Preliminary Examination of Gut Microbiota of *Pseudoetroplus maculatus* from Kadinamkulam Lake, Thiruvananthapuram, Kerala

DISSERTATION

Submitted to the University of Kerala

in the partial fulfilment of the requirement for the

BACHELOR OF SCIENCE IN

ZOOLOGY

Department of Zoology

TKM COLLEGE OF ARTS AND SCIENCE, KOLLAM - 5

2021-2024

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By

Candidate code	Name
25021142003	ANAGHA B.S.
25021142008	IRFANA I.
25021142012	REMYA KRISHNAN R.
25021142017	VISHNUPRIYA A.
25021142023	AKHILA S.
25021142027	ANSIYA S.
25021142033	SUBITHA S.
25021142020	ABHISHEK P.
25021142028	ARJUNKRISHNA M.

Department of Zoology
TKM COLLEGE OF ARTS AND SCIENCE, KOLLAM

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CERTIFICATE

This is to certify that the dissertation entitled '***Preliminary Examination of Gut Microbiota of Pseudoetroplus maculatus from Kadinamkulam Lake, Thiruvananthapuram, Kerala'*** is a bonafide work done by …….....under my supervision as partial fulfillment of the requirements for the ***Degree of Bachelor of Science in Zoology*** and this report has not been submitted earlier for the award of any degree or diploma or any other similar titles anywhere.

Dr. MUMTHAS Y

(Supervisor)
Assistant Professor,
Dept. of Zoology

Certified bonafide:

Dr. JASIN RAHMAN V. K.

Assistant Professor and HOD,
Dept. of Zoology

Examiners: 1.

2.

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DECLARATION

I do hereby declare that this dissertation entitled ***'Preliminary Examination of Gut Microbiota of Pseudoetroplus maculatus from Kadinamkulam Lake, Thiruvananthapuram, Kerala'*** is a bonafide work done by me under the supervision of Dr. Mumthas Y., Assistant Professor, Department of Zoology, TKM College of Arts and Science, Kollam as partial fulfilment of the requirements for the award of ***Degree of Bachelor of Science in Zoology***. No part of this has been presented earlier for any degrees or diploma of any university.

Name

Signature

Candidate Code

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CONTENTS

INTODUCTION

Fish are aquatic poikilothermic organisms that include a wide variety of animal kingdom vertebrates and invertebrates. Rich in omega-3 fatty acids, vitamins B2 and D, and minerals iron, calcium, phosphorus, iodine, magnesium, zinc, and potassium, fish are thought to be good sources of low-fat, high-quality protein. Since the middle of the 20th century, as the world's population has grown, so has the consumption of fish. Per capita fish consumption increased from 9.0 kg in 1961 to 20.2 kg in 2015, with an average annual growth rate of 1.5%, according to FAO (2020). In 2016 and 2017, the estimates were around 20.3 and 20.5 kg, respectively. The 170.9 MMT total fish production produced worldwide—90.9 MMT from capture fisheries and 80.0 MMT from aquaculture—contributed to this need. Together, the inland and marine aquaculture sectors produced 51.4 MMT and 28.7 MMT of fish, respectively, making up 46.8% of the total fish production.

According to Austin (2002), fish have bacterial flora on or in their skin and in several organs. The bacterial genera that are isolated are often associated with the fish habitat and vary depending on a number of characteristics, including salinity and the amount of bacterial communities present in the water. It's possible that the bacteria found on the fish's skin and gills are transient rather than permanent surface inhabitants. Fish gut microbiome appears to change in response to digestive system difficulties. There is evidence that certain animals have a distinct intestinal microflora. The microbiota found in the gut is a reflection of those from the surrounding environment or the food consumed that can survive and reproduce within the intestinal tract (Cahill, 1990). Fish growth, the lifespan, physiology, immunology, and defenses against infection can all be impacted by gut microbiota (Yan et al., 2016). Therefore, the elements that control the microbial invasion of fish guts will provide a foundational step forward in the prediction and management of fish illnesses (Xiong et al., 2019).

There is no single blue print for the alimentary canal of a fish; fish biology varies greatly with differing life histories, ecology and environmental factors. Filter feeders, parasites and predators as well as herbivorous and carnivorous fish exist and each has an appropriately adapted digestive system. Regardless of diet, the gut of some fish consists simply of a short tubular intestine, e.g., parrotfish, Scarus radicans (Horn et al., 2006). However, the majority of fish alimentary canals are divided into topographical regions with unique roles. All fish alimentary canals begin with the buccal and pharyngeal cavities of the head gut. From here, the gut can be loosely divided into the fore-, mid- and hind-gut which include various digestive organs that particular fish either possess or lack. The foregut, beginning at the posterior edge of the gills, often consists of the oesophagus, stomach and pylorus. However, it is estimated that 20% of fish species lack a true stomach (Wilson and Castro, 2010).

The fish gut microbiota may be impacted by several causes. Fish gut microbiota can vary in response to changes in external variables such as trophic levels, age, nutrition, and environment (Wang et al., 2018). The association of microorganisms is examined using a variety of ways in order to research the gut microbiomes in fish. A very small percentage of the important microorganisms were previously disclosed by the culture-dependent methods (Ringo et al., 2003), denaturing gradient gel electrophoresis, and temporal temperature gradient gel electrophoresis techniques (Reveco et al., 2014). A very small percentage of the important bacteria were previously discovered using the culture-dependent approaches (Ringo et al., 2003; Romero and Navarrete, 2006), denaturing gradient gel electrophoresis, and temporal temperature gradient gel electrophoresis techniques (Reveco et al., 2014). However, a large range of culture-independent techniques are now accessible for fish microbiota characterization (Tarnecki et al., 2017). Because we understand the significance of microbial communities in fish anatomy, we can work with these communities to optimize their expression in the body, thereby contributing to the health of fish.

Furthermore, evidence has demonstrated the significance of the gut microbiota for host nutrition and other physiological functions. It has been proven that the gastrointestinal tract's microbiota colonisation produces a variety of metabolites, amino acids, vitamins, and digestive enzymes that are critical to fish development and digesting (Sugita et al., 1997). For instance, large Aeromonas concentrations can facilitate digestion under steady circumstances by secreting several proteases. By producing byproducts such volatile fatty acids, which have been found in the largemouth bass's gut, anaerobic bacteria also aid in the digestive process and the absorption of nutrients (Nayak 2010).

