Isolation and identification of associated micro florasfrom gastrointestinal tracts of freshwater fish *Dawkinsiafilamentosa*(Valenciennes, 1844)

Comment [MF1]: Microbiota

ABSTRACT

Aims: To isolate and identify the gut associated micro-organisms present in the gastrointestinal tract of freshwater fish *Dawkinsiafilamentosa.*

Place and Duration of Study: Sampling site:Gadana river, Alwarkurichi, Tenkasi district, Tamilnadu, India. Place: Sri Paramakalyani Centre for Environmental Sciences, ManonmaniamSundaranar University, Azhwarkurichi, Tenkasi, Tamilnadu, India. Duration: Between December 2021 and April 2022

Methodology: The fish samples were anaesthetized and surface sterilized followed by aseptic dissection. The gut contents were removed and homogenized well and serial dilution was carried for isolation of Gut microbiome. The identification of gastrointestinal bacteria was done by performing various biochemical tests such as Gramstaining, Motility, Indoletest, Methylredtest, VogesProskauertest, Citrateutilizationtest, Triplesugartest, Catalasetest, Oxidasetest, Ureasetest, Nitratetest, Starchhydrolysis and Carbohydratefermentationtest. The fungi present in gastrointestinal tract is identified with lactophenol cotton blue staining followed by microscopic observations.

Results: The overall bacterial load of gastrointestinal material ranged from 47 $x10^{-7}$ to 219 $x10^{-5}$ CFU/g. A total of six bacterial strains and four fungi strains were isolated. Several biochemical tests were used to identify the bacterial isolates. The six bacterial isolates were tentatively identified as *Pseudomonas sp.,Aeromonas sp.,Staphylococcus sp., Bacillus sp.,Enterobacter sp.,* and *Vibrio sp.* and the four fungi species isolated from the gutwere *Aspergillusflavus, Aspergillusniger, Beauveriabasiana,* and *Penicillium sp.*

Conclusion: The current research revealed that, *Dawkinsiafilamentosa* has a variety of microbial flora in its gut. Microbial species from freshwater environments may be able to augment fish feed with these bacteria in commercial aquaculture operations. Additionally, these findings have aided in the development of possible remedies, enhanced knowledge of host-microbe interactions in other vertebrates, and enhanced aquaculture practises.

Keywords: Dawkinsiafilamentosa, Freshwater fishes, Gastrointestinal tract, Gut microbiota

1. INTRODUCTION

Fishes originated over 600 billion years ago in the earth; they are poikilothermic aquatic animals of the kingdom Animalia.They are considered as sources of low-fat and high-quality protein, over billions of people depend on fishes for their protein supplement; They are rich in omega-3 fatty acids, vitamins like B2, D and minerals such as iron, calcium, phosphorus, iodine, magnesium, zinc and potassium[1]. Fishes have various microflora in their body. The bacterial genera present in the fishes depends on their environment and they may vary by some of the factors such as salinity, temperature, bacterial communities in water. The study

of the fish GI microbes will help to manipulate the fish health and their productivity. The fish GI flora can reflect their diet preferences and physiological behaviors.

Our understanding of the intricate interactions that take place between microorganisms and host fish has increased in recent years as a result of studies on the microbial flora linked to fish guts. The gastrointestinal (GI) microbes of vertebrates play critical roles in nutrition, development, immunity and resistance against invasive pathogens. The earliest study of microbes in the fish intestine studied in the late 1910's[2, 3]. These efforts have been dedicated to describe the microbial communities present in the GI of the fish. Currently gastro intestinal tract microbes have been conducted in many species. The GI microbes of the fish have become a frontier field.

