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An insight into the gut microbiology of wild-caught Mangrove Red Snapper, *Lutjanus argentimaculatus* (Forsskal, 1775)

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ABSTRACT

Documenting bacteria present in healthy individuals forms the first step in understanding the effects of microbial manipulation in aquaculture systems. Among the commensal microflora, gut microbiota has attracted extensive attention owing to their role in host metabolism and health maintenance. Basic knowledge on normal gut microbes within a particular host species is thus essential to determine how successfully these microbes can be manipulated and engineered for sustainable aquaculture systems. In spite of the good aquaculture potential of Mangrove red snapper, *Lutjanus argentimaculatus,* the information on microbial communities associated with the gut of this fish, and their contribution towards digestive efficiency and disease resistance is scarce. Therefore, an attempt was made to elucidate the abundance and diversity of cultivable gut microbes of wild caught L. *argentimaculatus* along with their digestive exoenzyme profiles and prohibitory effect against fish pathogens. Results on abundance showed similar gut bacterial loads as that of other marine fish imposing the less contribution of microflora to the volume of gut materials in fish. Eleven distinct bacterial species including two proposed novel vibrios were identified. An incidental observation of *Morganella morganii* throughout samples is an alarming signal, emphasizing the need for immediate de-gutting to avoid histamine intoxication. Abundance of digestive enzyme producers and excellent enzymatic potential of some isolates suggested the contribution of digestive enzymes may supplement to the symbiosis between gut flora and host and the information is of interest to aquaculture nutritionists/commercial industries. Interestingly, some isolates demonstrated estimable co-aggregation with aquatic pathogens, indicating their involvement in disease resistance and the results correlated well with gut microbial diversity. These findings highlight the significant role of gut microbes towards nutritional physiology and disease resistance of this aquaculture candidate in natural ecosystem. The culturable microbiota profiles of wild fish generated in the study can be applied for measuring the quality of husbandry routines in aquaculture facility of this marine fish. Overall, the present study fetches insights on the gut microbiome of healthy L. *argentimaculatus* which forms a platform for follow-up studies. The study may also help in the development of "functional" fish feeds for L. *argentimaculatus*. The investigation also demonstrated some potential digestive enzyme-producing isolates having probiotic applications in commercial aquaculture.

1. Introduction

Aquaculture is emerging as alternative strategy to meet the increasing demand for fish, as the most capture fisheries worldwide are overfished with no room for further expansion of commercial fishing efforts (Food and Agricultural Organization, 2014). Anticipation from the World Bank (2013) also indicates that almost 63% of the world's food fish will be produced through aquaculture by 2030, necessitating the use of sustainable aquaculture practices. This need for sustainable aquaculture has led to an increase in the research across a range of areas such as fish nutrition, fish microbiology, environmental impacts, good management procedures and disease control research as evident by the recent gush of publications on the related field (Pal, 2015). In this scenario, manipulation of the microbial communities present in the healthy fish has attracted extensive concern (Sihag and Sharma, 2012). Documenting the bacteria present in healthy individuals is the first step

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to understand the impacts of microbial manipulation in aquaculture systems (Tarnecki et al., 2016). The knowledge on the gut bacterial abundance and diversity can provide insight to the health status of individuals, as there will be an increase in the abundance of the opportunistic pathogens with considerable decrement in bacterial diversity during the incidents of stress and disease (Boutin et al., 2013). Additionally, the gut microbial communities are demonstrated to play a large role in maintaining host health by increasing digestion efficiency, boosting immune system, and preventing attachment and proliferation of opportunistic pathogens (Perez et al., 2010). The studies on gut microbiome of certain fish have revealed the contribution of these intestinal micro-organisms to nutritional physiology of host through the microbial breakdown of various feed ingredients (Ray et al., 2010; Tanu et al., 2012).

Mangrove red snapper, *Lutjanus argentimaculatus* is one of the high-value marine fish with great potential for export (Coniza et al., 2012). This fish is considered to be a good candidate for aquaculture as it is a fast growing fish that can be reared easily in captivity and can survive well in all phases of culture (Coniza et al., 2012). However, the information on microbial communities associated with the gut of wild, healthy L. *argentimaculatus,* contribution of gut microbiota towards digestive efficiency and disease resistance is very scarce. Due to the paucity of knowledge on the above-stated parameters, a study was conducted to characterize the gut microbiota of L. *argentimaculatus*. The study elucidates the abundance and diversity of cultivable gut microbes along with their digestive *exo*-enzyme profiles and prohibitory effect against fish pathogens.