Orange chromide, *Pseudoetroplus maculatus* is a euryhaline fish with a wide distribution that can survive in fluctuating salinity. This species is endemic to brackish water, streams, lagoons, estuaries, and the lower reaches of rivers in peninsular India and Sri Lanka. Popularly known as Orange chromide, *Pseudoetroplus maculatus*, an omnivorous species, is widely distributed in almost all rivers and backwaters of Peninsular India and Sri Lanka (Jayaram, 1999). Its small size, bright orange colour and black spots on the body, calm nature etc., make them attractive candidates for the tropical aquariums. The colour and body patterns of the species make it an important fish for the ornamental trade (Bindu and Padmakumar, 2012).

Many of the indigenous ornamental fishes are being identified for potential use in ornamental fish industry and breeding technologies are being developed as pre-requisite to commercial exploitation. This has led to a spurt in freshwater ornamental industry and trade in Kerala during the last five years. However, many of the natural waters are subjected considerable organic pollution which might provide right kind of environment for fish. In this regard, we have analysed the prevalence of gut associated bacteria from indigenous freshwater ornamental fish *Pseudoetroplus maculatus* in order to get a snapshot of its viable count. In the present study, total viable count of bacteria in the gut of Orange chromide (*Pseudoetroplus maculatus*) collected from Kadinamkulam Lake, Thiruvanathapuram was analyzed and will provide insight into the microbial load present in the gut of Orange chromide.

AIM

To conduct a preliminary examination of gut microbiota of *Pseudoetroplus maculatus* that was collected from the Kadinamkulam lake in Thiruvananthapuram district of Kerala.

OBJECTIVES

To calculate the Total viable count of microbes, present in the gut of *Pseudoetroplus maculatus* in Kadinamkulam lake, Thiruvananthapuram.

To ascertain the Total plate count of Vibrio, present in the gut of *Pseudoetroplus maculatus* in Kadinamkulam lake, Thiruvananthapuram.

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REVIEW OF LITERATURE

Fish and other aquatic animals interact with their surroundings and the microorganisms that dwell there in a distinctive intimate way. The waters throughout the planet are brimming with microbes. The International Council for the Exploration of the Sea [ICES], 2011 states that the amount of virus particles may be 100 times bigger than the estimated $3.6 * 10^{30}$ microbial cells, which make up over 90% of the entire marine biomass. Fish and the microbes in their environment can have mutualistic or harmful relationships.

Research into the gut microbiota of fish dates back to the early half of the 20th century but more recently interest in this area has grown at a significant rate coinciding with the expansion of the aquaculture industry. Indeed, the first works on this topic were published in the late 1920's and 1930's (Reed and Spence, 1929; Gibbons, 1933) and investigated the intestinal and "slime flora" of fish. There were some further exploratory studies during the 1950's and 60's; Margolis (1953) investigated the effect of fasting on the intestinal flora, Colwell (1962) examined the intestinal flora of Puget Sound fish and Simidu and Hasuo (1968) examined the salt dependency of fish flora. In the following decade, the studies became more applied, with interest in how the gut microbiota changed with diet (Sera and Ishida, 1972), how the microbiota changed in farmed fish (Gilmour et al., 1976) and how animals succumbed to infection (Boulanger et al., 1977; Olivier et al., 1981). In the early 1990's the first reviews on this topic were published (Cahill, 1990; Ringø et al., 1995). They provided a comprehensive overview of the studies to date; however, they consequentially reported that bacterial levels in the gut of fish were low and appeared to be derived from the surrounding environment or diet (Cahill, 1990; Ringø et al., 1995). One of the limitations in the isolation and culture of gut microbiota using culture-dependent methods is that, only 10% of microorganisms has been studied.

Gut microbiota of freshwater fish

Considerable differences in the composition of intestinal microbial flora in marine and freshwater fish are described by Izvekova et al. (2007). The gut microbial composition of the freshwater fish differs due to the varying environmental conditions of their habitat. *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Lactococcus*, *Pseudomonas*, obligate anaerobes (*Bacteroides*, *Clostridium* and

Fusobacterium) and members of family *Enterobacteriaceae* dominate the gut of freshwater species (Gómez and Balcazar, 2008). A limited number of bacterial taxa found in the intestines of some fish species may indicate not only a low diversity of these bacteria but may also be due to insufficient knowledge about them.

Herbivorous and omnivorous freshwater fish shows shorter gut transit times with low levels of short chained fatty acids (SCFA) in the gut, which are produced during the conversion of unassimilable algal constituents by the gut microbes (German et al., 2010) their some of them marine counterparts. The highest level of SCFA was reported in the posterior intestine of *Oreochromis* sp. Free living Amoebae are ubiquitous in freshwater fish *Oreochromis niloticus* (Milanez et al., 2017) and its infection poses a public health problem due to possible human consumption.

Gut microbiota of marine fishes

The gut of marine fish is dominated by *Alcaligenes*, *Alteromonas*, *Aeromonas*, *Flavobacterium*, *Carnobacterium*, *Moraxella*, *Micrococcus*, *Pseudomonas* and *Vibrio* (Gómez and Balcazar, 2008). A summary of the major bacterial flora composing the gut microbiota of marine fish was reviewed by Llewellyn et al. (2014). A review of the intestinal microflora of fish larvae and fry of 24 marine and freshwater fish showed the most frequently reported bacteria were *Vibrio, Pseudomonas, Cytophaga*, *Flavobacterium* and the family *Enterobacteriaceae* (Ringø and Birkbeck, 1999). While the microbial community changes with life stage and habitat, a relatively stable gut microbiota are established within the first 50 days of life for many species (Larsen et al., 2014). Lactic acid bacteria (mainly *Lactobacillus* sp.) have also been found to be minor components of the gut microflora of both freshwater and marine fish (Izvekova et al., 2007).