The gut microbes of freshwater fishes may differ due to various environmental conditions. *Acetobacter* species*, Aeromonas* species*, Flavobacterium* species*, Lactococcus* species*, Pseudomonas* species*,* obligate anaerobes (Bacteroides, Clostridium and Fusobacterium) and members of family *Enterobacteriaceae* dominate the gut of freshwater species[4]. The microbial colonization may arise from the environment, eggs and their first feed. The newly hatched larva contains the low number of bacteria, after intake of water the bacteria may developed[5]. The microbes in GI of the fish play a critical role in the development and health of their organs. Moreover, the epithelial surfaces of the fish and all other vertebrates are colonized at birth by large number of microorganisms. Most intestinal bacteria are aerobic and facultative anaerobes. The distributions of aerobic microbes are grouped into gram- positive and gram- negative, as they have been observed in the gastro intestinal tract of freshwater fishes[6].The digestive enzymes secreted by intestinal microorganisms and the regulating functions of intestinal microorganisms on fish immunity are particularly important. The GI microbes have played an important role in the development of the fish immune system and the nutrient adsorption. Mostly the functional activities of the GI microbes include the immunity and digestion[7].

Studies have shown that more than 10^7 to 10^{11} bacteria per gram of intestinal content[8, 9].The fish GI has the trillions of bacteria, viruses and fungi. The GI microbiomes of fishes play an important role in mediating and stimulating host gastrointestinal (GI) development, aiding digestive function, maintaining mucosal tolerance[10]. By knowing the microbial community in the fish GI, one canidentify its host immune response and level of protection against gastro intestinal infections which helps to conserve and protect that particular species[11].

In this study we have identified, categorized the bacteria and fungi present in the GI of freshwater fish *Dawkinsia.filamentosa*, commonly known as black spot barb. It belongs to

the family Cyprinidae[12]. One important aim of GI microbiota studies in *D.filamentosa* is to give a scientific basis for developing effective strategies for manipulating GI microbial communities to promote the host health and improve the productivity.Hence forwarded, various biochemical tests performed for the identification of the bacteria and staining method for the identification of the fungi and we have highlighted the functions of the GI microbiomes of the fish.

2. MATERIAL AND METHODS

2.1. SampleCollection

Comment [MF2]: It is preferable to study the diversity of fish gut microbiome under several factors, like sex, seasons and diet.

Thefishsampleof*Dawkinsiafilamentosa*[Figure1] wascollectedfromtheGadanariver[Figure 2](latitude 8° 37' 0427" N; longitude 77° 18' 7243" E)located atthefoot of Western Ghats,Alwarkurichi,Tenkasi district, Tamil Nadu. Fishes were collectedusing cast net and the live fish samples were immediately transported to the laboratory of SriParamakalyani Centre of Excellence in EnvironmentalSciences, ManonmaniamSundaranarUniversity,Alwarkurichi, Tamil Nadu, India.