2. Materials and Methods

2.1. Sample collection and pre-processing

Red Snappers were collected from five sites, which are approximately 4–8 km west of Fort Kochi Beach, Kerala, India (Fig. 1). Hydrographic parameters such as depth, salinity, pH, temperature (sea surface temperature, SST and atmospheric temperature, AT) and turbidity were estimated at each site using hand held multiparameter tester (Eutech, Singapore) and dissolved oxygen (DO) was measured using Winkler's method (Winkler, 1888). Average physicochemical parameters at the sampling sites were as follows: Depth- 12.6 m, Salinity-35 ppt, Atmospheric temperature-29.4 °C, pH-7.24, Sea surface temperature-27.8 °C, Dissolved oxygen-6.78 mg L^{-1} and Turbidity-1.18 NTU (Table 1). A total of ten individuals (5 males and 5 females) were collected to minimize the inter-individual microbiota variability (Boutin et al., 2014; Tarnecki et al., 2016). Wild Red Snappers were caught using hook and line, kept in autoclaved sea water and transported to the laboratory within an hour. The weight (W) and total length (LT) of these fish were recorded (Mean weight: 366.3 ± 65.1 g: Mean total length of 266.7 ± 23 mm). The external surface of the fish was cleaned with 70% ethanol to avoid the contamination by surface microflora. After opening the ventral surface, the entire intestine was aseptically removed using clamps to prevent release of fecal material, and gut length (LG) was measured. Relative gut length was reported as the ratio of gut length to total length (L_G/L_T) (Kar and Ghosh, 2008). The average length of gut in the collected fish was 89.6 ± 12 mm and mean relative gut length was calculated as 0.335 ± 0.02.

All fish sampled in this study were handled in strict accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and the protocols were approved by ICAR- Central Marine Fisheries Research Institute, Kochi, India.

2.2. Enumeration of gut microbes

The gut along with intestinal contents of each fish were homogenized using sterile normal saline. Subsequently, 10 fold dilutions of each homogenate were prepared and each dilutions were spread on Zobell Marine Agar (ZMA), Thiosulphate citrate bile salt sucrose agar (TCBS) and Brain Heart Infusion Agar (BHIA) (Himedia, India) in duplicates and incubated at 30 °C for 48 h. Total viable count was expressed as the number of colony forming units (CFU) per gram (Hovda et al., 2007). The viable counts of presumptive vibrios (mesophilic Vibrionaceae and other closely related vibrios) were calculated after 48 h of incubation on TCBS agar (Bolinches et al., 1988).

2.3. Diversity of cultivable gut microbes

Each undiluted tissue homogenate was streaked on ZMA and BHIA plates in quadruplets and incubated at 30 °C. The plates were examined up to 5 days and morphologically unique colonies were selected for purification. Genomic DNA was isolated from the pure culture using CTAB method (Wilson, 1987) and characterized by *16S rRNA* gene sequencing using universal primers, NP1F 5'GAGTTTGATCCTGGC TCA-3′ and NP1R 5'-ACGGCTACCTTGTTACGACTT- 3′ (Nair et al., 2012). After verifying the PCR product on 1% submarine agarose gel electrophoresis, the amplicons were purified and sequenced at a custom DNA sequencing facility (Scigenom, India). The sequences obtained were edited and compiled using Editseq program (DNASTAR, Lasergene, USA). Pair alignments and sequence identity generation were performed using MegAlign program (DNASTAR, Lasergene, USA). The sequences were then subjected to homology search against GenBank database, NCBI using the BLAST search algorithm. Sequence identities of 99% and 97% were used as criteria for species and genus assignments, respectively (Janda and Abbott, 2007). The perplexity in assigning the species/genus was overcome by performing a set of biochemical tests (Bergey et al., 2012). The isolates showing a disparity in the results of biochemical and molecular characterization (*16SrRNA* gene sequence) were assigned as novel species (Janda and Abbott, 2007). The representative *16S rRNA* gene sequences of each species were then submitted to GenBank (NCBI).

2.4. Phylogenetic analysis

The overlapping 768 bp size segment of *16SrRNA* gene in the isolates of each species was used for phylogenetic study. The sequences were aligned using ClustalW and neighbor-joining (NJ) tree was constructed by MEGA version 7 using Kimura 2-parameter model (Kumar et al., 2016). The confidence in the NJ tree was estimated by 1000 bootstrap replicates.

