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ORIGINAL ARTICLE

Deep Sequencing Reveals Highly Variable Gut Microbial Composition of Invasive Fish Mossambicus Tilapia (*Oreochromis mossambicus*) Collected from Two Different Habitats

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Abstract Tilapia (*Oreochromis mossambicus*) is one of the most invasive fish found throughout the World and emerged as a major threat to the indigenous fishes in many countries. Investigating the gut microbial diversity of such fishes is one of the ways to understand its physiology. In the present study, we have explored the gut microbial community structure of tilapia using 16S rRNA gene sequencing on the Illumina Miseq platform. Our study showed significant differences in tilapia gut microbiota collected from different habitats (i.e. river and lakes) suggesting the influence of habitat on the gut microbial diversity of tilapia. This study gives a first insight into the mossambicus tilapia gut microbiota and provides a reference for future studies.

Keywords Mossambicus tilapia · Gut microbiota · Next generation sequencing · Invasive species · Host microbiota interactions

Introduction

Oreochromis mossambicus (tilapia) is the most commonly found Cichlid fish which is native to the eastern coast of Africa [1]. Tilapia has been introduced to almost 90 countries, including India for aquaculture, game fishing, pest control, etc. [2]. Due to its remarkable trophic plasticity and ability to obtain sexual maturity at an early age, they often get overpopulated and compete with other fishes for habitat and food. They can also tolerate polluted environments, different temperature ranges, poor quality water and low dissolved oxygen where most of the other fishes fail to survive [3–5]. This has not only made it difficult to eradicate this species, but also has consequently wiped out many native fishes [4, 6]. For these reasons, tilapia is included in the list of the 100 most invasive species of the World [7].

Studies have shown that microbes associated with fishes are known to affect its physiology and thus their survival [8, 9], Ye et al. [10] explored the gut microbial composition of invasive fish, *Hypophthalmichthys molitrixasian* (silver carp) to understand its physiology. Similarly, attempts were also made by others to investigate the gut microbial diversity of fishes [11–15]. However, despite its ecological significance, till now no attempts have been made to explore the gut microbial composition of tilapia. Although microbial composition of Nile tilapia (*Oreochromis niloctus*) has been explored [16], the knowledge about the gut microbial composition of mossambicus tilapia is still lacking. In this study, we have explored the gut microbial composition of tilapia using the Illumina Miseq platform for the first time. To understand the variation in the gut microbial structures of tilapia, samples were collected from two different habitats i.e. river and lake. Along with this, microbial community structure of the surrounding water was also explored to

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determine its possible role in structuring the microbial composition of tilapia.

Materials and Methods

The adult tilapia were collected from two sampling sites. The first sampling site was river Mula that flows through a human populated area at Aundh, Pune, Maharashtra, India (TR). The second sampling site was a natural lake located at Talegaon Dabhade, Maharashtra, India (TL). Details of sample collection are given in (Supplementary Table 1). Three individuals of adult tilapia were collected from each site. Water samples were also collected in triplicate from both the sites. Physiochemical parameters such as pH, temperature, total dissolved solids (TDS) conductivity, dissolved oxygen (DO), the ammonia and phosphate content of the water from collection sites were also measured using HANNA instruments (Italy) (Table 1). Gut contents of sampled fishes were removed aseptically and used for DNA isolation. The water (300 ml) from each sample was filtered using 0.2 µm pore sized hydrophilic Polyvinylidene fluoride durapore membrane filter (Millipore, India).

DNA from the gut contents and membrane filters was isolated using QIAamp DNA Stool Mini Kit (Netherlands) following manufacturer's information. Obtained DNA was sent to Xcelris Labs Ltd, Ahmedabad, India for exploration of microbial diversity using primers for V3–V4 region of 16S rRNA [17]. The high-throughput sequencing was performed using the Illumina Miseq platform. The adaptors and low quality sequences were removed using Trimmomatic v0.30 [18]. Paired ends were joined using QIIME [19]. Obtained raw reads were quality filtered using Mothur [20]. In brief, sequences with read length of more than 200 bp, q value of more than 20 with no ambiguity and a homopolymer length of less than 6 bp were selected. Sequences were then used for microbial diversity analysis using QIIME. In brief, sequences were clustered into Operational Taxonomic Unit (OTUs) at 97% similarity and OTU picking was done by an open reference method and chimera check was done using chimera-slayer. Taxonomic assignments were carried out using Greengene 13.8 database. The sequencing data generated here, were submitted to the MG-RAST (ID 4644287.3–4644298.3).