Figure 1: Experimental fish – *D. filamentosa.*

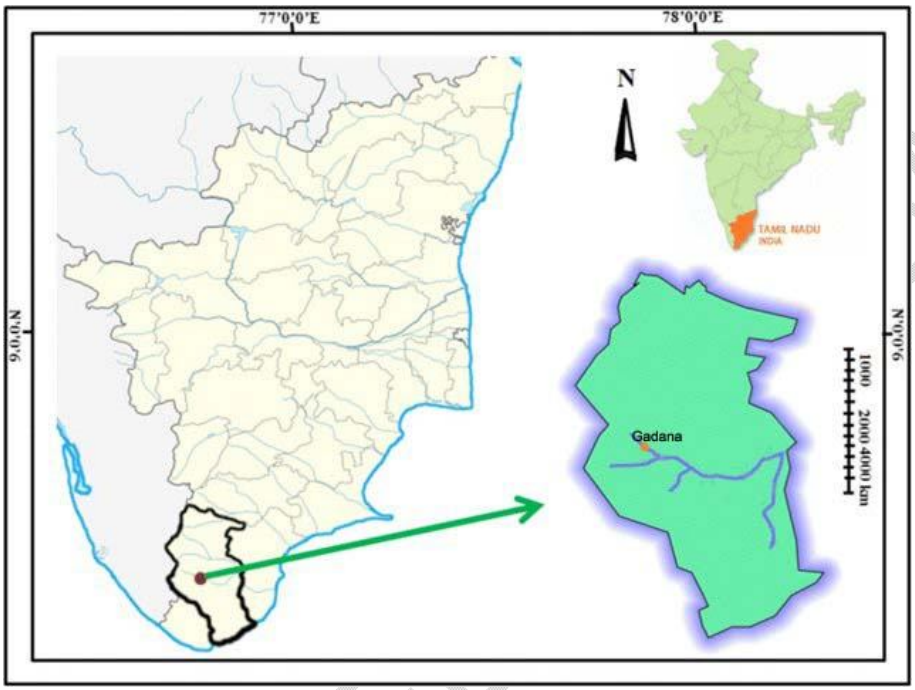

Figure 2: Map showing the study site – Gadana river

2.2. IsolationofGutandtheirhomogenization

The fish was anaesthetized by placing them in an ice bath for 5 to 10 min andthen surfacesterilizedbydippingthemin70% ethanolfor 30seconds.Its body surface was sterilized with 70% ethanol and its gut wasaseptic allydissected[Figure3]andplaced in 10ml sterilephosphate buffered saline solution (PBS). The gut contents werehomogenized well using mortor and pestle aseptically.The homogenized contentswere mixedwith100mlsteriledistilledwaterandthesamplewas saidtobediluted100times(10^{-2}dilution).Thenthesamplewasserially dilutedfrom10^{-2}to10^{-7}dilutions.

Figure 3: Dissected GI tract of *D. filamentosa*.

2.3. IsolationofGIbacteria

Fromtheseriallydilutedsample0.1mlwastakenfrom10^{-6}and10^{-7}dilutionsandfollowedthe spread plate technique in order to isolate the bacterialcolonies in nutrient agar plates. Then allthe plates wereincubated for 24 hours at 37°C.The colonies developedwere counted andexpressedasCFU/g.Furthertheisolatesfromthenutrientagarwerestreakedonthenutrientaga r slantstogetpure cultureand forstorage.

2.4. Identification of fish GI bacteria

Allthebacterialisolateswereretestedfortheirbiochemicalcharactersthrough the following biochemical tests[Figure4a –k], such as and Gramstaining, Motility, Indoletest, Methylredtest, VogesProskauertest, Citrateutilizationtest, Triplesugartest, Catalasetest, Oxidasetest, Ureasetest, Nitratetest, Starchhydrolysis and Carbohydratefermentationtest. The biochemical characterization ofbacterialisolateswasperformedasdescribedinBergeys'manualofDeterminativebacteriology[1 3].

2.5. Isolation of fungi

From the serially diluted samples 0.1 ml was taken from 10^{-6} and 10^{-7} dilutions and followed the spread plate technique in order to isolate the fungal colonies in Potato Dextrose Agar (PDA) plates. The plates were incubated for 24 – 48 hours at 28°C in an inverted position and the fungal growth can beobserved with distinct colonies after incubation.

2.6. Fungal observation by staining method

After incubation for 2 to 4 days, a drop of lactophenol cotton blue (LPCB) was placed on a microscopic slide. A small tuft of the fungus, preferably with spore and spore bearing structures were transferred into the drop and mixed gently. A cover slip was placed gently to avoid trapping of air bubbles in the stain. The preparation was observed under the low and high power (10X and 40X) objectives of the microscope.

3. RESULTS AND DISCUSSION

Comment [MF3]: Need to include some of culture -independent techniques (Molecular techniques) like q PCR to identify the unculturable microorganisms.

3.1. Bacteria identification

The total number of cultivable bacterial cells present in *D.filamentosa*gut were estimated after isolation and growth on nutrient agar plates. The total bacterial density was counted from 10^{-5},10^{-6} and 10^{-7} dilutions and the results were depicted in table 1, below. The bacterial density ranged from 47x10^{-7} to 219 x10^{-5} CFU/g of the gut sample and it was found to be the maximum at 10^{-4} dilution which is TNTC (too numerous to count) and minimum at 10^{-8} dilution which is TLTC (too low to count). A total of six bacterial strains and four fungi strains were isolated.

