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Microbiota from Intestinal Gut of *Dawkinsia filamentosa* (Valenciennes,1844) as Indicator of Environmental Condition

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To isolate and identify the gut associated micro-organisms present in the gastrointestinal tract of freshwater fish *Dawkinsia filamentosa.*
Place and Duration of Study: Sampling site: The fish samples were collected from the river Gadana, located at the foot hills of Western Ghats, Alwarkurichi, Tenkasi district, Tamil Nadu, India. Place: Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University, Azhwarkurichi, Tenkasi, Tamilnadu, India (Latitude 8°20'N; Longitutde 77°10'E). Duration: The research was done during December 2021 and April 2022.

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Methodology: The fish samples were anaesthetized and surface sterilized followed by aseptic dissectionby using sterile scalpel and needle. The gut contents were removed and homogenized well and isolation of Gut micro biome was done by means of serial dilution. The identification of gastrointestinal bacteria was done by performing various biochemical tests such as Gram staining, Motility, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Triple sugar Iron test, Catalase test, Oxidase test, Urease test, Nitrate test, Starch hydrolysis and Carbohydrate Fermentation Test. The fungi present in gastrointestinal tract is identified with lacto phenol 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 *Aspergillus flavus, Aspergillus niger, Beauveria basiana,* and *Penicillium* sp.

Conclusion: The current research revealed that, *Dawkinsia filamentosa* has a variety of microbiota 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 practices. The benefits of identifying gut microbiota in fishes is, it helps to study about the host nutrient catabolism and immune defence mechanism against the disease-causing pathogens.

Keywords: Dawkinsia filamentosa; 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 microbiota 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 gastro intestinalmicrobiota can reflect their diet preferences and physiological behaviors which could be identified through the fecal matter discharged into the water.

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 microbiota 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 can identify 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*, which is selected as it is commonly available fish species and popular for consumption. It is 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 micro biomes of the fish.

2. MATERIALS AND METHODS

2.1 Sample Collection

The fish sample of *Dawkinsia filamentosa* (Fig. 1) was collected from the Gadana river (Fig. 2) (latitude 8° 37' 0427" N; longitude 77° 18' 7243" E) located atthefoot of Western Ghats, Alwarkurichi, Tenkasi district, Tamil Nadu. Fishes were collected using cast net and the live fish samples were immediately transported to the laboratory of Sri Paramakalyani Centre of Excellence in Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi, Tamil Nadu, India.

2.2 Isolation of Gut and Their Homogenization

The fish was anaesthetized by placing them in an ice bath for 5 to 10 min and then surface sterilized by dipping the min 70% ethanol for 30seconds.Its body surface was sterilized with 70% ethanol and its gut was aseptically dissected (Fig. 3) and placed in 10ml sterile phosphate buffered saline solution (PBS). The gut contents were homogenized well using mortor and pestle aseptically. The homogenized contents were mixed with 100ml sterile distilled water and the sample was said to be diluted 10 times (10^{-2}). Then the sample was serially diluted rom10^{-2}to10^{-7}.

