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Ecological Indices of Microbes on Gastrointestinal Tract of Ornamental Fishes

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Abstract:
The aim of the study was to determine the ecological indices of an apical dominance and diversity rate of bacteria on gastrointestinal (GI) tract of some ornamental fishes. The GI tract bacterial strains were characterized by biochemical methods. Eleven species of bacterial strains were isolated belonging to the Phyla Proteobacteria (45%), Firumicutes (10%) and Actinobacteria (45%). Out of the eleven strains, *Aeromonas* sp., *Micrococcus* sp. and *Vibrio* sp., predominantly occurred in the GI tract. Total viable count of *Vibrio metschnikovii* ($5.93x10^7 \pm 0.2x10^7$ CFU/ml) was significantly higher in *Tanichthys albonubes* and *Micrococcus varians* ($9.42x10^2 \pm 0.75x10^2$ CFU/ml) was the least in *Parachromis managuensis* compared to other strains. Ecological indices of diversity and dominance have shown that out of ten ornamental fishes, *Tanichthys albonubes* has higher dominance of 0.993 and *Cichla ocellaris* has higher diversity and species richness of 0.509 and 0.806, respectively. The obtained results will create an impact on the aqua environment thereby decreasing an anthropogenic consequence of fishes. In future, the aforesaid positive aspect will be taken into consideration for formulating probiotics from gut microflora which probably will increase the endurance and health of fishes.

Keywords: Ecological Indices, Fish, Microflora, Probiotics, Gastrointestinal Tract

INTRODUCTION

In recent days, an unseen national resource of gut microbial diversity is entitled to a greater attention. Microbial diversity in the gastrointestinal (GI) tract of fishes plays an important role in understanding more about the microbial ecology and their evolution (Martinez-Porchas, and Vargas-Albores, 2017). The GI tract of fish is a complex ecosystem containing a large number of microbial species. Diversity indices are used in microbial ecology studies to understand the relationship between environmental conditions and distribution of the microbial community (Bargiela et al., 2015; Stach et al., 2013).

The microbial diversity gives a skeletal frame of structural, metabolic, genetic and morphological diversity of bacteria present in GI tract. Normally microbes of fishes enter externally by having contact with faecal or sewage wastes present in the water, which will naturally affect the skin, eyes and gills of the fish (El-Shafai et al., 2004). Similarly, aqueous environment and choice of food taken by the fish greatly influences the bacteria found in the GI tract of fish (Nieto et al., 1984). Many

reports have demonstrated that, the gut microbiota are dominated by endogenous microbiota, obligate anaerobic bacteria and lactic acid bacteria (Huber et al., 2006; Ringo et al., 2006; Kapetanovic et al., 2005; Hovda et al., 2007; Kim et al., 2007). Few reports are available on microbial communities present in the GI tract of ornamental fishes (Romero et al., 2014). Bacterial community present in the GI tract of fish creates an impact upon the overall health of fishes (Di Maiuta et al., 2013). Probiotics, as dietary feed supplements aids in the proper growth of fish without causing damage to the existing microbial flora. The highlights of the present work focus on the ecological indices of microbiota to understand the distribution and diversity of microbes in the GI tract of fish.

MATERIALS AND METHODS

Isolation of Gut Microbiota

Ten ornamental fishes *Cichla ocellaris, Barbonymus schwanenfeldii, Parachromis managuensis, Cyprinus carpio, carssius auratus, Pterophylum scalare, Aulonocara nyassae, Cichla orinocensis, Tanichthys albonubes* and *Labeo chryophekadion* were bought from Kolathur fish farm, Chennai. Each fish was individually transported to the laboratory with aerated polythene bag. Fishes were transferred to the aquarium tank for acclimatization. After 24 hr,. Fishes were anesthetized with benzocaine and their GI tracts were extracted by dissecting the fish in sterile conditions after washing them for several times with sterile saline solution. The extracted GI tract was homogenized using tissue homogenizer. The homogenates of the intestinal samples were transferred to sterile 0.9% saline solution. 1 ml aliquot of the GI homogenate was spread onto nutrient agar. The plates were incubated at 35 – 37ºC for 24 to 48 hr and examined for distinct isolated colonies (Ghosh et al., 2014).