Table 1: Bacterial load in gut of *Dawkinsiafilamentosa*

Dilution	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Bacteria load (CFU/g)	TNTC*	219	144	47	TLTC**

*TNTC - Too Numerous To Count**TLTC – Too Low To Count

All the six bacterial isolates were tested for their biochemical characteristics (Table 2). Gram staining tests shows that two strains are gram negative rod, one is gram positive cocci and one is gram positive rod. Through biochemical characterization [Figure4a - k] and colony morphology, the isolated bacteria from the GI of *Dawkinsiafilamentosa*, the isolates were tentatively identified as strain 1- *Pseudomonas sp.,* strain 2- *Aeromonas sp.,* strain 3- *Staphylococcus sp.,* strain 4- *Bacillus sp.,* strain 5 – *Enterobacter sp.,* and strain 6 - *Vibrio sp.*

Comment [MF4]: four Strains were gram negative rod
Pseudomonas
Aeromonass
Enterobacter
And Vibrio

Figure 4: Biochemical characterization (A) Indole test, (B) Methylred test (C) Voges Proskauer test (D) Citrate utilization test (E) Triple sugar iron test (F) Urease test (G) Nitrate test (H) Carbohydrate fermentation test (I) Catalase test (J) Starch hydrolysis test (K) Oxidase test.

Through a series of biochemical tests, the strain 1 was identified as *Pseudomonas sp.,* which is a gram-negative, rod-shaped, motile bacteria, it shows positive results for Methyl red test, Citrate Utilization, Catalase, Oxidase, Nitrate and Carbohydrate fermentation tests, negative for Indole, Voges Proskauer, Urease and Starch hydrolysis test, and it involves in alkaline production and no gas production for the Triple sugar iron test. And the strain 2 was found to be the Gram-negative, motile rod - *Aeromonas sp.,* which shows positive results for Indole, Methyl red, Voges Proskauer, Citrate Utilization, Catalase,

Oxidase, Urease, Nitrate, Carbohydrate fermentation and Starch hydrolysis tests, it involves in acid and gas production for the Triple sugar iron test.

The strain 3 was identified as a Gram positive, non-motile Cocci - *Staphylococcus sp.,* as it shows positive results for Methyl red test, Voges Proskauer, Citrate Utilization, Catalase, Urease Nitrate and Carbohydrate fermentation tests, negative for Indole, Oxidase, and Starch hydrolysis test, and it involves in acid production with no gas or H_2S formation for the Triple sugar iron test. And the bacterial strain 4 was identified as - *Bacillus sp.* which is a gram-positive, rod-shaped, motile bacteria, it shows positive results for, Voges Proskauer, Citrate Utilization, Catalase, Nitrate, Starch hydrolysis test and Carbohydrate fermentation tests, negative for Methyl red test Indole, Oxidase, and Urease tests, and it involves in alkaline production and no gas or H_2S formation for the Triple sugar iron test.

The strain 5 was identified as a Gram negative, Rod - *Enterobacter sp.,* as it shows positive results for motility test, Voges Proskauer, Citrate Utilization, Oxidase, Nitrate and Carbohydrate fermentation tests, negative for Indole, Methyl red, Catalase, Urease and Starch hydrolysis test, and it involves in acid production with gas and no H_2S formation for the Triple sugar iron test. And the bacterial strain 6 was identified as - *Vibrio sp.* which is a gram-negative, curved rod-shaped, motile bacteria, it shows positive results for, Voges Proskauer, Catalase, Urease, Nitrate, Starch hydrolysis test and Carbohydrate fermentation tests, negative for Indole, Methyl red, Citrate utilization, and Oxidase tests, and it involves in alkaline production in slant and acid production in butt with H_2S and gas formation for the Triple sugar iron test.

Table 2: Biochemical test results for bacterial isolates

S.No	Biochemical tests	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
1	Shape	Rod	Rod	Cocci	Rod	Rod	Rod (slightly curved)
2	Gram's staining	-	-	+	+	-	-
3	Motility	+	+	-	+	+	+
4	Indole	-	+	-	-	-	-
5	Methyl red	+	+	+	-	-	-
6	VogesProskauer	-	+	+	+	+	+
7	Citrate utilization	+	+	+	+	+	-
8	Triple sugar iron	K/K	A/A	A/A	K/A	A/A	K/A
	Gas	-	+	-	-	+	+
	H_2S	-	-	-	-	-	+
9	Catalase	+	+	+	+	-	+
10	Oxidase	+	+	-	-	+	-