2.5. Contribution of cultivable gut microbes towards digestive enzymes

The abundance of each digestive enzyme (amylase, protease and cellulose) producers was calculated by enumeration on specific substrate embedded media using a previously described protocol (Kar and Ghosh, 2008). Concurrently, three additional digestive enzyme producers (lipase, pectinase and chitinase) were also enumerated in duplicates (Jayashankar and Graham, 1970; De Boer et al., 1998; Kanchana et al., 2011).

Three representative isolates from each identified species were individually tested for their enzymatic activities (Nair et al., 2012). Diameter of the clear zone formed in each assay was measured and enzymatic index (EI) was calculated for scoring the enzyme production potential, as the potential isolates will be showing an EI value ≥1 (Fungaro and Maccheroni Jr., 2002). Potential producers for each enzyme, and enzymatic versatility of each isolates were then analyzed.

2.6. Contribution of cultivable gut microbes towards disease resistance

The isolated bacteria were screened *in-vitro* for antagonistic activity against 7 fish pathogens (Table 2) by spot-on-the-lawn approach with slight modifications (Rojo-Bezares et al., 2007). Briefly, 24 h old cultures of these pathogens were inoculated (10^8 cells/100 µl) separately

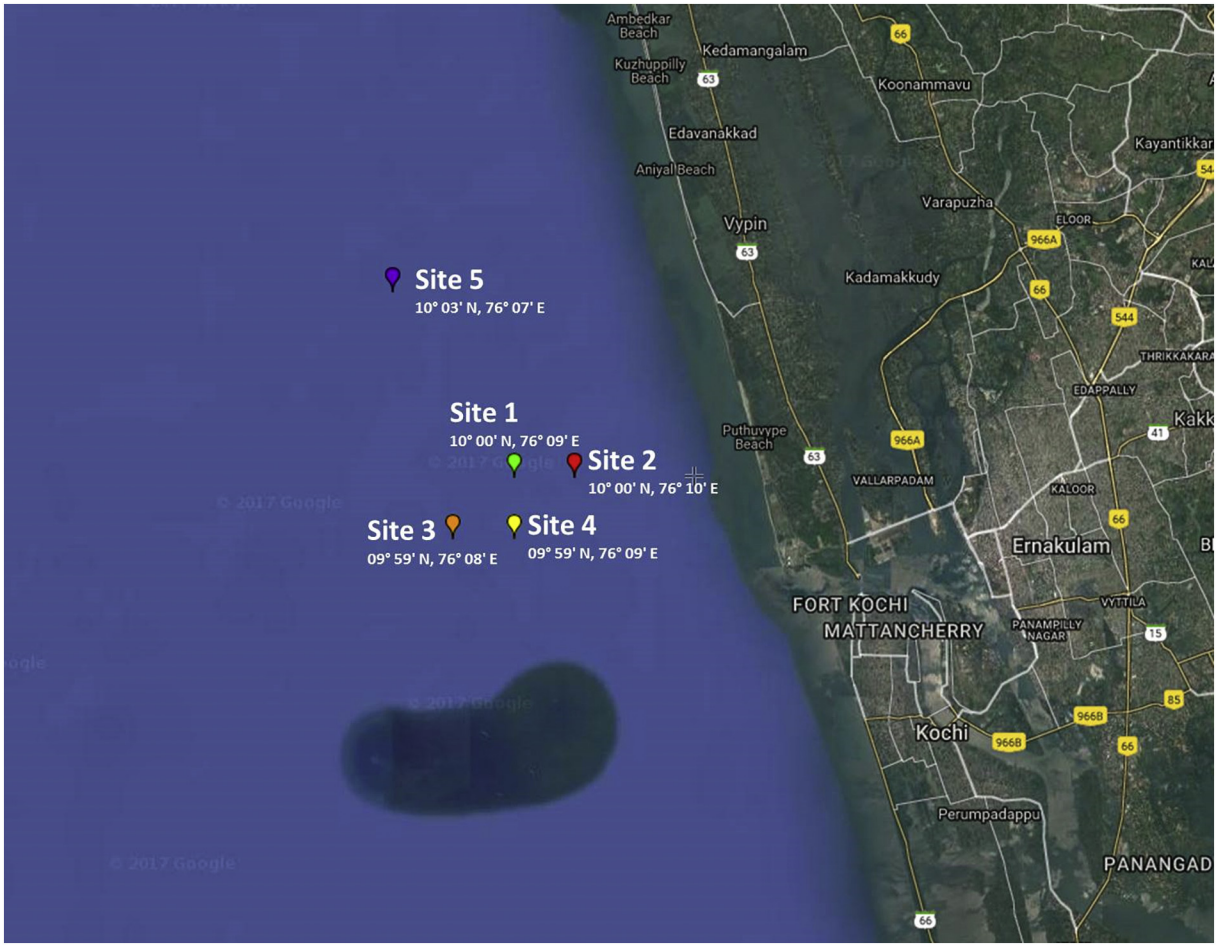

Fig. 1. Sampling locations of the present study.

on Mueller Hinton Agar media (HiMedia) by pour plating. Subsequently, the pure culture of each isolate was spotted onto these agar plates. The plates were incubated at 30 °C overnight and zones of inhibition were measured and recorded.