Importance of gut microbiomta in disease resistance

Gut microbiota are the microorganisms that are colonizing the digestive tract, enveloping the entire scope of the biochemical cycle, and incite a resistant arrangement of the host life form (Gómez and Balcazar, 2008). Numerous beneficial bacterial strains have been created to treat microorganism prompted bacterial maladies, and this current strategy's adequacy has been demonstrated (Verschuere et al., 2000). Some beneficial microbes can create microorganisms that stifle or even destroy the inhibitory compounds (Teplitski et al., 2009). *Lactococcus lactis* isolated

from marine fish produced bacteriocin nisin Z, which can restrain the development of the fish microbe *Lactococcus garvieae* at 5 AU mL-1 made it a promising option in the prevention of lactococcosis (Sequeiros et al., 2015). A bacteria *Centroscyllium fabricii* isolated from the deep sea shark was found to have an antagonistic activity in the fish gut (Bindiya et al., 2015).

Gut microbiota with respect to the life stages in fish

The colonization of fish gut begins early in the larval stage and is driven towards the achievement of a complex assemblage of gut associated microorganisms (Nayak, 2010). Microbial colonization of fish larvae originates from the eggs, the environment and the first feed. The microbiota of the surrounding water dictates which bacteria encounter the eggs and consequently have the opportunities to colonise. Upon hatching, sterile larvae intake the chorion-associated bacteria, which are the first colonisers of the developing gastrointestinal tract (GIT) (Egerton et al., 2018). The GIT of the newly hatched larvae tends to contain a few bacteria. Subsequent bacterial habitats are acquired in the fish larvae for the first time when they begin to drink water to control osmoregulation and the microbiota then becomes further diversified through feeding (Hansen and Olafsen, 1999).

Numerous studies have shown that diet plays a major role in shaping the gut microbial community and from first feeding; cause to substantial diversification (Lauzon et al., 2010). Around 108 bacterial cells having a place with more than 500 distinct species are accounted to populate the fish gut, which is overwhelmed by aerobes or facultative anaerobes (Romero and Navarrete, 2006). The diversity of the gut microbiota generally increases as the fish diet changes from predatory to omnivorous and omnivorous to herbivorous (Liu et al., 2016). The gut colonization can be either driven by stochastic (neutral assembly) or deterministic (non- neutral model). Stochastic deduced from random dispersion of microorganisms or events that land the microorganisms into the intestine that are responsible for the final shape of the gut microbial community and in deterministic, the assembly is acquired by the host selective factors, active dispersal by the host and microbe and microbe-microbe interactions (Talwar et al., 2018). Over a formative time, the colonization of gut was started by seeding from the surrounding environment, then progressively determined by the non-neutral factors as the fish matures from larvae to adult (Yan et al., 2016). Therefore, the studies suggested stochastic factors as a determinant in colonization of the GI tract. The gut microbial community can change with a variety of factors affecting the host, including

changing environmental conditions such as temperature and salinity (Macfarlane and Englyst, 1986), developmental stage (Romero and Navarrete, 2006), digestive physiology (Cahill, 1990) and feeding strategy (Uchii et al., 2006). Some of the gut microfloras appear to be temporary, while other bacterial floras seem to be permanent residents (Kim et al., 2007).

Herbivorous fish like pinfish *Lagodon rhomboides* under-goes an ontogenetic diet shift, while transitioning from carnivorous juveniles to either omnivorous or herbivorous adults (Gallagher et al., 2001). Likewise, the growth, development and migration in anadromous Atlantic salmon *Salmo salar* involve a radical shift across an ecological and trophic spectrum (Orlov et al., 2006). Accompanying the behavioural, physiological and dietary adaptations are necessary to cope up with the transition between freshwater and marine environments (McCormick et al., 2013). The ecological succession of gut microbial communities during development and migration of wild teleost is an excellent system to explore the contribution of host and environmental factors in shaping the microbiome recruitment, particularly in euryhaline species (Schmidt et al., 2015). The study of Xia et al. (2014) provided the first perception into the fish gut microbiota and its changes during starvation. A detailed study on interactions between gut microbiota and hosts under such dynamic conditions will through new light on how the hosts and microbes respond to the dynamic environment. Nikouli et al. (2020) provided evidence on adult farmed fish in the Mediterranean Sea have a divergent and species-specific gut microbiota profile, that are shaped independently of the similar environmental conditions under which they grow.

Herbivorous marine fish species having higher intestinal short-chain fatty acid concentrations depend on the intestinal microbiota to convert the unassimilable algal constituents to metabolically useful short-chain fatty acids (White et al., 2010), and these fish displays metabolic specializations to the hindgut fermentation (Willmott et al., 2005). Absorption of such short-chain fatty acid in fish is driven by an osmotic gradient between the intestine and blood (Titus and Ahearn, 1992), and so the concentration of these end products of anaerobic metabolism of microbiome in the posterior gut can serve as a rough indicator on potential importance of microbial digestion.

Proteobacteria has far and wide presence in the gut microbiota of the aquatic invertebrates and are dominant in crustacean gut (Rungrassamee, 2014; Holt et al., 2020). The phylum proteobacterium is highly diverse in genetics, morphology and physiology (Stackebrandt et al., 1988). Crustaceans predominantly consist of *Vibrio* and *Photobacterium* spp. which have additionally classified

sequences attributed to other high-level taxa: Bacteroidetes, Firmicutes, Fusobacteria and Actinobacteria in *Penaeus monodon* (Rungrassamee et al., 2014).

Numerous *Vibrio* spp. produce chitinolytic enzymes (Sugita and Ito, 2006), which may express their strength in a chitin-rich environment like crustacean gut by giving a niche substrate for their use. However, the enzymatic capability of a few *Vibrio* spp. may contribute to negative impacts on the carapace of the animals and other health implications such as red disease, tail necrosis, loose shell syndrome (Jayasree et al., 2006). Microbial profiles are likely impacted by the longitudinal axis of the gut itself as various morphologies and functions along the gut will induce differential pressures on the microbial selection (Holt et al., 2020). These interior variations show comparable taxa in the gut of wild and farmed *P. monodon* (Rungrassamee et al., 2014). *Penaeus vannamei* guts from various farms were more likely similar to each other despite differences in the microbial community structure of their respective rearing environment (Zoqratt et al., 2018).