11	Urease	-	+	+	-	-	+
12	Nitrate	+	+	+	+	+	+
13	Starch hydrolysis	-	+	-	+	-	+
14	Carbohydrate fermentation	+	+	+	+	+	+
15	Genus Identified as	*Pseudomonas sp.*	*Aeromonas sp.*	*Staphylococcus sp.*	*Bacillus sp.*	*Enterobacter sp.*	*Vibrio sp.*

3.2. Fungi identification

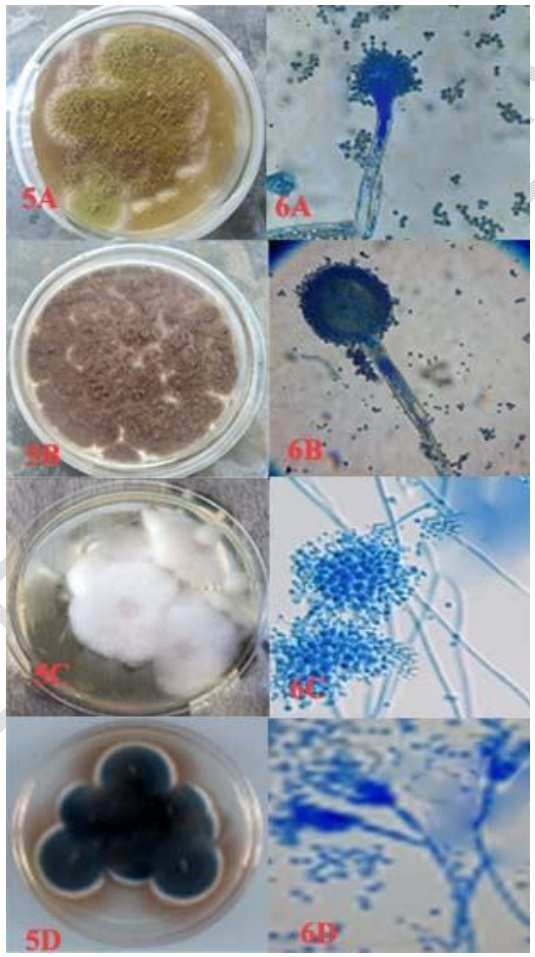

Figure 5 and 6 : Macroscopic and microscopic (40x) identification of fungi species such as (5A, 6A) *A. flavus*, (5B, 6B) *A. niger*, (5C, 6C) *B. basiana* and (5D, 6D) *Penicillium sp.* encountered in the GI of *D. filamentosa.*

The investigation of fungal diversity within the gastrointestinal tract of *Dawkinsiafilamentosa*results in identification four different fungal species namely, *Aspergillusflavus, Aspergillusniger, Beauveriabasiana,* and *Penicillium sp.* They were identified by their colony morphology and by observing mycelium and their spores under microscope.**3.3. Macroscopic characteristics**

The isolated fungal colonies that grew on the Potato Dextrose Agar plates showed the following morphology. *Aspergillus flavus* - Green spores that appear to be powdery are visible on the upper surface while the lower surface is reddish-yellow in color[Figure5A].*Aspergillusniger* - The colony appears velvety with white and black spores on the surface. The lower surface is yellow and heavily furrowed [Figure 5B]. *Beauveriabasiana*- White colony with hyphae resembling floss in cotton. The colony elevation were thick and raised [Figure 5C]. *Penicillium sp.* -The surface color of the colony is dark green with a velvety texture. The lower surface has a high edge, a shallow centre, and a colourless to creamy appearance [Figure 5D].

3.4. Microscopic Characteristics

The fungal colonies that were stained with LPCB stain showed the following characteristics which were observed under 40X objective in light microscope. *Aspergillus flavus* – Hyphal growth produces mycelia. The conidiophores are seen rough, colorless and thick walled bearing the vesicles. Flask shaped phialides are seen. The vesicles are sub globose in shape [Figure6A]. *Aspergillusniger* – Filamentous fungi, consist of smooth colorless conidiophore which produces dark brown spores from its conidial head. The conidial heads are globose and dark brown in colour [Figure6B]. *Beauveriabasiana*– Its conidiophores are supported by long zig zag like transparent hyphae that produce short spike like structures that gives a convex appearance to the cells [Figure6C]. *Penicillium sp.* - the fungal hyphae are simple, elongated and unbranched, each end bears a cluster of flask shaped phialides are present. Matured spores occupy the apex of phialides and immature spores occupies at the bottom [Figure6D].