Further, co-aggregation assays were performed to assess the ability of these gut isolates to prevent the colonization of pathogens using a previously described protocol (Del Re et al., 2000). Briefly, the gut isolates and fish pathogens (Table 2) were cultured on LB broth (Himedia) individually for 24 h in a shaker incubator at 30 °C and the cells were pelleted by centrifugation for 15 min at 8000 rpm. After washing twice in sterile PBS, the pellets were re-suspended in same buffer to adjust the OD 600 to 0.25 ± 0.05 (Collado et al., 2008). Equal volume (1 ml) of each gut bacterial suspension were then mixed with each pathogen suspension by vortexing for 15 s. Absorbance of upper portion of the mixed suspension was measured after 0 h, 1 h, 2 h, 4 h and 24 h of incubation at room temperature (Kos et al., 2003). The co-aggregation percentage was expressed as A% = (1 - A_{mix}/A_0) X 100, where A_{mix} represents the absorbance at time t = 1, 2, 4 and 24 h; A_0 represents the absorbance at t = 0 (Zhang et al., 2013). Each assay was performed in triplicate to precise the intra-assay variation.

Table 1
Sampling locations, hydrographic parameters and characteristics of L. *argentimaculatus* under study.

Sampling locations	Cordinates	10° 00′ N 76° 09′ E		10° 00′ N 76° 10′ E		09° 59′ N 76° 08′ E		09° 59′ N 76° 09′ E		10° 03′ N 76° 07′ E	
	Date	12-12-2016		15-12-2016		21-12-2016		23-12-2016		29-12-2016	
Hydrographic parameters	Depth (m)	10	10	11	11	13	13	14	14	15	15
	Salinity (ppt)	35	35	35	35	35	35	35	35	35	35
	AT (°C)	28	28	29	29	30	30	30	30	30	30
	DO (ppm)	5.4	5.4	6.1	6.1	8.1	8.1	7.2	7.2	7.1	7.1
	pH	7.17	7.17	7.18	7.18	7.24	7.24	7.29	7.29	7.31	7.31
	Turbidity (ntu)	1.83	1.83	0.95	0.95	0.66	0.66	1.22	1.22	1.24	1.24
	SST (°C)	27	27	27	27	29	29	28	28	28	28
Fish collected	Snapper ID	1	2	3	4	5	6	7	8	9	10
	Sex	M	F	F	F	F	M	M	F	M	M
	W (g)	487	323	353	450	256	335	337	364	368	390
Gut parameters	LT	30	25.2	26.8	30	25.7	24.1	23.2	28.4	25.3	28
	LG	10.2	8.3	9.4	10.5	8.2	8.0	7.2	10.5	7.8	9.5
	L_G/L_T	0.34	0.33	0.35	0.35	0.32	0.33	0.31	0.37	0.31	0.34

Table 2
Different bacterial pathogens used in the study.

Bacteria	Source
Bacillus cereus MTCC 430	MTCC, Chandigarh
*Aeromonas hydrophila*MTCC1739	MTCC, Chandigarh
Vibrio alginolyticus Strain 101	Central Institute of Brackish water Aquaculture, Chennai
Vibrio anguillarum O1	Central Institute of Brackish water Aquaculture, Chennai
Vibrio parahemolyticus MTCC 451	MTCC, Chandigarh
Vibrio vulnificus MTCC 1145	MTCC, Chandigarh
Vibrio harveyi Strain 102	Central Institute of Brackish water Aquaculture, Chennai

2.7. Statistical analysis

One-way ANOVA was used to find out and compare the means with p value < 0.05 set to represent the significant difference and a value $p < 0.01$ set to represent the highly significant difference. After one way ANOVA, Tukey's Honest Significant Difference test was used for post-hoc analysis (SPSS software program ver. 16). Ordination analysis performed through non-Multidimensional Scaling (non-MDS) using PRIMER v6 (Primer-E Ltd., Plymouth, UK) to compare the similarity of microbial community composition among 10 individual fish. Diversity of isolated bacterial species from the gut of all samples were analyzed in terms of Simpson Index, D (Simpson, 1949), Shannon's index, H (Shannon and Weaver, 1949) and Pielou's evenness, J (Pielou, 1975). The one-way multivariate ANOVA was used to determine the differences among diversity indexes between fish samples. After this, Tukey's Honest Significant Difference test was used for post-hoc analysis (SPSS software program ver. 16).