Factors affecting GI microbiota of fish

In fish, it is well known that GI microbiota is affected by a range of factors, including host factors (e.g. genetics, gender, weight, age, immunity and intestinal motility) (Li et al., 2012; Li et al., 2015; Stephens et al., 2016), environmental factors (e.g. water, diet and medicine/antibiotics) (Sullam et al., 2012; Ringø et al., 2016; Dehler et al., 2017), microbial factors (e.g. adhesion capacity, enzymes and metabolic capacity) (Prakash et al., 2011) and displayed individual variations and day-to-day fluctuations (Sugita et al., 1987; Sugita et al., 1990; Ringø et al., 1995; Ringø & Birkbeck, 1999). In addition, a recent study revealed that the intestinal microbial communities of wild largemouth bronze gudgeon (*Coreius guichenoti*) were significant different between male and female fish (Li et al., 2016). Stephens et al., (2016) demonstrated stage-specific signatures in the zebrafish and extensive inter-individual variation.

The regional difference of GI microbiota

The microbial density varies in different regions of the GI tract of fish depending on the physico-chemical conditions (Zhou et al., 2007). Generally, a progressive increase in bacterial levels from the stomach to the posterior intestine was observed in fish (Trust & Sparrow, 1974; MacDonald et al., 1986; Cahill, 1990; Molinari et al., 2003). Navarrete et al. (2009) analysed the bacterial composition of stomach, pyloric caeca, and intestine from ten juveniles (30 g) Atlantic salmon,

and the average total bacterial density was $1*10^7$, $8*10^6$ and $5*10^7$ CFU g^{-1}, respectively. Ye et al. (2014) investigated the microbiota composition in the foregut and hindgut of gizzard shad (*Dorosoma cepedianum*) and Asian silver carp (*H. molitrix*). The results showed that gizzard shad hindgut samples exhibited the highest alpha-diversity indices followed by Asian silver carp foregut (n = 15), gizzard shad foregut (n = 9) and Asian silver carp hindgut (n = 24). Tao et al. (2013) investigated the microbial communities of eight parts of brown croaker (Miichthys miiuy) digestive tract and revealed that the intestine harbours the highest number of bacterial cells, followed by midgut (27.4%), foregut (25.2%), hindgut (22.9%), stomach (21.4%), pylorus (15.6%), proventriculus (2.2%) and oropharyngeal cavity (3%).

Methods used to assess the bacterial communities

It is generally accepted that the GI microbiota of fish plays an important role in nutrition and immunity. In-depth knowledge of the structure and relationships between GI microbiota and their host fish can provide insight into both the function and dysfunction of the host organism. For this purpose, a comprehensive and detailed view of fish GI microbiota, including both taxonomic composition and genetic potential, is a prerequisite. In the past few decades, most of the studies on the intestinal microbiota of fish were carried out by conventional culture-dependent methods (Cahill, 1990; Ringø & Gatesoupe, 1998). However, the fish GI microbiota has been reported to be of low cultivability; it only represents <0.1% of the total microbial community in the GI tract of some fish species (Romero & Navarrete, 2006; Navarrete et al., 2009; Zhou et al., 2014; Ghanbari et al., 2015). Recently, with the development of DNA sequencing technologies and bioinformatic analysis, a wide range of molecular ecology methods based on the 16S and 23S rRNA genes have become more commonly used. These culture-independent molecular-based techniques have substantially improved our knowledge of the structure and diversity of bacterial communities within the gut of fish (Austin, 2006; Kim et al., 2007; Namba et al., 2007; Wu et al., 2010, Zhou et al., 2014; Parma et al., 2016; Ringø et al., 2016). Zhou et al. (2014) reviewed the methodological approaches which have been used in evaluations of fish gut microbiota. The main methodologies utilized have depended on the aim of the studies: (i) clone libraries have been used to identify the microbiota composition; (ii) finger printing methods such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE) have been used to analyses microbial community structure and diversity; (iii) quantitative real-time PCR

(qPCR) and fluorescent in situ hybridization (FISH) have been used to determine the abundance of particular taxa or total microbial levels; and (iv) FISH and immunohistochemistry have been used to assess bacterial-host interactions at the mucosal brush border.

The majority of research on the intestinal microbiota of fish has been descriptive thus far, focusing solely on the make-up of the microbial community. It is necessary to conduct more research to determine the roles played by the microbiota's subpopulations and, eventually, by the species themselves. This will aid in the creation of new probiotics for fish usage and direct the development of prebiotics with a more sensible design that targets the advantageous subgroups of the gut microbiota. Moreover, indigenous fishes must be studied for establishing microbiota composition.

MATERIALS AND METHODS

Study site

The fishes were collected from Kadinamkulam Estuary of Trivandrum, Kerala. The location of lake is 8° 36' 46" N to 76° 49' 3" E. It opens into the sea at Perumathura by a temporary bar mouth. The Vamanapuram River flows into the sea through this opening. It is connected to the Anchuthengu Backwater on the north and the Veli Lake on the south.

Fish collection and examination

The fish samples were collected using cast net from the study site. The fishes were transported to the laboratory in live condition with the help of an aerator. The fishes were brought and acclimatized into a rearing tank in the laboratory and were kept starved for up to 48 hrs to remove any non-adherent microbes from the gut (Ray et al., 2010).

Postmortem examination

The fishes were sacrificed and the gut was removed aseptically. The gut was cleaned carefully three times with 0.9% sterile NaCl solution using an injection syringe in order to remove non-adherent (allochthonous) microflora (Ghosh et al, 2010). The gut segments homogenised with 10 parts of sterilised, prechilled 0.9% NaCl solution (Rengipat et al., 2008).

Isolation of bacterial strains

Each homogenised gut segment samples were subjected to serial (1:10) dilution (Beveridge et al, 1991). To determine the culturable heterotrophic autochthonous aerobic 0.1 ml of the diluted samples were spread onto sterilised LB agar plates (Himedia) enriched with 1% NaCl. The samples were also plated on to TCBS Agar (Himedia) for assessing the *Vibrio* colonies. The plates were incubated at 37^{o} C for 48 hrs. After 24-48 hrs they were examined for development of bacterial colonies. The colonies were counted and live viable count (LVC) was estimated per gram weight of the gut.