Morphological and Biochemical Characterization

The cultured bacterial colonies were counted using colony counter and expressed as CFU / ml. The purity of the isolates was checked by streaking them individually onto fresh agar plates of the isolation media, followed by microscopic examinations. Characterization of the pure isolates was performed by colonial characteristics, cell morphology, motility test and biochemical tests (gram reaction, catalase test, glucose, sucrose and lactose utilization, citrate test, indole test, urease test, hydrogen sulfide production, gas production, methyl red test, Vogues Proskauer test, coagulase test and spore staining). These tests were done to identify the isolates to generic level as mentioned in the Bergey's manual of bacteriology (Buchanan and Gibbons, 1974).

Statistical Analysis and Ecological indices

The data was subjected to statistically analyze using SPSS 21.0 ver. The significant differences between microbial counts were calculated by one-way analysis of variance (ANOVA). Post hoc test, Duncan Multiple Range test was employed the mean difference of the variables ($P < 0.05$). $P<0.05$ is considered as significant. The ecological indices such as index of dominance (Simpson, 1949), index of diversity (Shannon and Weaver, 1949) index of evenness (Pielou, 1966) and index of similarity (1948) were performed.

Index of Dominance

$c = \Sigma (ni/N)^2$

where ni = number of individual for each species

N = total number of individuals

Shannon Index of General Diversity

$H = -\Sigma (ni/N) \log_e (ni / N)$

where ni = number of individual for each species

N = total number of individuals

Evenness Index

$e = H / \log_e S$

where H = Shannon index

S = number of species

Index of Similarity

S = 2C / (A+B)

Where A = number of species in Sample A
B = number of species in Sample B
C = number of species common to both samples

Index of Dissimilarity

D = (1-S)

Where S = index of dissimilarity

The euclidean cluster analysis was performed to study the similarity of distribution and Principal Component Analysis was carried out to analyze the correlation between the bacterial strains using PAST 3.29ver.

RESULTS

The microbial flora was isolated from GI tract of *Cichla ocellaris, Barbonymus schwanenfeldii, Parachromis managuensis , Cyprinus carpio, carssius auratus, Pterophylum scalare, Aulonocara nyassae, Cichla orinocensis, Tanichthys albonubes* and *Labeo chryophekadion* The biochemical characteristics and morphology of different species of microbial colonies were given in table 1.

Table 1: Biochemical Characterization of Isolated Bacteria from Gastrointestinal Tract of Ornamental Fishes

Biochemical Tests	Microbial community										
	Serratia liquifaciens	***Staphylococcus. saprophyticus***	***Aeromonas veronii***	***Aeromonas schubertii***	***Micrococcus luteus***	***Micrococcus lylae***	***Micrococcus halobius***	***Micrococcus varians***	***Micrococcus roseus***	***Vibrio metschnikovii***	***Vibrio cincinnatiensis***
Gram stain	Gram -ve	Gram +ve	Gram +ve	Gram +ve	Gram –ve	Gram -ve	Gram +ve	Gram +ve	Gram +ve	Gram –ve	Gram –ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
Motility	Motile	Non-motile	Non Motile	Non motile	Motile	Motile	Motile	Non motile	motile	Motile	Motile
Citrate	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
Growth in 6.5% NaCl	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl Red	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
Voges-Prausker	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
Indole	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
H_2S	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Esculin hydrolysis	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
Glucose	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
Lactose	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
Sucrose	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve
Mannitol	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve
Sorbitol	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Arabinose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Rafinose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

The total viable count of bacterial colonies among the different ornamental fishes is depicted in table 2. Highest GI microbiota was observed with *Vibrio metschnikovii* ($5.93x10^7 \pm 0.2x10^7$ CFU/ml) in *Tanichthys albonubes* while, least was observed with *Micrococcus varians* ($9.42x10^2 \pm 0.75x10^2$) in *Parachromis managuensis*.

