, Assam, India. Then, the fish were sacrificed and rinsed with sterile distilled water. The fish were then dissected and the gut was taken out and placed on a sterile plate. Fish gut was prepared according to a modified protocol of Amin et al. (2022). The digestive tract was weighed and then homogenized and mixed with a sterile phosphate-buffered saline (pH 7.2) solution.

The sample was serially diluted using a spread plate technique in a nutrient agar medium. The isolates were purified by repeated streaking of each distinct colony on the medium (Beveridge et al. 1991).

Molecular characterization of the isolates

Genomic DNA was isolated from overnight bacterial cultures using a CTAB/phenol-chloroform protocol. Cell pellets were resuspended in 500 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with lysozyme (20 mg mL^{-1}) and incubated at 37 °C for 30 min, followed by 1% sodium dodecyl sulphate and proteinase K (200 µg mL^{-1}) at 56 °C for 1 h. CTAB/NaCl solution (10% CTAB in 0.7 M NaCl; 100 µL) was added and incubated at 65 °C for 10 min. Lysates were extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with 0.7 volumes isopropanol, washed with 70% ethanol, air-dried, and dissolved in 50 µL nuclease-free water with RNase A (10 µg mL^{-1}, 37 °C, 15 min). DNA quality and concentration were assessed by $A_{260/280}$ and agarose gel electrophoresis.

16S rRNA gene sequencing is a common method for identifying, classifying, and counting microbes in complex biological samples, like environmental or gut microbiomes. The PCR amplification reactions were prepared in a final volume of 25 µL. Each reaction mixture contained 2.5 µL of 10× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of the forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'), 1.25 U of Taq DNA polymerase, and 10 – 50 ng of template genomic DNA, with nuclease-free water added to make up the final volume. The reactions were set up on ice to avoid non-specific amplification, and a master mix was used to minimize pipetting errors. For difficult templates, additives such as bovine serum albumin (0.1 mg mL^{-1}) or dimethyl sulfoxide (2 – 3%) were included, and the $MgCl_2$ concentration was optimized between 1.5 and 2.5 mM when necessary. Negative controls, which excluded the DNA template, and positive controls, containing known bacterial DNA, were included in each run to validate the amplification.

PCR was started with an initial denaturation at 95 °C for 5 min. This was followed by 25 cycles of denaturation at 95 °C for 40 s, annealing for 2 min, and extension at 72 °C for 1 min. The process was concluded with a final extension at 72 °C for 7 min. The confirmation of the amplified products was done by using agarose gel electrophoresis and they were stored at −20 °C for later sequencing.

Next, single-molecule real-time sequencing was used to sequence the 16S rRNA amplicons. The full 16S sequence was then compared with reference databases to find sequence variations, which helps in in phylogenetic analysis and microbial identification (Tanasupawat et al. 2004; Bertolo et al. 2024).

A BLAST search against the NCBI GenBank database identified the top ten matching sequences based on maximum identity scores. These sequences were aligned using CLUSTAL W, followed by distance matrix generation and phylogenetic tree construction using MEGA XI for evolutionary analysis.

Morphological characterization

All bacterial isolates obtained in this study were identified at the molecular level by 16S rRNA sequencing. Isolate M2 (*Macrococcus canis*) was chosen for detailed characterization because it was comparatively less studied in the context of fish gut microbiota. The strain was inoculated in agar medium at 35 °C for 4 to 8 h. The bacterial colonies were observed under a microscope for respective colour, texture, type of margin, growth pattern, consistency, and pigmentation properties as per Bergey's Manual.

Biochemical tests

Following the procedures described in Bergey's Manual, the bacterial strains were put through a series of biochemical tests, including those for catalase, indole, oxidase, nitrate, urease, citrate, methyl red, and Voges-Proskauer.