Fig. 1. Experimental fish – *Dawkinsia filamentosa*

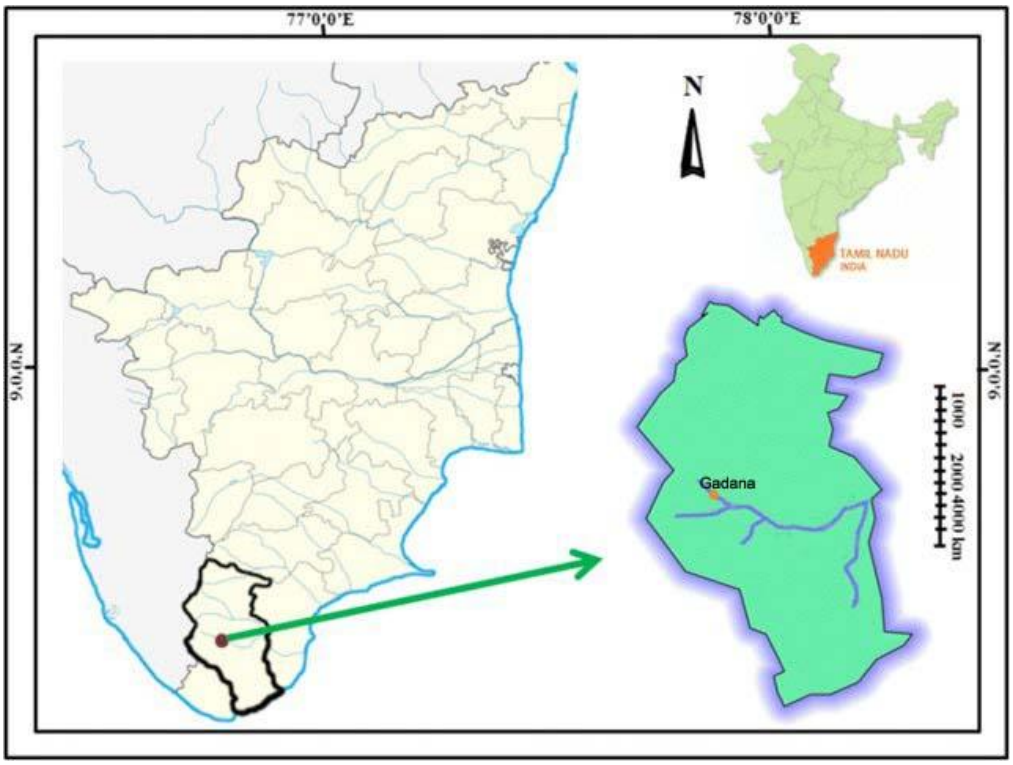


Fig. 2. Map showing the study site – Gadana river

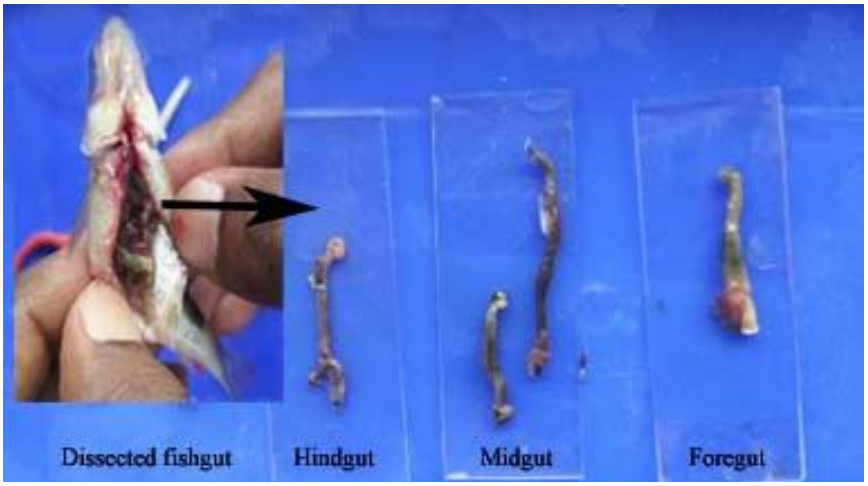


Fig. 3. Dissected GI tract of *D. filamentosa*

2.3 Isolationand Identification of GItract Microorganisms

2.3.1 Isolation and identification of GI bacteria

From the serially diluted sample 0.1 ml was taken from 10^{-6} and 10^{-7} dilutions and followed the spread plate technique in order to isolate the bacterial colonies in nutrient agar plates as the nutrient agar contains nutrients that made them suitable for sub culturing a wide range of cultural microorganisms. Then all the plates were incubated for 24 hours at 37^{o}C. The colonies developed were counted and expressed as CFU/g. Further the isolates from the nutrient agar were streaked on the nutrient agar slantstogetpure culture and for storage.

All the bacterial isolates were tested for the is biochemical characters through the following biochemical tests, such as and Gram staining,

Motility, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Triple sugar Iron test, Catalase test, Oxidase test, Urease test, Nitrate test, Starch hydrolysis and Carbohydrate fermentation test. The biochemical characterization of bacterial isolates was performed as described in Bergey's manual of Determinative bacteriology [13].

2.3.2 Isolation and fungal observation

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 Sabouraud Dextrose Agar (SDA) plates. The plates were incubated for 24 – 48 hours at 28^{o}C in an inverted position and the fungal growth can be observed with distinct colonies after incubation. The fungal morphology selected for further study includes powdery, furrowed, velvety and cottony textured with green, brown, white and black colored colonies.

After incubation for 2 to 4 days, a drop of lacto phenol 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 [14]. The preparation was observed under the low and high power (10X and 40X) objectives of the microscope.

3. RESULTS AND DISCUSSION

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. 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). The dilutions which contain 30-300 colonies per plate were used to count. The plate that contains less than 30 colonies were referred as TLTC and the plate that contains more than 300 colonies were referred as TNTC. A total of six bacterial strains and four fungi strains were isolated.

All the six bacterial isolates were tested for their biochemical characteristics (Table 2). Gram staining tests shows that four strains are gram negative rod, one is gram positive cocci and one is gram positive rod. Through biochemical characterization and colony morphology, the isolated bacteria from the GI of *Dawkinsia filamentosa*, 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*.*

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, Citrate Utilization, Catalase, Oxidase, Nitrate tests and specifically they can grow in blood agar. And the strain 2 was found to be the Gram-negative, motile rod - *Aeromonas* sp*.,* which shows positive results for Catalase and Oxidase which were performed in MacConkey agar in absence of fermentable sugar.