Statistical analysis: Alpha diversity indices such as Chao1, observed_species, Shannon and Good's coverage were calculated using QIIME. ANOVA was used to determine the differences in the different categories using PAST software [21]. Two-dimensional non-metric multidimensional scaling (NMDS) analysis was also performed using PAST to plot the differences in the microbial community structure.

Table 1 Physiochemical parameters measured for the water samples collected from the two habitats

Physiochemical parameters	TRwater	TLwater	*p* value
Temperature (°C)	23	26	<0.0001
pH	7.4	7.1	<0.0001
Conductivity (mS/cm)	653 (±3.33)	481 (±6)	0.0001
TDS (mg/L)	336 (±3.05)	228 (±6.11)	0.0006
DO (mg/L)	4.56 (±0.03)	5.93 (±0.06)	0.0003
Ammonia (mg/L)	3	0.7	<0.0001
Phosphate (mg/L)	5.18 (±0.09)	1.7 (±0.11)	<0.0001

The measurements were done in triplicates, mean value are shown here and standard error in parenthesis

p value are calculated using *T* test in PAST software

Results and Discussion

After quality filtering and removing of singletons, 2,073,739 good quality sequences were obtained from 12 samples ranging from 83,065 to 227,418 sequences per sample (Table 2). The OTUs observed in tilapia gut were ranged from 1788 to 3901 per samples. The number of OTUs observed in the tilapia gut was higher than those reported in common carp [22] and eastern African cichlid fishes [23]. However, OTUs found in tilapia gut was low as compared to OTUs of its respective water samples in both the cases. It was noted that, there was significant ($p = 0.006$) differences in the OTUs of TRgut samples and its respective water samples (TRwater), same was also true for TLgut and TLwater samples ($p = 0.003$). These observations are in agreement with the previous studies done by Wu et al. [24] showing higher microbial diversity in water samples than the fish gut microbial communities.

The gut microbial composition of the most fishes is mainly dominated by *Proteobacteria*, *Firmicutes*, *Fusobacteria* and *Cyanobacteria.* However, abundances of these bacterial phyla vary with the phylogenetic origin of the fishes [25]. Interestingly, in the present study, tilapia collected from different habitat showed notable differences in the gut microbial compositions. Tilapia gut samples collected from the river (TRgut) were mainly dominated by *Fusobacteria* (49–57%) and *Proteobacteria* (22–31%) while sample collected from the lake (TLgut) were dominated by *Actinobacteria* (35–36%), *Cyanobacteria* (21–25%), *Planctomyctes* (21–25%) and *Proteobacteria* (17–20%). Phylum *Bacteroidetes* is one of the major phyla found in many fish gut communities [10] however, in this study, the abundance of *Bacteroidetes* was very low in TRgut (1%), while in TLgut it was completely absent albeit its presence in the surrounding water (Fig. 1). On the other hand, bacteria belonging to *Fusobacteria* were abundantly present only in TRgut sample, but it was absent in the

Table 2 Summary of species richness estimators of the samples studied

No.	Sample code	Good quality sequences	Chao1[a]	Observed species[a]	Shannon[a]	Goods coverage (%)[a]
1	TRgut1	195,577	5616	3594	5.75	98
2	TRgut2	176,202	5163	3901	6.89	98
3	TRgut3	145,212	3069	2144	4.96	99
4	TRwater1	178,048	10,514	7151	9.01	96
5	TRwater2	102,269	8908	7606	10.62	98
6	TRwater3	193,268	8832	6381	9.32	97
7	TLgut1	194,504	3230	2227	6.32	99
8	TLgut2	216,663	5701	3609	7.05	98
9	TLgut3	209,645	2581	1788	5.84	99
10	TLwater1	227,418	11,368	7165	8.82	96
11	TLwater2	151,868	10,731	7713	9.80	96
12	TLwater3	83,065	6472	6059	9.52	99