A loopful of bacterial isolate was combined with 3% hydrogen peroxide for the catalase test; the formation of bubbles signified a successful outcome (Reiner 2010). By inoculating Christensen's medium and watching for a pink tint on the slant, which signifies urease production, urease activity was measured (Brink 2010). In the nitrate reduction test, cultures were cultivated in nitrate broth and treated with reagents A and B; the presence or absence of a reddish-yellow tint or color following the addition of zinc signified nitrate reduction (Buxton 2011). Kovac's reagent was added after cultures were incubated in Tryptone Broth as part of the indole test; a red ring signified a successful outcome (MacWilliams 2012). Cultures in MR-VP broth were treated with particular reagents for the methyl red and Voges-Proskauer tests; Voges-Proskauer reaction was indicated by red coloration, while a negative result was indicated by yellow (McDevitt 2009). Simmon's citrate medium was used to test citrate utilization; growth that changed colour from green to blue indicated a successful outcome (MacWilliams 2009). The oxidase test used filter

paper that had been saturated with oxidase reagent; a dark blue or purple hue within 10 to 30 s indicated a successful result (MacFaddin 2000).

Enzyme assays

Isolates were screened for various enzymatic activities using standard protocols. Amylolytic properties were assessed through the starch hydrolysis test on starch agar plates, as described by Jacob et al. (1960), and visualized by flooding the plates with Gram's iodine.

Cellulolytic activity was evaluated by inoculating isolates on nutrient agar plates impregnated with carboxymethyl cellulose, following the protocol of Hankin et al. (1977), and detecting hydrolysis zones by flooding the plates with Congo red. Similarly, chitinase activity was screened using nutrient agar plates containing colloidal chitin as a substrate, as per Kuddus et al. (2013), with visualization also done using Congo red.

Lipase activity was tested using tributyrin as a substrate, following the method of Bairagi et al. (2002). Protease activity was determined according to Sarath et al. (1989), using casein as a substrate, with hydrolysis zones visualized by flooding the plates with Coomassie Brilliant Blue.

Results

Isolation of bacteria and molecular identification

From the gut of *Macrognathus* species, we isolated seven morphologically distinct bacterial strains based on differences in colony size, shape, texture, and opacity. Seven different bacterial species (M1, M2, M3, M4, M5, M6, M7) were found when each isolate was put through molecular characterization for accurate identification. Among the bacterial isolates obtained, the one designated M2 was identified as *Macrococcus canis*. This was determined through a phylogenetic analysis of the 16S rRNA gene sequences (Fig. 1). The analysis revealed that M2 clustered with the aforementioned bacterial species (99.43% sequence similarity with NR_156154.1), confirming its identification as *Macrococcus canis*. This bacterium is a rare and uncommon species found in the fish gut environment.

We carried out a thorough investigation to describe the biochemical and morphological characteristics of *Macrococcus canis*, because there is a dearth of information on these aspects. Its discovery in *Macrognathus* is especially significant because it broadens the ecological niche and known host range of *Macrococcus canis* and could offer important new information about its possible function in fish health and gut ecology.

Morphological characterisation of Macrococcus canis

The isolated *Macrococcus canis* was found to be cocci-shaped, Gram-positive bacterium with unique colony traits (Fig. 2). It produced round, yellowish orange colonies that grew to a size of around 2 mm. The colony margin was entire with a smooth texture, a convex elevation, and an opaque appearance. This non-motile bacterium showed best growth at temperatures between 28 and 37 °C. Furthermore, the ideal pH range for its growth was 6 to 9 (Table 1).

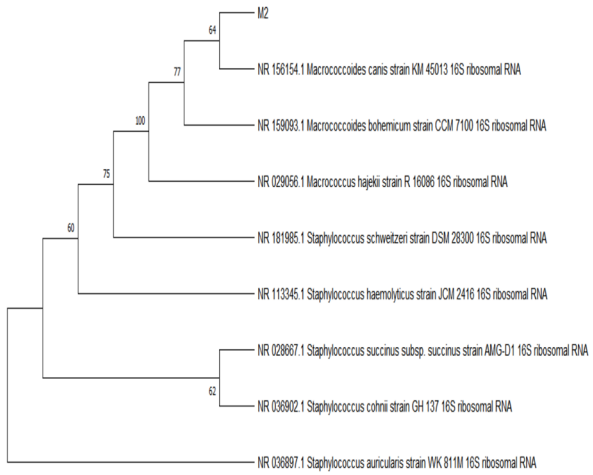

Fig. 1. Phylogenetic tree of isolate M2 constructed using the maximum likelihood method based on 16S rRNA gene sequences, showing its closest relationship with *Macrococcus canis*.