Table 2: Microbial Load of Isolated Bacteria from Gastrointestinal Tract of Ornamental Fishes (CFU/ml)

Organism	Ornamental fishes									
	Cichla ocellaris	***Barbonymus schwanenfeldii***	***Parachromis managuensis***	***Cyprinus carpio***	***Carssius auratus***	***Pterophylum scalare***	***Aulonocara nyassae***	***Cichla orinocensis***	***Tanichthys albonubes***	***Labeo chrysophekadion***
S. liquefaciens	–	$4.9X10^4 \pm 0.15X10^{4(b)}$	$2.99X10^5 \pm 0.18X10^{5(b)}$	–	$1X10^5 \pm 0.2X10^{5(b)}$	$1.05X10^4 \pm 0.8X10^{3(c)}$	–	$3.19X10^5 \pm 0.2X10^{5(b)}$	–	
S. saprophyticus	$2.33X10^5 \pm 0.2X10^{5(b)}$	–	–	–	–	–	$2.33X10^5 \pm 0.2X10^{5(b)}$	–	–	$3.13X10^4 \pm 20.3X10^{4(c)}$
A. veronii	–	$3.03X10^4 \pm 0.1X10^{4\ (a)}$	–	–	$4.86X10^5 \pm 0.5X0^{5(c)}$	$1.31X10^4 \pm 0.19X10^{4\ (d)}$	–	–	–	$6X10^5 \pm 0.2X10^{5\ (d)}$
A. schuberti	$4X10^5 \pm 0.2X10^{5(c)}$	$4.07X10^5 \pm 0.13X10^{5\ (c)}$	–	–	–	–	–	$5.01X10^5 \pm 0.17X10^{5(c)}$	–	–
M. luteus	–	$2.9X10^4 \pm 0.1X10^{4\ (a)}$	–	$4.30X10^3 \pm 0.15X10^{3(a)}$	$1.03X10^3 \pm 0.4X10^{3(a)}$	$1.1X10^3 \pm 0.1X10^{3\ (a)}$	$5.06X10^5 \pm 0.15X10^{5(b)}$	–	–	$3.2X10^4 \pm 0.2X10^{4\ (b)}$
M. lylae	–	–	–	$1.1X10^3 \pm 0.3X10^{3\ (a)}$	$1.1X10^3 \pm 0.2X10^{3(a)}$		$2.0X10^3 \pm 0.14X10^{3(a)}$	$1.9X10^3 \pm 0.2X10^{5(a)}$	–	–
M. halobius	–	–	$3.9X10^3 \pm 0.1X10^{3\ (a)}$	–	–	$3.9X10^3 \pm 0.1X10^{3(b)}$	–	–	$5.8X10^3 \pm 0.2X10^{3(a)}$	–
M. varians	–	–	$9.42X10^2 \pm 0.75X10^{2\ (a)}$	–	–	–	–	–	$2.0X10^3 \pm 0.2X10^{3(a)}$	–
M. roseus	–	–	–	$7.6X10^3 \pm 0.19X10^{3(a)}$	–	–	–	$1.0X10^3 \pm 0.15X10^{3(a)}$	–	$2.33X10^5 \pm 0.2X\ 10^{5\ (a)}$
V. metschnikovii	$3.23X10^4 \pm 0.3X10^{5(a)}$	–	–	–	–	–	$5.9X10^6 \pm 0.2X10^{6(d)}$	–	$5.93X10^{7\ \pm} 0.2X10^{7(b)}$	–
V. cincinnatiensis	$4.01X10^5 \pm 0.19X10^{5\ (c)}$	–	$4.08X10^5 \pm 0.1X10^{5\ (c)}$	$4.98X10^5 \pm 0.22X10^{5(b)}$	–	–	–	–	$1.99X10^5 \pm 0.25X10^{5(a)}$	–

Mean ± SD values are in triplicate (n=3)
Different superscripts in parenthesis in the same column shows significant difference at $P < 0.05$ level.
Anova followed by DMRT's test
- No strain recorded

Anova for microbial load of GI tract showed significant difference ($P < 0.05$) between microbial loads (Table 3). DMRT's test showed that mean difference of the bacterial strains significantIt difference ($P < 0.05$) between the isolated groups.