The strain 3 was identified as a Gram positive, non-motile, Cocci - *Staphylococcus* sp*.,* as it shows positive results especially for coagulase test which could be detected by either slide or tube coagulase test. The strain 4 was identified as - *Bacillus* sp. which is a gram-positive, rod-shaped, motile bacteria, it shows positive results for, enzyme hydrolysis tests specifically for Catalase, Nitrate, oxidase, and gelatin hydrolysis tests.

The strain 5 was identified as a Gram negative, Rod - *Enterobacter* sp*.,* as it shows positive results for motility test, and it has the ability to grow in SIM agar which is a differential media for members of Enterobacteriaceae. The 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, Starch hydrolysis tests, and it can grow well in TCBS (Thiosulphate Citrate Bile Salt) agar, which is the selective medium for *Vibirio* sp.

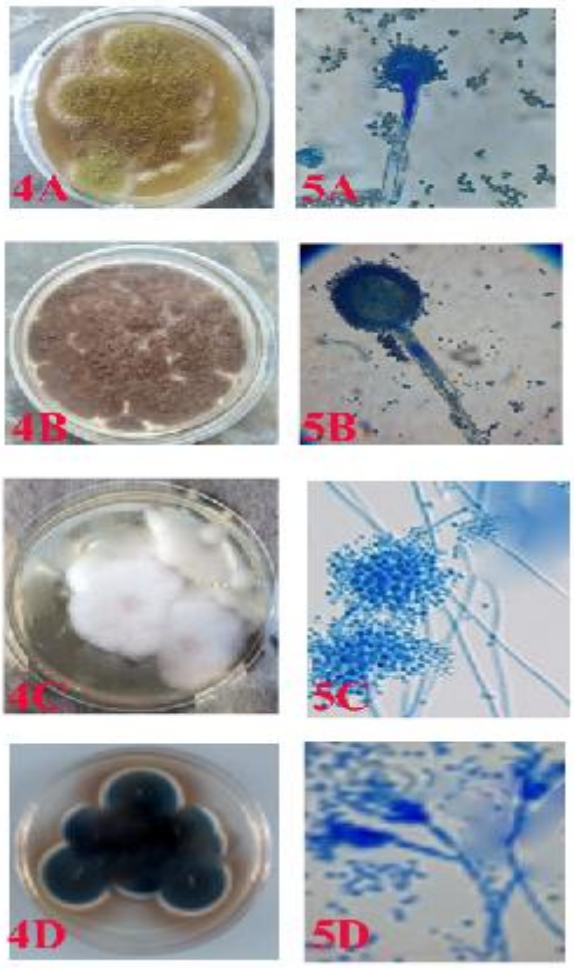
Table 1. Bacterial load in gut of *Dawkinsia filamentosa*

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(>300 colonies per plate); **TLTC – Too Low To Count (<30 colonies per plate)*

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.



Figs. 4 and 5. Macroscopic and microscopic (40x) identification of fungi species such as (4A, 5A) *A. flavus*, (4B, 5B) *A. niger*, (4C, 5C) *B. basiana* and (4D, 5D) *Penicillium* sp. encountered in the GI of *D. filamentosa*

3.2 Fungi Identification

The investigation of fungal diversity within the gastrointestinal tract of *Dawkinsia filamentosa* results in identification four different fungal species namely, *Aspergillus flavus, Aspergillus niger, Beauveria basiana,* 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 ([Fig. 4A). *Aspergillus niger* - The colony appears velvety with white and black spores on the surface. The lower surface is yellow and heavily furrowed (Fig. 4B). *Beauveria basiana*- White colony with hyphae resembling floss in cotton. The colony elevation were thick and raised (Fig. 4C). *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 (Fig. 4D).

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 (Fig. 5A). *Aspergillus niger* – 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 (Fig. 5B). *Beauveria basiana*– 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 (Fig. 5C). *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 [Fig. 5D].

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 [15]. 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. In healthy fishes, the gut microbiome performs a wide range of beneficial tasks, including obtaining energy through the metabolism of nondigestible food substances, defending the host against pathogenic attack and immunomodulation. The gut microbiota is thought to contribute in a variety of ways by increasing the effectiveness of digestion, increasing its capacity to survive on diets that are not optimal and provision of certain nutrients and vitamins [15]. The GI microbiota in the freshwater fish, *Dawkinsia filamentosa*, 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 *Aspergillus flavus, Aspergillus niger, Beauveria basiana,* 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 [16]. Filamentous algae are their primary diet [17,18].One of the elements that could contribute to the GI microbiota 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. [19] 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 GI lining, 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 [20]. 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 [21]. The bacterial and fungal biota were screened from *Oreochromis niloticus'*s intestine, gills and skin. *Klebsiella, Citrobacter* and *Erwinia* were the common bacterial species present among them [22]. 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 [10,23,14]. 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 microbiota in the gut of *Dawkinsia filamentosa*, 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 have great significantly helps to better understanding sustainable interaction on micro biota 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 micro biota 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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