The alpha diversity indices were calculated with 3% distance cut-off

[a] These values were calculated after all samples were randomly subsampled with 83,065 sequences per samples as lowest number of sequences were found in TLwater3 sample

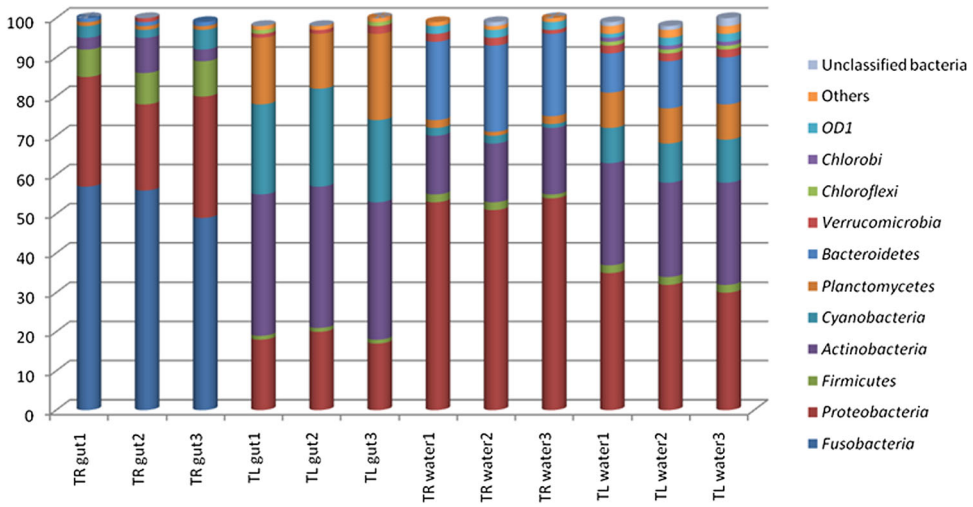

Fig. 1 The relative abundance of different bacterial phyla in the samples studied. The abundance of the phyla is plotted on *Y axis*. The category 'others' includes OTUs with <1% relative sequence abundance

surrounding water. Furthermore, Statistical Analysis of Metagenomic Profiles (STAMP) [26], showed a significant difference in the gut microbial composition, of the samples collected from different habitats, even though they belonged to the same species (Supplementary Fig 1). Such high variation in the gut microbial community structure at the phylum level was also observed in Trinidadian guppies despite their same taxonomic origin [27].

Along with biotic factors, various abiotic factors found in different habitats could also potentially influence the gut microbiota of fishes [28]. The water collected from the river showed high TDS, conductivity, phosphate and ammonia content compared to the water collected from the lake (Table 1). Mula river receives sewage polluted water from the surroundings and that could be the reason for differences in these ecological parameters (except temperature and DO). These variable abiotic factors of the water could also be responsible for the highly divergent microbial composition of tilapia gut. The difference in the microbial community of the samples was more evident from NMDS analysis implemented in PAST software [21] (Fig. 2). Although we observed a variation between the gut microbial community of the individuals collected from the same locality, all the individuals of the TRgut sample formed distinct clade and was separated from the cluster formed by TLgut sample, suggesting habitat specific separation of the gut microbiota. The water microbial composition of the respective habitat also showed significant difference from each other and tilapia gut microbial community (Fig. 2). It was noted that the difference in the microbial community structure of tilapia gut within the individuals collected from the same locality was not

Table 3 The ANOVA of the bacterial communities

Comparisons	*p* value
Within TRgut	0.99
Within TLgut	1
Within TRwat	0.99
Within TLwat	0.99
Between TRgut and TRwat	0.0001*
Between TLgut and TLwat	<0.0001*
Between TRgut and TLGut	<0.0001*
Between TR water and TLwater	0.0001*

* Significant values

significant. However, there was a significant difference between the microbial community structure of tilapia gut collected from different localities (Table 3). This is also supported by the analysis of similarities (ANOSIM, based on the weighted unifrac distances) using QIIME, showing significant differences between the microbial communities of TLgut and TRgut sample ($p = 0.003$).