Biochemical characterisation of Macrococcus canis

The isolate tested negative for indole, citrate, methyl red, and urease, indicating that it does not produce indole, cannot utilize citrate as its sole carbon source, does not perform mixed acid fermentation, and lacks urease activity (Table 1). However, positive results were observed for the Voges-Proskauer, oxidase, catalase, and nitrate reduction tests, suggesting its ability to produce acetoin, utilize oxygen in respiration, break down hydrogen peroxide, and reduce nitrate to nitrite or other nitrogenous compounds.

Enzyme assays for Macrococcus canis

The enzyme activity of *Macrococcus canis* (isolate M2) was evaluated through qualitative assays to determine its potential functional role in the gut environment. The isolate

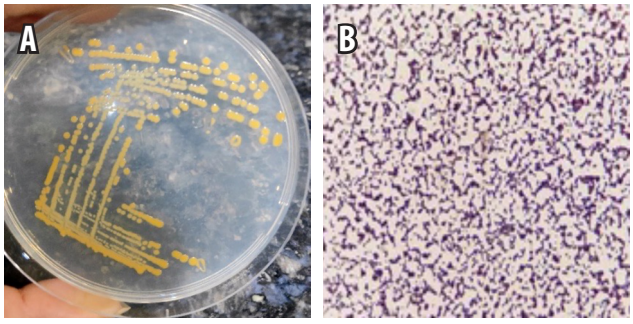

Fig. 2. Pure culture of *Macrococcus canis* (isolate M2) on nutrient agar (A) and Gram-stained microscopic image showing Gram-positive cocci (B).

tested negative for amylase, cellulase, and chitinase activity, indicating a lack of carbohydrate-degrading enzyme production (Table 1). However, it exhibited positive results for lipase and protease activity, suggesting a significant role in lipid and protein metabolism (Fig. 2). These findings highlight the metabolic versatility of *Macrococcus canis*, particularly in degrading complex macromolecules like fats and proteins, which could influence nutrient processing and microbial interactions within the fish gut.

Discussion

The genus *Macrococcus* is Gram-positive, non-motile cocci that is frequently isolated from a variety of animal hosts, such as dogs, cattle, and marine environments, and is closely related to *Staphylococcus* (Kloos et al. 1998). According to 16S rRNA phylogenetic analysis, the strain we recovered for this investigation was *Macrococcus canis* (M2) from fish gut, which exhibited a 99.43% similarity to *Macrococcus canis* (accession NR_156154.1). This conclusion contradicts the findings of Gobeli et al. (2017), who found that *Macrococcus canis* from dog skin was more closely related to *Macrococcus caseolyticus*. However, the strain that we isolated showed a closer phylogenetic relationship with the *Macrococcides bohemiceum* strain CCM7100.

The bacterial *Macrococcus canis* isolate M2, was found to have similar morphology to staphylococci and other Gram-positive cocci. Because they divide into two planes, *Staphylococcus* are spherical bacteria with a diameter of 0.5 to 1.0 µm that grows in clusters, pairs, or short chains. We observed that the *Macrococcus canis* we found, on the other hand, mainly forms clear, smooth, circular, yellowish-orange colonies (~2 mm) with a convex elevation and an entire edge, while it also appears as cocci. Both are non-motile and grow in the same pH (6 to 9) and temperature range (28 to 37 °C). *Staphylococcus* are identified by their clustered cocci form, whereas streptococci grow in chains. To achieve accurate identification, observations should be made in broth cultures rather than solid media because *Streptococcus* can grow as clumps on solid surfaces. According to Foster (1996), these physical characteristics are essential for differentiating *Macrococcus canis* from closely similar Gram-positive cocci such as *Streptococcus* species and *Staphylococcus aureus*.

Both the oxidase and catalase tests came out positive for our isolate, which is consistent with Gobeli et al. (2017)'s findings for strain KM 45013T. Our isolate's morphology, which was defined by the shape of its cocci, also agrees with findings of Gobeli et al. (2017). The pigmentation of the strain, however, is a distinguishing characteristic that was similar to the results obtained by Masalnova et al. (2017) for *Macrococcus caseolyticus* subsp. *hominis*.