Table 3: **Anova for Microbial Load of Isolated Bacteria from Gastrointestinal Tract of Ornamental Fishes (CFU/ml)**

Organism	Ornamental fishes									
	C. ocellaris	*B. schwanenfeldii*	*P. managuensis*	*C. carpio*	*C. auratus*	*P. scalare*	*A. nyassae*	*C. orinocensis*	*T. albonubes*	*L. chrysophekadion*
df	3, 8	3, 8	3, 8	3, 8	3, 8	3, 8	3, 8	3, 8	3, 8	3, 8
F value	190.327	79.372	2260.746	1184.861	2215.933	1033.519	2431.211	1439.818	302.027	2348.497
P value	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

* - Significantly different at $P < 0.05$

Table 4: Ecological Indices of Isolated Bacteria from Gastrointestinal Tract of Ornamental Fishes

	Ornamental Fishes									
Ecological indices	*C. ocellaris*	*B. schwanenfeldii*	*P. managuensis*	*C. carpio*	*C. auratus*	*P. scalare*	*A. nyassae*	*C. orinocensis*	*T. albonubes*	*L. chrysophekadion*
Index of Dominance	0.331	0.637	0.505	0.95	0.712	0.364	0.797	0.521	0.993	0.733
Index of Diversity	0.509	0.323	0.313	0.061	0.21	0.488	0.182	0.301	0.01	0.232
Index of evenness	0.8069	0.5256	0.5142	0.2879	0.4051	0.7691	0.3806	0.4996	0.256	0.4269

Index of dominance, diversity and evenness of bacterial strains was depicted in table 4. Ecological indices showed that highest dominance was recorded in the fish, *Tanichthys albonubes* (0.993), whereas diversity and richness were recorded in the fish *Cichla ocellaris* (0.509 and 807) (Table 4). Dissimilarity matrices of microbial load of GI tract in ornamental fishes showed maximum in the range between 0.76 – 1.00. There was no dissimilarity recorded in the range of 0.01 – 0.25 of bacterial strain distribution in GI tract of ornamental fishes (Figure 1).

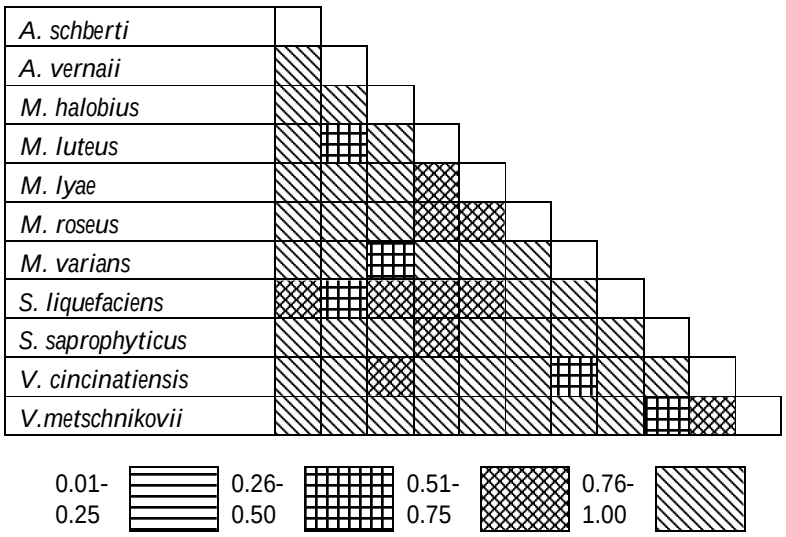

Figure 1: Dissimilarity Matrices of Isolated Bacteria from Gastrointestinal Tract of Ornamental Fishes

Cluster analysis of fish intestinal microbes represented that *Micrococcus varians* and *Micrococcus halobius* form a single cluster to the nearest neighbor with *V. metschinikovii*. *Micrococcus luteus, Staphylococcus. saprophyticus, Serratia liquifaciens* and *Aeromonas schubertii* were least relations to preceded group (Figure 2). Principal component analysis (PCA) of microbial relation between the fishes showed that *Aeromonas veronii* and *S. liquefaciens* were positively related to *A. schuberti*, *Vibrio cincinnatiensis,* while, other strains were negative relations (Figure 3)

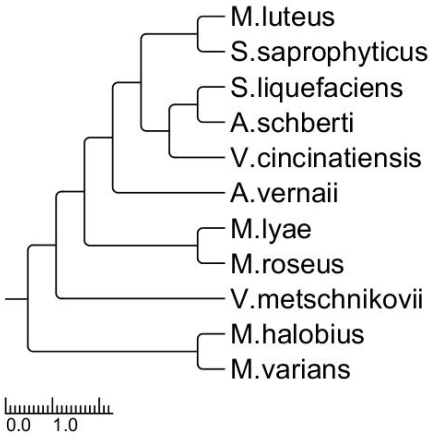

Figure 2: Cluster Analysis of Isolated Bacteria from Gastrointestinal Tract of Ornamental Fishes