3. Results

3.1. Enumeration of bacteria in fish gut

Three different media were used for the enumeration of cultivable heterotrophic gut bacteria from L. *argentimaculatus*. Data were presented as log colony forming units per gram of gut tissue (log CFU) ± SE (Fig. 2). Over the observation period of one month, log CFU/g ± SE in the gut of healthy wild caught red snappers collected from the different locations in the Fort Kochi, Kerala, India were 7.633 ± 0.144, 6.70 ± 0.9 and 2.5 ± 0.13 in ZMA, BHIA and TCBS respectively. A Tukey *post hoc* test revealed that there was a significant difference in CFU value between three different growth media and mean CFU was statistically significantly higher in ZMA followed by BHIA and TCBS respectively ($p < 0.05$).

Table 3
Different bacterial species identified from the gut of L. *argentimaculatus*.

Sl. No.	Isolate ID	Species assigned	GenBankAcession number
1	1G	*V. alginolyticus*	MF171027
2	3 G	*V. natriegens*	MF171028
3	7G	*V. hepatarius*	MF171029
4	20G	*S. haliotis*	MF171030
5	21G	*P. damselae* subsp. damselae	MF171031
6	32G	Unidentified Vibrio sp. I	MF171032
7	33G	Unidentified Vibrio sp. II	MF171033
8	38G	*M. morganii*	MF171034
9	45G	*V. parahaemolyticus*	MF171035
10	52G	*B. megaterium*	MF171036
11	53G	*P. dispersa*	MF171037

3.2. Diversity of cultivable gut microbes

The diversity of gut microbiota in wild caught L. *argentimaculatus* was examined through culture dependent methods using three different media. A total of 128 bacterial isolates were selected for *16S rRNA* gene sequencing based on colony morphological characteristics. It was found that out of the three media used, ZMA displayed the highest density (Fig. 2) and morphological diversity. The isolates were characterized up to species level based on conventional microbiological tests and *16S rRNA* gene sequencing). The representative *16S rRNA* gene sequences of each species were submitted to GenBank (NCBI) and assigned with accession numbers (MF171027-MF171037).

Overall, we could identify 11 distinct bacterial species (Table 3) from 6 genera, which belonged to two major phyla namely Proteobacteria (97.7%) and Firmicutes (2.3%). The phylum Firmicutes was represented by a single bacterial species, *Bacillus megaterium*. All identified bacterial species were classified in 4 different families in which, *Vibrionaceae* (74.2%) was the most abundant, followed by *Enterobacteriaceae* (14.06%), *Shewanellaceae* (9.4%) and *Bacillaceae* (2.34%) (Fig. 3). The genera wise abundance was in the order of *Vibrio* (68%) > *Morganella* (11.7%) > *Schewanella* (9.4%) > *Photobacterium* (6.2%) > *Pantoea* (2.34%) > *Bacillus* (2.3%). Altogether, the most abundant bacterial species was *V. alginolyticus* (37.5%) which was followed by *V. natriegens* and *M. morganii* with an abundance of 17.19% and 11.7% respectively (Fig. 4). The bacterial species *V. alginolyticus* and *M. morganii* were present in all the ten fish under study. Ordination analysis through non-MDS indicated individual variations in gut microbial distribution of L. *argentimaculatus* (Fig. 5). Biodiversity indices like Simpson's index, Shannon's index and Pielou's evenness from different individuals were estimated and summarized in Table 4. In the present study, the value of Shannon's index varied from 1.16 to 1.96 indicating a relatively low to moderate diversity. Simpson richness