S. aureus is distinguished from other staphylococci by phenotypic markers such as thermonuclear, coagulase, and catalase tests; 84% of isolates test positive for thermostable nuclease, 92% coagulase, and 100% catalase. *Macrococcus canis* has a lower potential for pathogenicity than *S. aureus*, since it is coagulase-negative and does not have haemolytic activity (Karmakar et al. 2016).

Table 1. Characterization of *Macrococcus canis* isolated from fish gut

Parameter	Characteristic
Morphological	
Gram stain	Positive
Cell shape	Cocci
Colony colour	Yellowish orange
Colony shape	Circular
Colony size (mm)	2
Margin	Entire
Elevation	Convex
Texture	Smooth
Opacity	Opaque
Cell size	Small
Motility	Non-motile
Growth temperature range (°C)	28 – 37
Optimum pH range	6 – 9
Biochemical	
Indole	Negative
Citrate	Negative
Methyl red	Negative
Voges-Proskauer	Positive
Oxidase	Positive
Catalase	Positive
Nitrate	Positive
Urease	Negative
Enzymatic	
Amylase	Negative
Cellulase	Negative
Chitinase	Negative
Lipase	Positive
Protease	Positive

The *Macrococcus canis* we isolated tested negative for indole, citrate, methyl red, and urease, but positive for

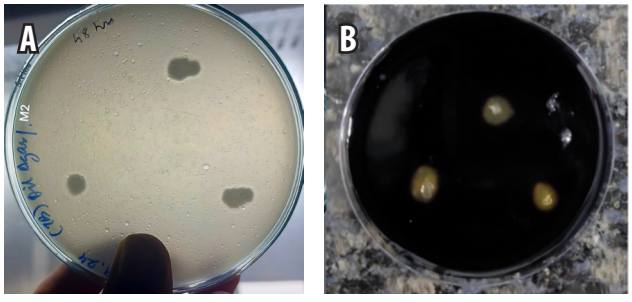

Fig. 3. Enzyme activity of *Macrococcus canis* (isolate M2) showing positive results for lipase (A) and protease (B) production as indicated by clear zones around colonies on respective media.

Voges-Proskauer, oxidase, catalase, and nitrate reduction in biochemical assays. These findings are in line with earlier research showing that *Macrococcus canis* has significant oxidase and catalase activity, suggesting aerobic respiration, but lacks urease activity and does not use citrate. Similar biochemical traits for *Macrococcus caseolyticus* subsp. *hominis* were previously reported by Masalnova et al. (2017).

Protease and lipase, which are necessary for hydrolyzing proteins and lipids, are produced by the isolate we found. While *S. aureus* showed a greater prevalence of enzymatic activity, with 81% of isolates producing protease and 51% producing lipase, *Macroccoccus canis* revealed favourable results for both enzymes in the current investigation (Karmakar et al. 2016).

These results imply that *Macrococcus canis* might be involved in the gut microbiota of fish, possibly aiding in the metabolism of proteins and fats. However, further research is required to fully comprehend its ecological and physiological implications in aquatic habitats, including whole-genome sequencing and functional testing. It is interesting to note that little research has been done on the gut microbiota of *Macrognathus* spp., the omnivorous freshwater fish species from which *Macrococcus canis* was identified. This study suggests a possible new habitat for *Macrococcus canis* in aquatic habitats, as *Macrococcus* species are mainly associated with animals.

Earlier research has also shown that the fish gut microbiome contains *Macrococcus* species. For instance, among other bacterial taxa, *Macrococcus* was found in a study on the gut microbiome of hilsa fish (*Tenualosa ilisha*) (Kawser et al. 2024). Furthermore, studies on bioluminescent bacteria from the intestines of marine fish by Burtseva (2020) revealed that *Macrococcus* strains formed biofilms, indicating a possible function for them in the gut environment.

Conclusions

The current work focused on the molecular, morphological, biochemical, and enzymatic characterization of isolate of *Macrococcus canis* from a fish gut. The findings provide insights into the ecological plasticity of *Macrococcus canis*, its potential role in nutrient metabolism within the fish gut, and contribute to a broader understanding of host-microbe interactions in freshwater fish. The findings of this investigation can be used as a base to study further ecological adaptation of this bacteria.

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