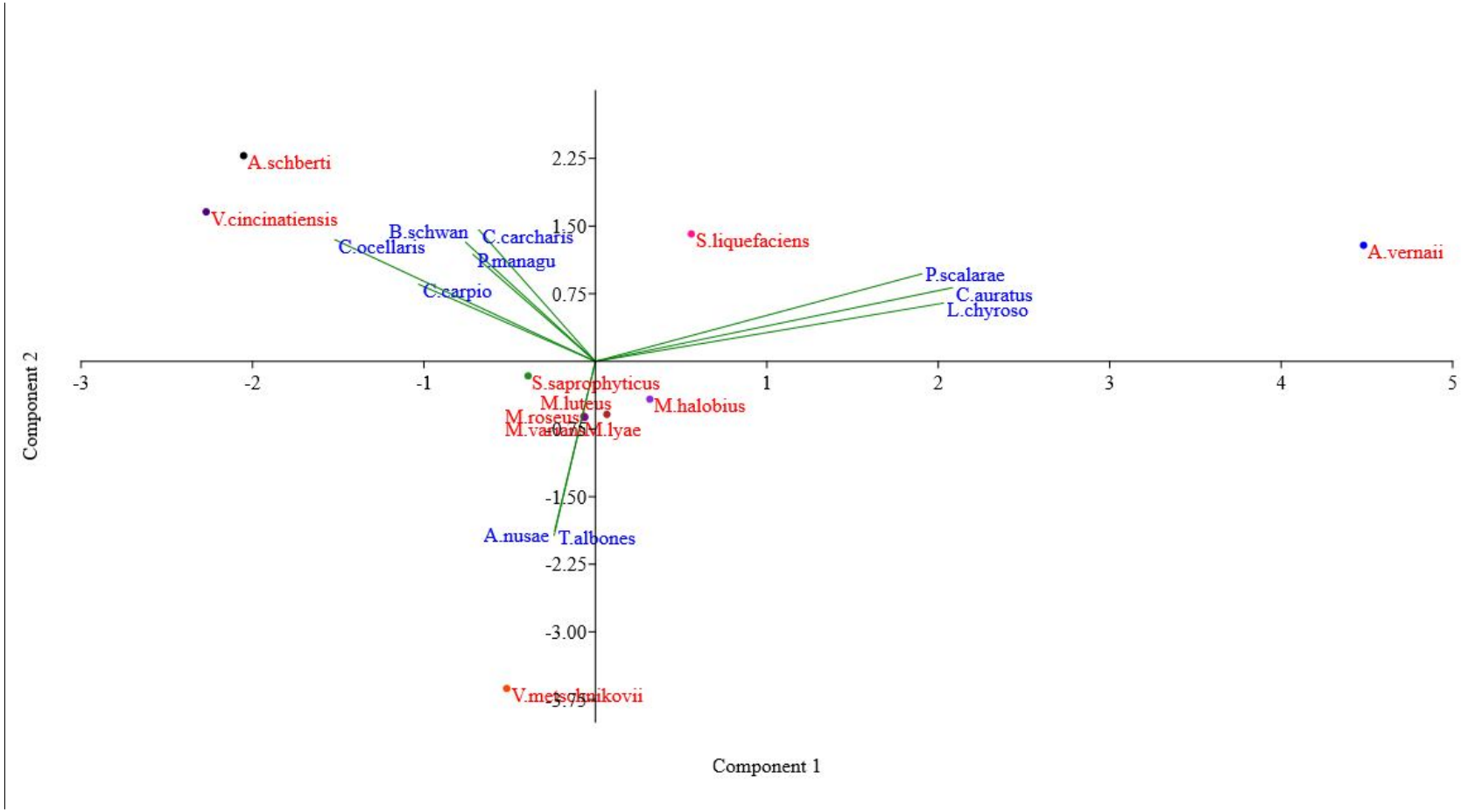

Figure 3: Principal Component Analysis of isolated bacteria from gastrointestinal tract of ornamental fishes