Fig 1 : Kadinamkulam Lake

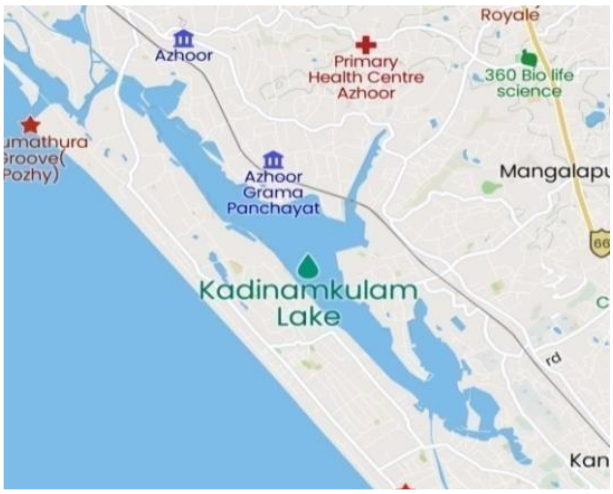


Fig 2: Site Map

(Sourcehttps://mapcarta.com/W383753872/Map)

Fig 3: Measuring *Pseudoetroplus maculatus*

Fig 4: Dissected *Pseudoetroplus maculatus*

Fig 5. Sterilised LB Agar plates

Fig 6. Spreading of samples

Taxonomic classification

Pseudoetroplus maculatus (Bloch, 1795)

Kingdom	**: Animalia**
Phylum	**: Chordata**
Subphylum	**: Vertebrata**
Infraphylum	**: Gnathostomata**
Superclass	**: Actinoptergii**
Class	**: Teleostei**
Superorder	**: Acanthoptergii**
Order	**: Perciformes**
Suborder	**: Labroidei**
Family	**: Cichlidae**
Genus	**: Etroplus**
Species	**: *Pseudoetroplus maculatus* (Bloch, 1795)**

Source: WoRMS-, http://www.marinespecies.org version (08/2021)

The orange chromide, *Pseudoetroplus maculatus* is a species of cichlid fish that is endemic to freshwater and brackish streams, lagoons and estuaries in southern India and Sri Lanka (Loiselle, P.V., 1995). It is locally known as 'Pallathi'. It reaches a length up to 8cm. This species is popular with fishkeeping hobbyists, and is kept frequently in aquariums. The species co-occurs throughout its range with the green chromide (*Etroplus suratensis*). Orange chromides prey on the eggs and larvae of the green chromide and also act as a "cleaner fish" removing parasites from the larger green chromides in a cleaning symbiosis. The species also feeds on zooplankton and algae (Fish base, 2017). They spawn in shallow water, on a soft depression excavated by both parents. About 200 eggs are laid and hatch after 5 days, during which time the parents tend, and if necessary fan them.

Statistical analysis

The relevant data were processed and analyzed manually and MS Excel Office 2016 version was used for computer-based analysis.

RESULTS

Activities such as the digestion and absorption of feeds occur into the gastrointestinal tract (GIT), which also serves to excrete waste products of digestion. These processes occur thanks to the different species of microorganisms inhabiting the GIT, the microbiota, which contribute to the health status of fish by providing metabolic benefits and counteracting pathogen infection. The microbiota is affected by environmental conditions and by the dietary habits of fish species. To get a preliminary figure of its viable count, we have examined the abundance of gut associated bacteria in native freshwater ornamental fish, *Pseudoetroplus maculatus*.

The numbers of cultivable bacterial cells present in fish gut were estimated after isolation and growth on nutrient agar (NA) plates incubated at room temperature at 37°C for 48 Hrs. Of total 3 samples analysed Total viable count (TVC) of heterotrophic bacteria ranged between 3.99 to 7.25 in the gut of *Pseudoetroplus maculatus*. The bacterial population in the gut of the fish generally varies due to the hydrobiological fluctuations occurring in the natural systems (Rheinheimer, 1985). The total heterotrophic bacterial load had mean values of Log viable count as final Total viable count (Table 1, Table 2, Table 3). Sample 1 and Sample 2 detected with *Vibrio sps* in TCBS. There was two type of activity in TCBS by *Vibrio sps* that is forming yellow colonies and green colonies which is notorious for its pathanogenicity.

Dilution	CFU	LVC	TOTAL VIABLE COUNT
10^{-5}	17681729	7.25	6.52±0.65
10^{-4}	2161100	6.33	
10^{-3}	982318	5.99	
10^{-2}	9823	3.99	4.02±0.04
10^{-1}	11198	4.05	

Table 1. Viable count of bacteria in different dilutions for Sample

Dilution	CFU	LVC	TOTAL VIABLE COUNT
10^{-5}	117647059	8.07	6.89±1.09
10^{-4}	4705882	6.67	
10-3	823529	5.92	
10^{-2}	35294	4.55	4.55

Table 2. Viable count of bacteria in different dilutions for Sample 2

Dilution	CFU	LVC	TOTAL VIABLE COUNT
10^{-5}	8928571	6.95	
10^{-4}	2232143	6.35	6.45±0.46
10^{-3}	1116071	6.05	