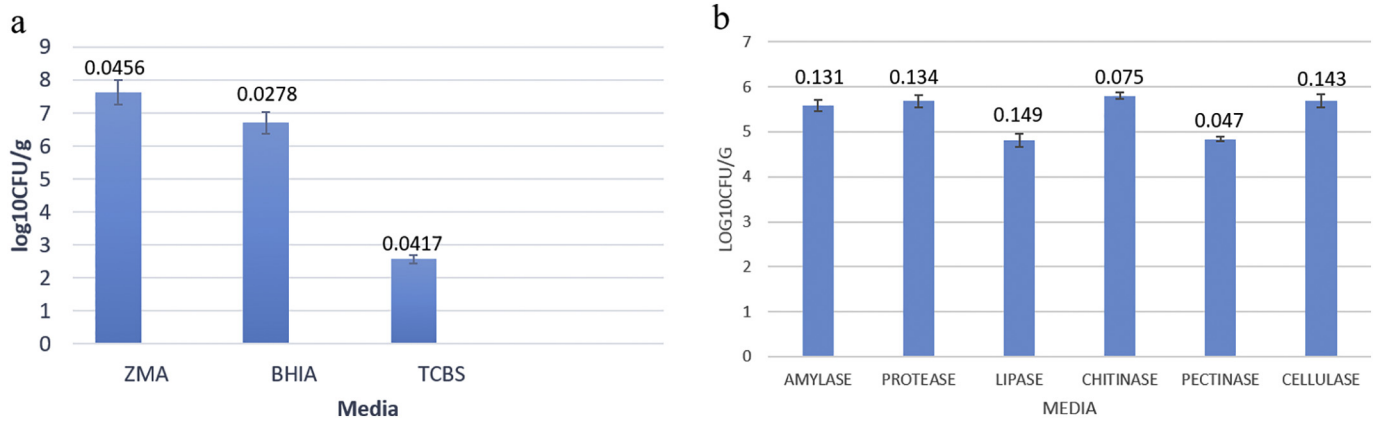

Fig. 2. Average log colony forming units per gram of gut tissue (log CFU) ± SE. a: Enumeration of total viable gut heterotrophs; b: Enumeration of digestive enzyme-producers.

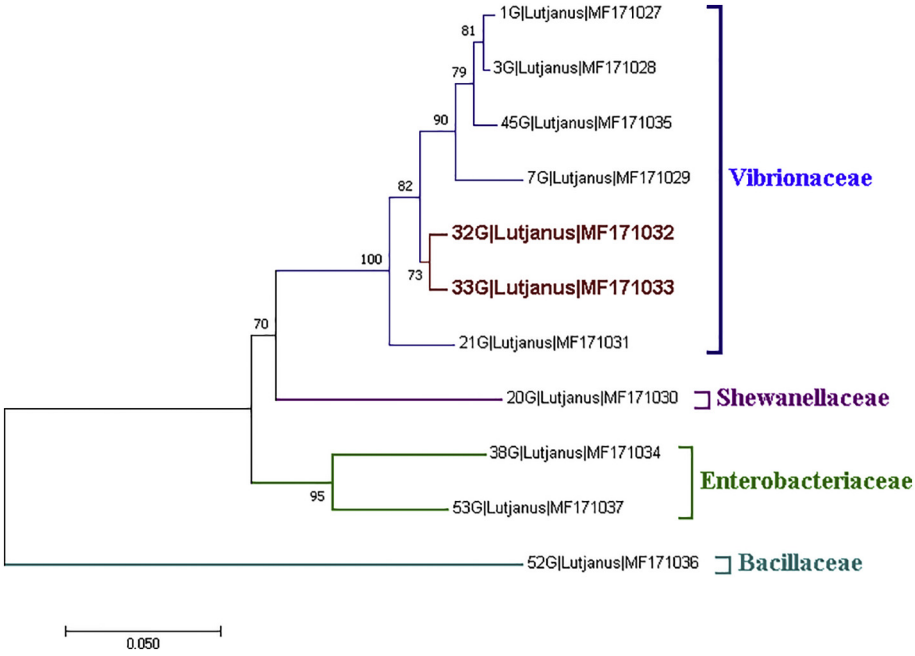

Fig. 3. Neighbor-joining tree of gut isolates based on *16SrRNA* gene sequence. The ID of representative gut isolate are followed by the source of bacteria and GenBank accession numbers. The bacteria which could not be identified upto species level in the present study are represented by red coloured bold letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ranged from 0.67 to 0.96 and Pielou's evenness varied from 0.75 to 0.97, signifying a moderate to high richness and comparatively higher evenness respectively. There was a statistically significant difference in biodiversity indices between fish samples ($p < 0.0005$).