4. Discussion

Due to aquatic habitat, fishes have more physical contact with the ambient micro-biota than terrestrial species. Bacterial and fungal spores from the aquatic environment are continuously consumed along with water and food[14]. Because of this, invading microbes likely to be engaged in more regular and significant interaction with the fish digestive systems in comparison to the land-based animals. The GI microbiota in the freshwater fish, *Dawkinsiafilamentosa*, was examined in the current study. *Pseudomonas sp.*, *Aeromonas sp.*, *Staphylococcus sp.*, *Bacillus sp.*, *Enterobacter sp.* and *Vibrio sp.* were

the bacterial species with the total bacterial load ranged from 47 $x10^{-7}$ to 219 $x10^{-5}$ CFU/g of the GI sample and *Aspergillusflavus, Aspergillusniger, Beauveriabasiana,* and *Penicillium sp.* were the fungal species isolated from its gastro intestinal tract of the fish. The GI composition was discussed above. The *Dawkinsiafilamentosa*is an omnivore species whichfeeds on algae, tiny green plants, aquatic insects, worms and organic debris[15]. Filamentous algae are their primary diet[16, 17].One of the elements that could contribute to the GI flora in these fishes are their habitat for feeding. Although the host GI tract offers habitat for microorganisms, it has been shown that some adaptations are necessary for this possession.

Ley et al.,[18]documented the requirements of microbiota by the Gastrointestinal tract, including the need for cell surface molecules for attachment for the microorganisms to adhere resolutely to the mucosal epithelium of the GIlining, the production of enzymes for the effective utilisation of all micro and macro nutrients, and the need for genetic makeup in the microbes to adapt towards the ecological plasticity provided by the GI tract and for immunity against bacteriophage[19]. The most adaptable bacteria can survive and thrive in the GI tract and occupy the majority of niche spaces to establish themselves permanently are the autochthonous microbes, While, some are allochthonous, they are the GI visitors who derive from their surroundings[20].The bacterial and fungal biota were screened from*Oreochromisniloticus'*s intestine, gills and skin. *Klebsiella, Citrobacter* and *Erwinia* were the common bacterial species present among them[21]. Their mycological investigation revealed the presence of *Aspergillus, Pencillium*and*Fusarium*species. Our mycological investigation results are in partial consistent with the Mahmoud et al., [11].These results are the indication of infection in fishes that may have more impact on human health due to high consumption. There are few evidences that the microbial makeup varies along the GI tract, with the foregut microbial populations being very different from the hindgut microbial populations[10]. But more information is needed to be fully understand how these two demographic categories differ from one another.

5. Conclusion

The current research revealed that there is various microflora in the gut of *Dawkinsiafilamentosa*, and that microbes from the freshwater environment may help to incorporate these bacteria in commercial aquaculture practices as a supplement for fish feed. Several researches done so far, has provided significant perspectives into the mechanisms by which these communities are able to control the fish host. These insights have also helped to improve aquaculture practices, gain a better understanding pertaining to host-microbe interactions among other vertebrates and develop potential treatments.Hence, best research practices in this field havegreat significantly helps to better

understandingsustainable interaction on microbiota with the aquatic organism like fishes.For the enhanced sustainability of the mankind, it is essential to mine the hidden resources from the universe of microbiota like bacteria and fungi. It opens up a variety of study fields for the advancement of aquaculture, fulfilling the need for wholesome food across the world.