DISCUSSION

Microbial ecological studies in aquaculture focus on understanding the relationship between the host and microbial community. Bacteria plays a predominant role in the gut of the fish and it has become a frontier research field so far (Rombout et al., 2011). In the present study, gut microbiota of ten ornamental fishes was dominated by eleven bacterial species belonging to families of Proteobacteria, Firmicutes and Actinobacteria. Predominant bacterial genera present in gut microbiota were Proteobacteria (45%) and Actinobacteria (45%) followed by Firmicutes (10%). A similar study was reported from the intestinal lumen of Rainbow trout and the bacterial groups of Proteobacteria (57%), Actinobacteria (0.2%) and Firmicutes (12%) (Lyons et al., 2015). Numerous studies reported the dominance of bacterial communities such as Firmicutes, Proteobacteria and Actinobacteria are present in the gut of fish (Luo et al., 2001; Huang et al., 2009; Smith et al., 2012; Ingerslev et al., 2014; Song et al., 2016). Apart from this, the predominant bacteria play an important role in the digestion of complex dietary substances through the production of digestive enzymes (Tyagi and Singh, 2017). The gut microbiota has a major impact on the anatomical, physiological, and immunomodulation of the host (Rawls et al., 2004).

The average microbial counts in the gut of different ornamental fishes ranges from $9.42x10^2 \pm 0.75x10^2$ CFU / ml to $5.93x10^7 \pm 0.2x10^7$ CFU / ml. Our results were in accordance with previous study (Martin-Antonio et al., 2007), which showed the counts of *Solea senegalensis* within the range of $2.3x10^5$ – $6.7x10^6$ CFU / g. Many authors reported that different feeding rates alters the bacterial load in different species of microbes (Ringo et al., 2006; Ye et al., 2014; Ringo et al., 2016; Vatsos., 2016; Rimoldi et al., 2018). Bacterial load present in the intestine of common carp was reported with the value of $1.9x10^9$ CFU / g (Hagi et al., 2004).

Ecological diversity indices are widely used to compare diversity among microbial communities. Species richness represents the number of different types of species present in a community and species evenness represents their abundance distribution (Hill et al., 2003). The present study results were similar to the study on *Cyprinus carpio*, which had highest dominant bacterial load of 0.870 (Sivakumar et al., 2015), whereas the *Barbonymus schwanenfeldii* had the highest microbial diversity of 0.861. Similarly, (Dieguez et al., 2014, Li et al., 2015), reported dominant and distinguished bacterial diversity in the intestine mucus of European sea bass (*Dicentrarchus labrax*) and grass carp (*Ctenopharyngodon idellus*). Furthermore, differences in the microbial diversity of intestinal lumen and mucosal layer of fish have been reported previously (Merrifield et al., 2009, Wu et al., 2010).

Our results showed that *Micrococcus* was the most abundant genera suggesting that it was the core species in the gut of ornamental fishes, while the least was *Serratia liquefaciens*. The difference in bacterial dominance load, diversity and richness of bacteria indicates that GI tract of each ornamental fishes are influenced by feeding habits, water quality and various other environmental factors (Talwar et al., 2018).

The bacterial strains predominantly recorded in the fish, *Tanichthys albonubes* showed the index of dominance of about 99%. And *Cichla ocellaris* has shown high diversity range of 50% when compared to other species. The similar research has been carried out in gills of catfish, in which the dominant rate of bacterial species was 16% more prevalent with *A. hydrophila*, *S.putrefaciens*, and *V. cholera* (Uddin and Al-Harbi, 2012).

CONCLUSION

Microbial diversity is the measure of bacterial community structure, which may increase or decrease based on the prevailing physical, chemical and biological factors. High Diversity indicates a balanced and stable distribution of microorganisms in the GI tract. Our present study contributes to the aquaculture industry in determining the pathogenic strains as well as beneficial strains microbial community. The study on fish intestinal microbiota will assist the improvement of effective strategies for manipulating GI microflora to promote the health of fish and productivity. Further, research in

this field will enable the selection of probiotics with potentials to improve the gut homoeostasis and health of fish, which are alternatives of antibiotics that have been inhibited for use in food animals.

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