Table 3. Viable count of bacteria in different dilutions for Sample 3

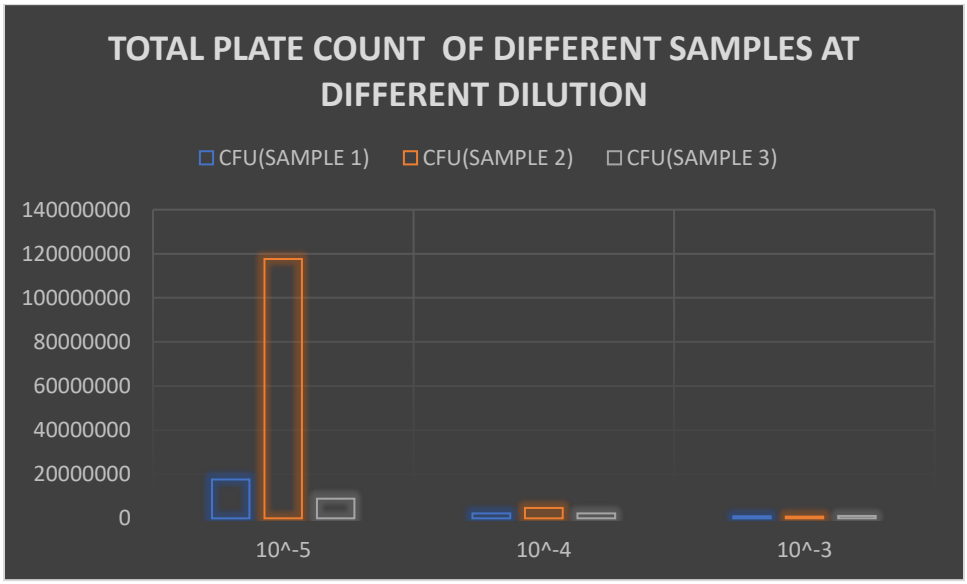


Fig 7. Total plate count in different dilutions

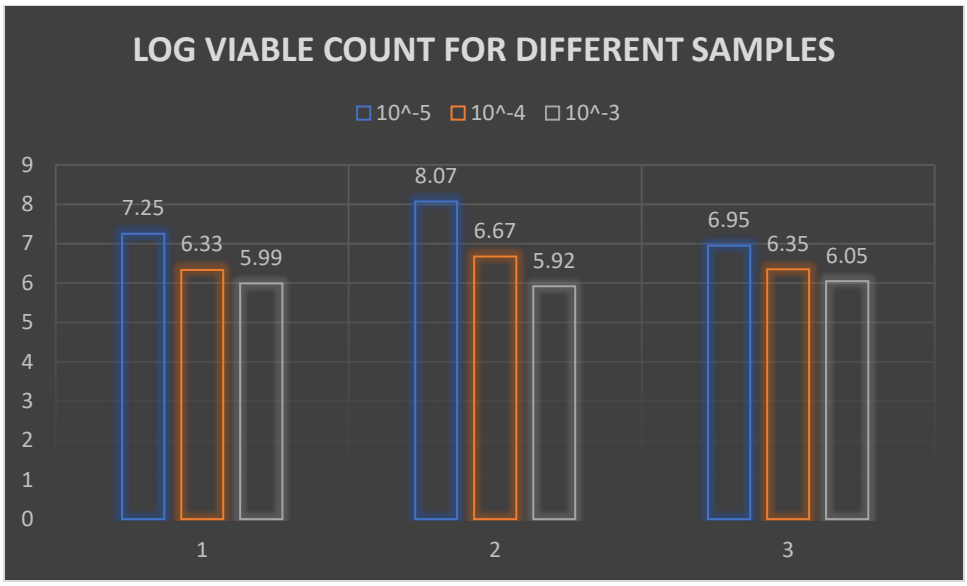
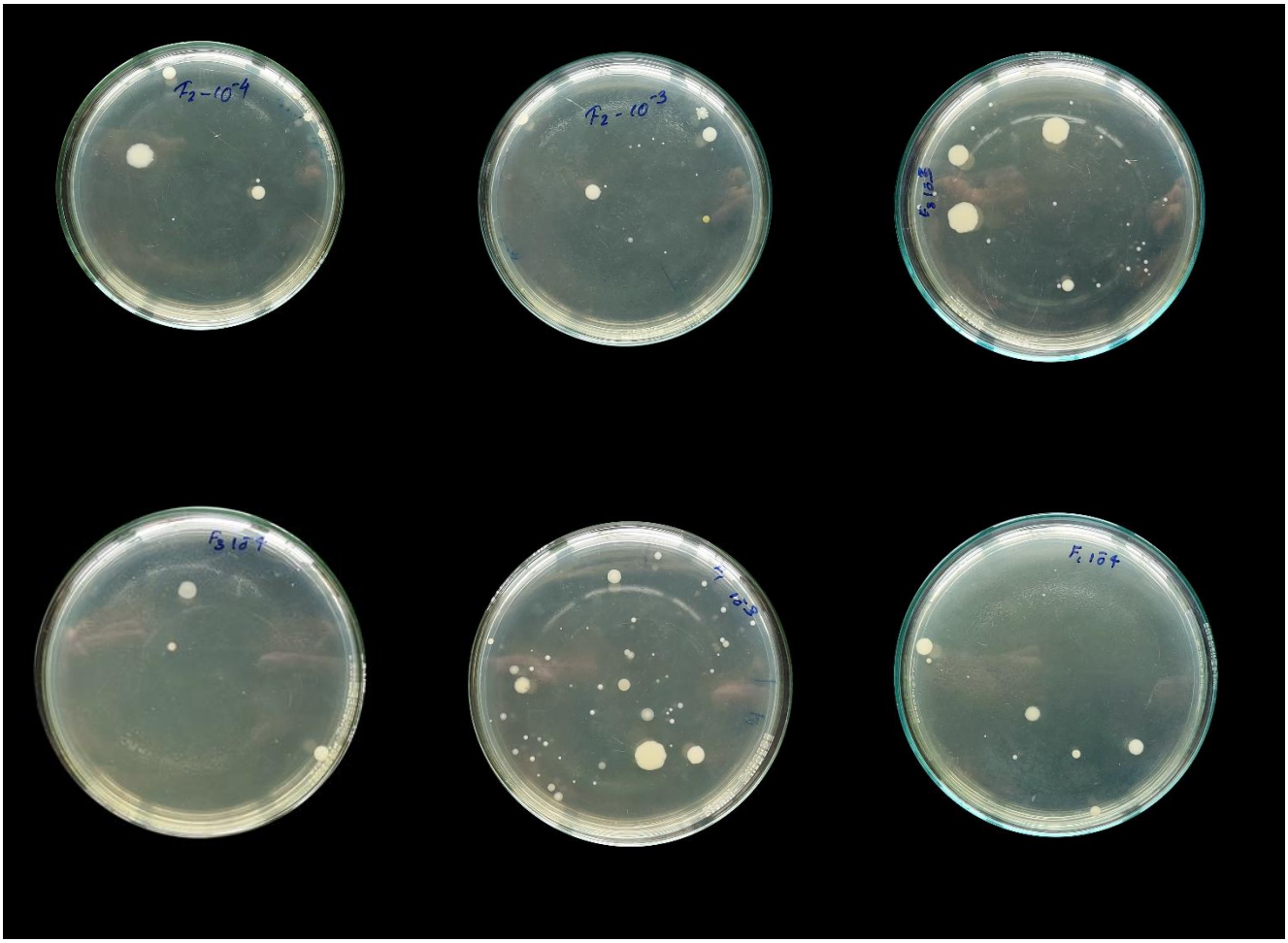