6. References

1. Kanthimathi M, Kanimozhi S, Muralidharan M and Soranam R. Molecular characterization of selected *Mystus* species using COI gene as DNA barcode. Journal of Xi'an shiyou university, Natural Science Edition. 2023. 19(2): 436 - 440.
2. Hartviksen M, Vecino JLG, Ringø E, Bakke AM, Wadsworth S, Krogdahl Å et al. Alternative dietary protein sources for Atlantic salmon (Salmo salar L.) effect on intestinal microbiota, intestinal and liver histology and growth. Aquac. Nutr. 2014;20:381–98.
3. Reed GB, Spence CM. the Intestinal and Slime Flora of the Haddock: a Preliminary Report. Contrib. to Can. Biol. Fish. 1929;4:257–64.
4. Givens CE, Ransom B, Bano N, Hollibaugh JT. Comparison of the gut microbiomes of 12 bony fish and 3 shark species. Mar. Ecol. Prog. Ser. 2015;518:209–23.
5. Uma A, Subash P, Abraham TJ. Importance of gut microbiota in fish – A review. Indian J. Anim. Heal. 2020;59:181–94.
6. Karlsen C, Tzimorotas D, Robertsen EM, Kirste KH, Bogevik AS, Rud I. Feed microbiome: confounding factor affecting fish gut microbiome studies. ISME Commun. 2022;2:1–4.
7. Hoseinifar SH, Mirvaghefi A, Amoozegar MA, Sharifian M, Esteban MÁ. Modulation of innate immune response, mucosal parameters and disease resistance in rainbow trout (Oncorhynchus mykiss) upon synbiotic feeding. Fish Shellfish Immunol. 2015;45:27–32.
8. Romero J, Ringø E, Merrifield DL. The gut microbiota of fish. Aquac. Nutr. Gut Heal. Probiotics Prebiotics. 2014;9:75–100.
9. Talwar C, Nagar S, Lal R, Negi RK. Fish Gut Microbiome: Current Approaches and Future Perspectives. Indian J. Microbiol. 2018;58:397–414.
10. Ye L, Amberg J, Chapman D, Gaikowski M, Liu WT. Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. ISME J. 2014;8:541–51.
11. Zaky MMM, Ibrahim ME. Screening of Bacterial and Fungal Biota Associated with

Oreochromis niloticus in Lake Manzala and Its Impact on Human Health. Health (Irvine. Calif). 2017;09:697–714.

12. Arumugam S, Abul Asan Sathali MS, Ramaiah S, Krishnan G. Diversification of Dawkinsia filamentosa (Valenciennes, 1844) and their growth conditions by the impact of toxic metals in the river Tamiraparani. Ecotoxicology. 2021;30:1043–55.

13. Sinclair CG. Bergey's Manual of Determinative Bacteriology. 1939. doi:10.4269/ajtmh.1939.s1-19.605.

14. Da Costa PM, Loureiro L, Matos AJF. Transfer of multidrug-resistant bacteria between intermingled ecological niches: The interface between humans, animals and the environment. Int. J. Environ. Res. Public Health. 2013;10:278–94.

15. Mahadevi, Felix S, Ahilan B, Rajagopalasamy CBT, Samuel Moses TLS. Induced Breeding and Developmental Biology of Endemic WesternGhatsFishDawkinsiafilamentosa(Valenciennes,1844) under Captive Conditions. Indian J. Anim. Res. 2020;54:1069–77.

16. Kanthimathi M, Soranam R. Morphological Characterization of Two Species of the Freshwater Fish Genus Dawkinsia (Osteichthyes: Cyprinidae), with Emphasis on Stream Habitat Characteristics. Res. Rev. J. Ecol. 2016;5:8–21.

17. Sajen SH, Sabaridasan A, Devi KSS, Kanthimathi M, Palanikani R, Soranam R. Environmental Factors Associated with Fish Diversity in Two Tributaries of River Chittar, Southern Western Ghats, Tamil Nadu, India. Asian J. Fish. Aquat. Res. 2022:10–23.

18. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124:837–48.

19. Saleh A, Shawky R, Sabae Z, Abou El-Gheit EN. Microbiological water quality and bacterial infections among Tilapia fish of Lake Manzala, Egypt. Int. J. Environ. water. 2013;2:75–85.

20. Nayak SK. Role of gastrointestinal microbiota in fish. Aquac. Res. 2010;41:1553–73.

21. Yoshida M aki, Tanabe T, Akiyoshi H, Kawamukai M. Gut microbiota analysis of Blenniidae fishes including an algae-eating fish and clear boundary formation among isolated Vibrio strains. Sci. Rep. 2022;12:1–13.