3.3. Contribution of the gut microbes towards digestive enzymes

Enumeration of digestive enzyme-producers showed that amylolytic, cellulolytic, chitinolytic, lipolytic, pectinolytic and proteolytic bacteria were abundantly present in the gut (Fig. 2b). Overall, there was statistically significant difference in mean CFUs of different enzymatic producers ($p < 0.05$). A Tukey *post hoc* test revealed that chitinolytic (LVC- 5.80 ± 0.24), proteolytic (LVC- 5.68 ± 0.43), cellulolytic (LVC- 5.69 ± 0.45) and amylolytic strains (LVC-5.5862 ± 0.131) were statistically significantly higher in abundance than lipolytic and pectinolytic microorganisms (LVC- 4.81 ± 0.47 and LVC- 4.84 ± 0.15 respectively) ($p < 0.05$). However, there was no statistically significant difference between the abundance of lipolytic and pectinolytic strains ($p = 1.00$). Similarly, there was no statistically significant difference between the abundance of amylolytic and proteolytic strains ($p = 0.992$), amylolytic and chitinolytic strains ($p = 0.811$), amylolytic and cellulolytic strains ($p = 0.990$), proteolytic and chitinolytic strains ($p = 0.985$), proteolytic and cellulolytic strains ($p = 1.00$) as well as chitinolytic and cellulolytic strains ($p = 0.987$). Afterwards, three representative isolates from each identified species were tested individually to assess their enzyme production potential in terms of enzymatic index (EI) as the potential isolates will be having an EI value ≥1 (Fungaro and Maccheroni Jr., 2002). The highest EI observed for various enzymes *viz.*, amylase, cellulase, lipase, pectinase, chitinase and protease were 2.2, 1.2, 1.5, 2, 3 and 1.2 respectively (Fig. 6). In terms of digestive enzymatic versatility of representative gut isolates, four isolates showed maximum versatility producing 4 different digestive enzymes. Overall, the isolates investigated in the study showed diversified enzymatic patterns. However, the capacity of digestive enzyme production was found to be strain-specific rather than a species-specific property. By combining the data in Fig. 4a and Fig. 6, it was clear that bacteria having the highest EI for chitinase, protease, amylase, cellulase, pectinase and lipase belonged to species *V. alginolyticus/S. haliotis*, *M. morganii/S. haliotis, V. alginolyticus, V. natriegens, M. morganii* and *V. natriegens/M. morganii* respectively, the four bacterial species occupying the first four positions in terms of abundance.

3.4. Contribution of gut microbes towards disease resistance

When the gut isolates were screened *in-vitro* for their antagonistic activity against aquatic pathogens, none of the isolates except two

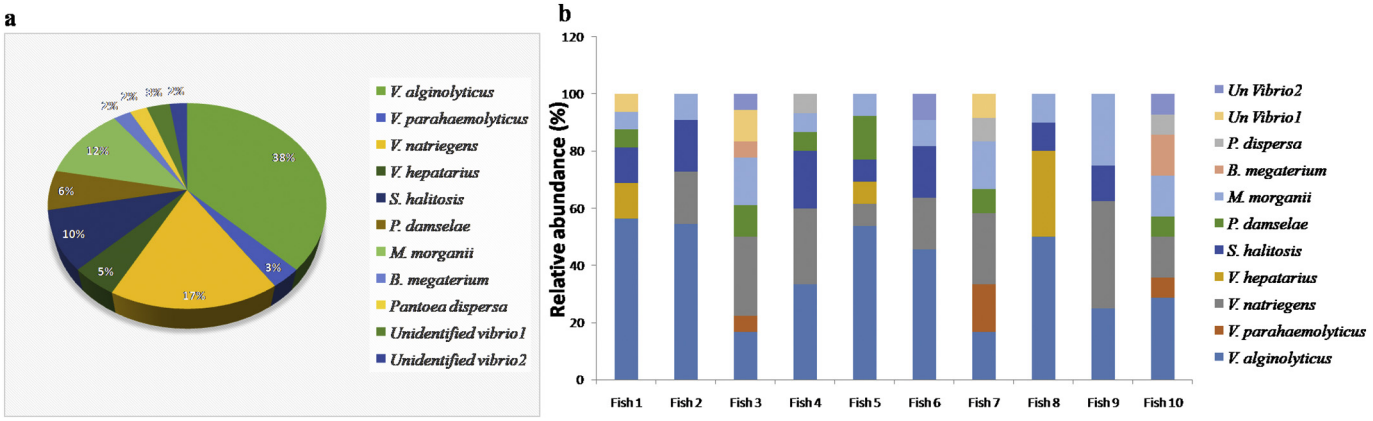

Fig. 4. Abundance of identified bacterial species in the gut of L. *argentimaculatus*. a: Overall relative abundance; b: Relative abundance in individual fish.