Fig 8. Log Viable count in different dilutions



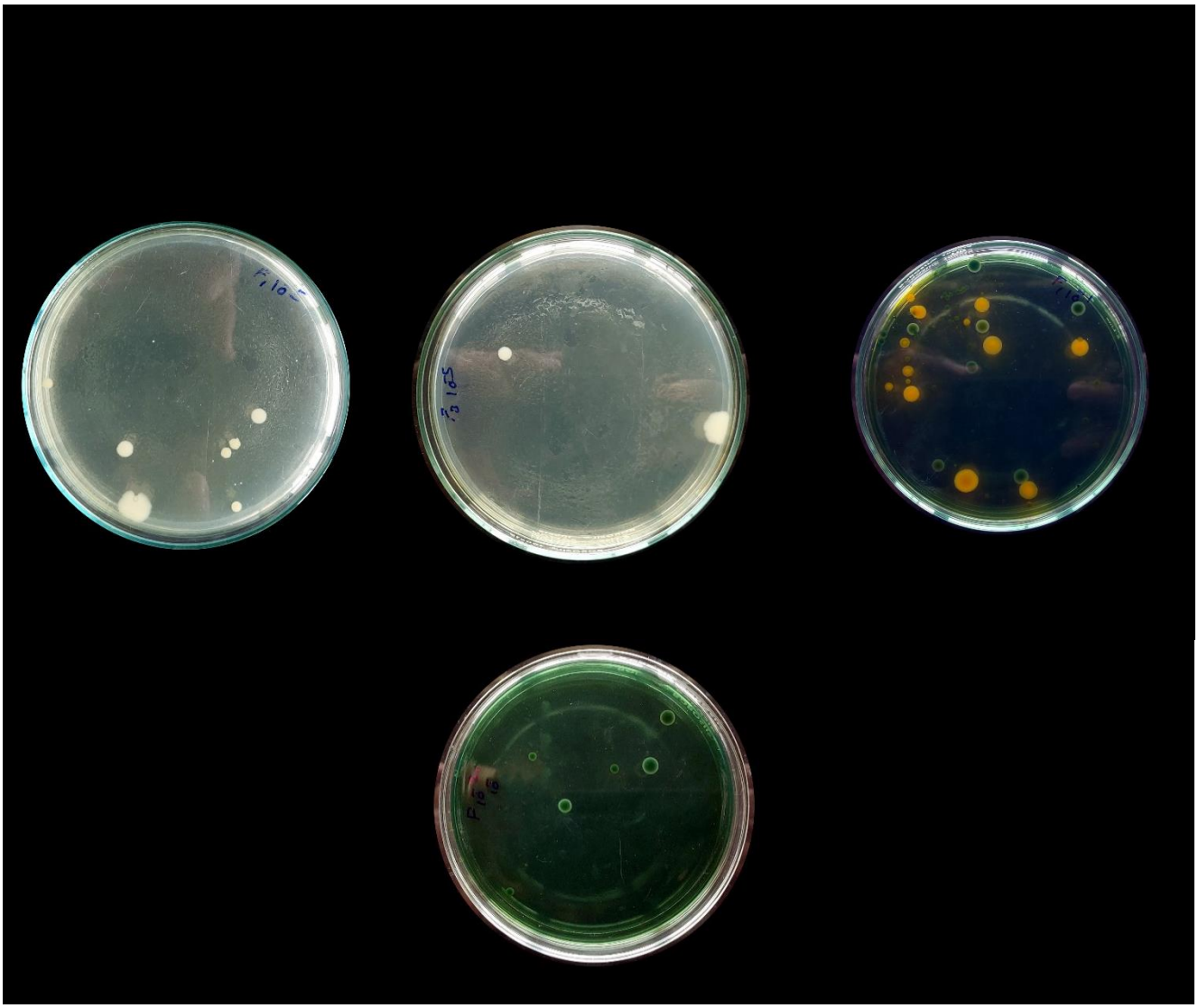


Plate 1. Microbial colonies formed in different dilutions

DISCUSSIONS

In fish, there are several possible sources for the intestinal microbiota, and it is generally believed that the processes of bacterial colonization in early developing fish larvae are complex and depend upon the microbiota of: (i) eggs, (ii) the larval rearing water and (iii) the live feed. In the early development stage, fertilized eggs are released into the water. Both cultivation and ELISA studies have revealed that the gut microbiota of larvae rapidly established after hatching, and based on cultivation, the colonization of the larval intestine seems to follow a two-step pattern (e.g. Strøm & Ringø 1993; Bergh et al., 1994; Ringø et al., 1996; Ringø & Vadstein 1998), with a stable indigenous microbiota forming at the metamorphosis and post-larval stage (Eddy & Jones, 2002). Fish larval uptake feeds from water through the gill and mouth prior to the complete development of GI tract. Romero and Navarrete (2006) showed the stable microorganisms are established after first feeding stages, and its major components are acquired from the environment at hatching.

Early studies have demonstrated that GI bacteria of non-fed marine fish larvae originate from the resident egg epiflora at the time of hatching (Olafsen 1984; Hansen & Olafsen 1989). As the larval gut is sterile at the time of hatching, it is rapidly colonized by microbiota present in the environment, as well as those originally present on the chorion (Hansen & Olafsen 1989). Moreover, the studies of Fernandez et al., (1996) have demonstrated that the dominant Pseudomonas species of the bacterial flora of yolk-sack larvae of milkfish*, Chanos chanos* , were similar to those of the rearing water. Additionally, several studies have revealed that, once feeding begins, the intestinal microflora was derived from the live feed ingested rather than the bacteria present in water (Muroga et al., 1987; Tanasomwang & Muroga 1988; Munro et al., 1993, 1994; Bergh et al., 1994; Bergh 1995; Griez et al., 1997).