Water is also known to influence, the microbial colonization of the fish gut [9, 29]. In this study, tilapia gut microbial community structure is significantly different from the microbiota of the respective water samples (ANOSIM, $p = 0.005$, Table 3; Supplementary Fig 2 and 3). It was also noted that bacterial phyla and their abundance found in tilapia gut were different from its respective water samples (Fig. 1). Furthermore, the distribution of the ten most abundant OTUs of tilapia gut, in its respective water samples suggested that they are either absent or present in low abundance (Supplementary Table 2). Such highly divergent microbiota of the fish gut compared to water microbiota from the same locality suggest that water has less influence on the gut microbial composition of tilapia. Thus, our data revealed that sources other than water could be playing important role in structuring the tilapia gut microbial community. Our findings were supported by the study on three-spine stickleback [28] and grass carps [24] suggesting that surrounding water has little or no role in shaping the gut microbial community structure.

Differences in the microbiota of the tilapia population could also be due to the acquisition of microbes from different diet. In the present study, tilapia samples were collected from completely different habitat i.e. river and lake and therefore types of diet available in both the habitats would be completely different. Preference of tilapia for different diets in different habitats is well documented in the past. Nevertheless, colonization of diverse bacteria due to consumption of different diet has been already well documented in fishes [30]. The genetic divergences among different population and diversity of major histo-compatibility (MHC) complex are also thought to be one of reasons for highly variable microbial community structures in fishes [28]. Polymorphism in the MHC complex [31] and genetic diversity among different populations of tilapia [32] have been already reported in the past. Studies linking such genetic diversity and gut microbiota of tilapia would shed more light on the variable gut microbial community structure.

The OTUs contributing to the differences in TRgut and TLgut samples were determined using similarity percentage (SIMPER) analysis implemented in the PAST software [21] (Table 4). Our data based on the SIMPER analysis