In fish, it is well known that GI microbiota is affected by a range of factors, including host factors (e.g. genetics, gender, weight, age, immunity and intestinal motility) (Li et al., 2012, Navarrete et al., 2012; Bolnick et al., 2014; Li et al., 2015, 2016; Stephens et al., 2016), environmental factors (e.g. water, diet and medicine/antibiotics) (Sullam et al., 2012; Ringø et al., 2016; Dehler et al., 2017), microbial factors (e.g. adhesion capacity, enzymes and metabolic capacity) (Prakash et al.,

2011) and displayed individual variations and day-to-day fluctuations (Sugita et al., 1987a, b; Sugita et al., 1990; Ringø et al., 1995; Ringø & Birkbeck 1999). In addition, a recent study revealed that the intestinal microbial communities of wild largemouth bronze gudgeon (Coreius guichenoti) were significant different between male and female fish (Li et al., 2016). Stephens et al., (2016) demonstrated stage-specific signatures in the zebrafish and extensive inter-individual variation. Furthermore, we elaborated the influence of fish microbiota by water and diet, which have been mostly studied as the environmental factors affecting the fish microbiota.

In water environment, water temperature and salinity are two main factors that affect fish GI microbiota. Hagi et al., (2004) reported that the intestinal lactic acid bacteria (LAB) composition varied with seasons in four fish species, that is silver carp (*Hypophthalmichthys molitrix*), common carp (*C. carpio*), channel catfish (*Ictalurus punctatus*) and deep bodied crucian carp (*Carassius cuvieri*). It was revealed that abundance of predominant LAB depended on the water temperature, irrespective of fish species. Seasonal variations in the intestinal microbiota have also been revealed in farmed Atlantic salmon (Hovda et al., 2012). Zarkasi et al., (2014) revealed that the intestinal composition of LAB within Atlantic salmon also varied with seasons. Al-Harbi and Uddin (2004) analysed the total viable counts (TVC) of bacteria in the intestine of hybrid tilapia (*Oreochromis niloticus, Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia, and the results showed that the TVC of bacteria varied in different seasons (autumn, summer and winter). Recently, Neuman et al., (2016) showed that number of bacteria generally increases with water temperature, when not considering the influence of diet.

As early as 1953, it was reported that fasting has influence on fish intestinal bacteria (Margolis 1953). Currently, a number of studies have demonstrated that diet could strongly influence the fish GI microbiota (Campbell & Buswell 1983; Sugita et al., 1987; Onarheim et al., 1994; Ringø & Birkbeck 1999; Ringø et al., 2006, 2016; Uchii et al., 2006; Martin-Antonio et al., 2007; Muegge et al., 2011; Sullam et al., 2012; Xia et al., 2014; Ye et al., 2014). The GI tract of fish is colonized at an early stage and guided in new and different directions depended on diet type (Brunvold et al., 2007; Reid et al., 2009). Ingerslev et al., (2014) examined the gut microbiota change in rainbow trout (On. mykiss) during the onset of first feeding, and the authors revealed that microbial abundance and diversity increased after first feeding. Furthermore, Firmicutes dominated the gut

of fish fed plant source oils while Proteobacteria was the dominant phyla in fish oil fed fish, which is consistent with previous reports (Desai et al., 2012).

Moreover, feeding habit is also an important factor influencing GI microbial diversity, and an increasing trend in diversity was observed following the order of carnivores, omnivores and herbivores (Ward et al., 2009; Larsen et al., 2014; Li et al., 2014; Miyake et al., 2015). The study of He et al., (2013) revealed that herbivorous grass carp (*C. idella*) possessed more bacterial species than the exclusively omnivorous gibel carp and black bream and carnivorous black carp under the same rearing environment. Furthermore, feeding habit also influences the structure and composition of GI microbiota. Recently, research has reported that cellulose-degrading bacteria Clostridium, Citrobacter and Leptotrichia were dominant in the herbivores, while Cetobacterium and protease-producing bacteria Halomonas were dominant in the carnivores (Liu et al., 2016). The gut microflora of freshwater fish species is generally comprised of *Aeromonas* sp., *Pseudomonas* sp., Flavobacterium/Cytophaga species, *Enterobacter* sp., and/or *Acinetobacter* sp. (Trust et al., 1979; Cahill 1990; Ringø et al., 1995; Ringø and Birkbeck 1999). Marine species harbor a different assemblage featuring *Vibrio* spp., *Pseudomonas* sp., *Acinetobacter* sp., *Achromobacter* sp., Enterobacteraceae, Flavobacterum, and/or *Micrococcus* Sp. Diet and trophic level have presented as clear influencing factors of fish gut microbial composition. It has been shown that Clostridium is linked to an herbivorous diet while Vibrio and Photobacterium are commonly found in carnivores. (Liston 1957; Colwell 1962; Newman et al., 1972; Sera and Ishida 1972a; Sugita et al., 1988; Cahill 1990; Onarheim et al., 1994; Ringø et al., 1995; Ringø and Birkbeck 1999; Izvekova et al., 2007).

The gut immune system, also named gut-associated lymphoid tissues (GALT), not only protects GI tract from infectious agents but also regulates immune system in the GI tract. The GI microbes play a critical role in the development and maturation of GALT, which in turn mediate a variety of host immune functions. (Bates et al., 2006)

SUMMARY

Similar to mammals, the gut microbiota of fish can be recognized as an organ, in itself responsible for key physiological functions which aid health maintenance of its host. Knowledge of its composition and exact functional role in health and disease is vital given the environmental changes to which fish are being exposed, particularly in light of the growth of the aquaculture industry and rising sea temperatures as a result of climate change. Many of the natural waters are subjected considerable organic pollution which might provide right kind of environment for fish. To get an initial estimate of its viable count, we have examined the frequency of gut-associated bacteria in native freshwater ornamental fish, *Pseudoetroplus maculatus* in this study. In the present study, total viable count of bacteria in the gut of Orange chromide (*Pseudoetroplus maculatus*) collected from Kadinamkulam Lake, Thiruvanathapuram was analyzed and will provide insight into the microbial load present in the gut of Orange chromide. Of total 3 samples analysed Total viable count (TVC) of heterotrophic bacteria ranged between 3.99 to 7.25 in the gut of *Pseudoetroplus maculatus*. Sample 1 and Sample 2 detected with *Vibrio sps* in TCBS.

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