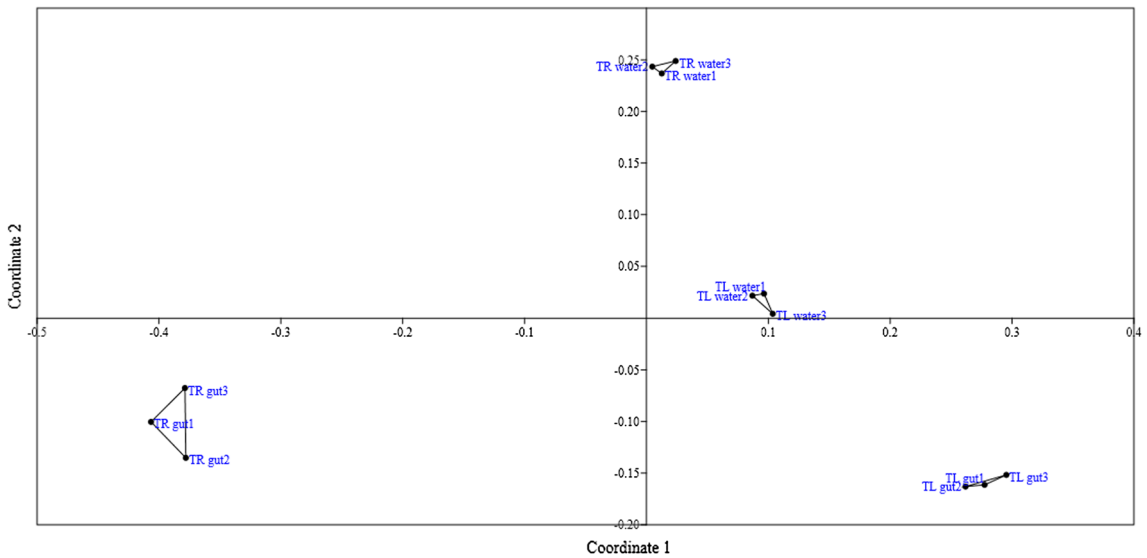

Fig. 2 Two dimensional non-metric multidimensional scaling (NMDS) of the bacterial communities of the samples studied. NMDS plot is based on the Bray-curtis similarity index. A convex hull encloses all samples of the same category forming distinct cluster. There was significant difference between TRgut and TLgut samples and their respective water (TRwater and TLwater, respectively) samples

Table 4 SIMPER analysis showing the principal OTUs responsible for the differences between TRgut and TLgut samples

No.	OTUs	Dissimilarity contribution (%)	Abundance in TRgut (%)	Abundance in TLgut (%)
1	*Cetobacterium*	32.35	53.7	0
2	*Mycobacterium*	13.45	3	25.3
3	Unclassified *Cyanobacteria*	8.635	0	14.3
4	*Pirellulaceae*	6.234	0	10.3
5	*Aeromonadaceae*	5.398	9	0
6	*Synechococcus*	3.413	0	5.67
7	*Gemmataceae*	3.016	0	5
8	*Enterobacteriaceae*	3.013	5	0
9	*Rhizobiales*	2.604	2.67	7
10	*Microthrixaceae*	2.007	0	3.33
11	*Lactococcus*	1.803	3	0
12	*Aeromonadaceae*	1.801	3	0
13	*Solirubrobacterales*	1.608	0	2.67
14	*Aeromonas*	1.413	2.33	0
15	*Methylocaldum*	1.403	1	3.33
16	*Clostridium*	1.006	1.67	0

identified 15 OTUs (those contributing more than 1% of dissimilarity) responsible for observed differences in the tilapia gut microbial communities. OTUs belonging to *Cetobacterium* (from TRgut) and *Mycobacterium* (from TLgut) contributed most (32.35 and 13.45%, respectively) to the dissimilarity between two habitats. OTUs belonging to the unclassified *Cyanobacteria, Pirellulaceae, Synechococcus, Gemmataceae, Rhizobiales* and *Microthrixaceae* were abundantly present in TLgut samples and thus contributed in observed differences.

Although, OTUs present in the tilapia gut were specific to the habitat, they are routinely found in other fish gut microbial communities. *Cetobacterium* is the member of core gut microbiota of many fish species and known to be involved in vitamin B12 synthesis [33, 34]. Presence of *Mycobacterium* in high abundance is not surprising as mycobacteriosis is very common in many fishes [35] including mossambicus tilapia [36]. Similarly *Aeromonas* is also a common pathogen of fishes [29]. Presence of *Rhizobiales, Lactococcus, Clostridium* and *Planctomyces* surely needs further investigation as they are known to be involved in many beneficial activities such as nitrogen fixation [37], cellulose degradation [10] and as a probiotic [8]. Although *Methylocaldum* is known to be found in the fish gut [24], the presence of this group in such high abundance in tilapia gut is intriguing and surely needs further investigations as it is known to be involved in methane utilization [38]. Furthermore, the presence of OTUs belonging to *Cyanobacteria* in high abundance suggests its utility as the food source for fishes [10].

Our results revealed that gut microbial diversity of tilapia collected from two different habitats showed highly divergent microbiota, implicating possible role of local environment, diet and or genetic factors in microbiota differentiation. Significant differences in the microbial community structure of tilapia gut observed in this study, suggest its ability to acquire different bacteria from different habitat and these microbes could be helping the host to survive in such diverse habitats. Kowalski et al. [39] suggested that the understanding the microbiota of such fishes would also enable us employ strategies to manage them. Further studies are also needed to investigate the role of intrinsic genetic factors responsible for acquiring these microbes that would assist us to develop strategies for control and management of such species.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interests.

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