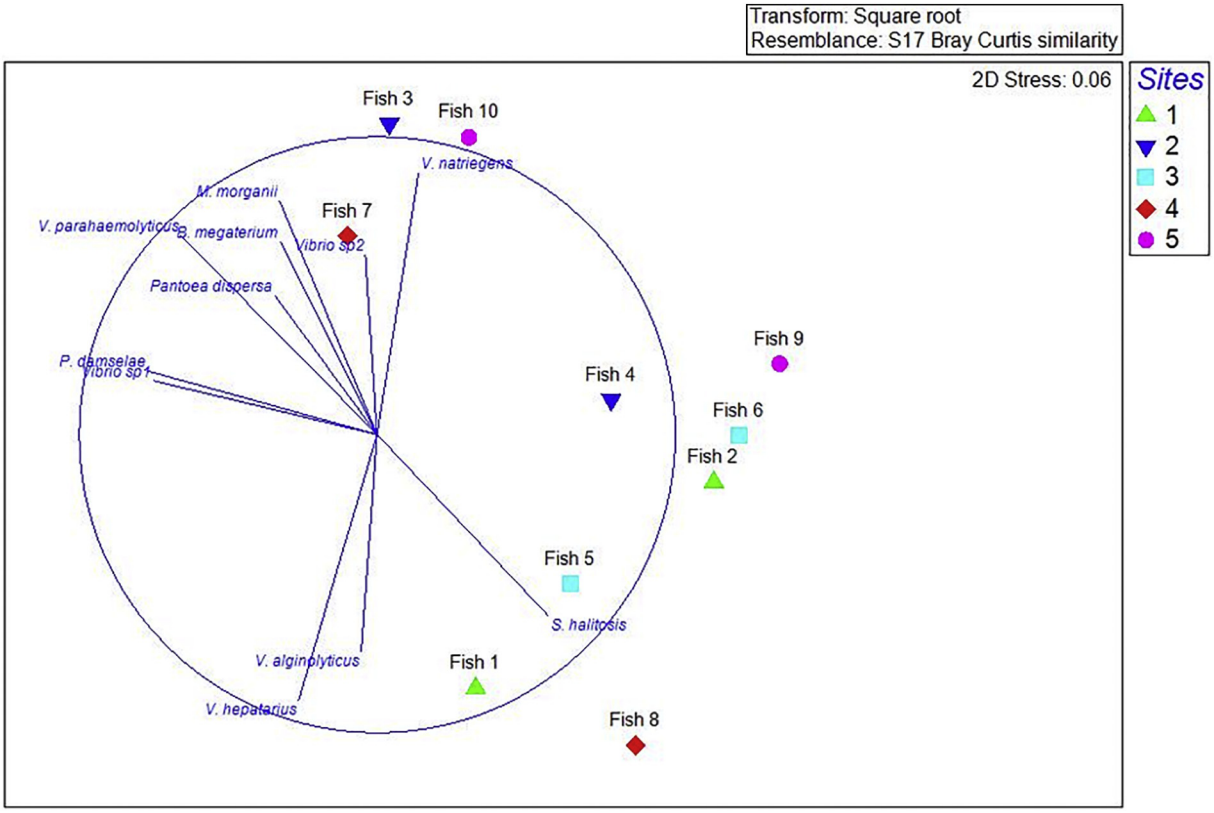

Fig. 5. Compositional variation in microbial communities between fish under study. NMDS ordination between microbial communities of fish under the study where, each point represents an individual site.

showed antagonistic activity. The isolates 42G and 45 G (*V. natriegens* and *V. parahaemolyticus* respectively) had weak inhibition to the growth of *V. anguillarum* (only 2 mm). Further, co-aggregation of the gut isolates with the pathogens was examined to assess their ability to prevent the gut colonization of pathogens. The results were expressed as relative percentage reduction in the absorbance of a mixed suspension after 1, 2, 4 and 24 h compared to the initial absorbance (Fig. 7). Some of the gut isolates exhibited commendable co-aggregation towards pathogens, and co-aggregation percentage increased with incubation time. Among the total 33 representative isolates tested for co-aggregation with pathogens 2, 3, 4, 5 and 7 isolates displayed co-aggregation with *V. vulnificus*, *V. anguillarum, V. parahaemolyticus, V. harveyi* and *A. hydrophila* respectively. At the same time none of the isolates showed co-aggregation against *B. cereus* and *V. alginolyticus*. The maximum co-aggregation percentage was observed for *V. harveyi* (70.59% by 47G) and *A. hydrophila* (65.42% by 35G) at 24 h of incubation. Interestingly, one strain of *V. hepatarius* (7G) showed co-aggregation against three pathogens (*V. vulnificus, V. anguillarum* and *A. hydrophila*). Nevertheless, the ability of co-aggregation was found to be strain-specific rather than a species-specific property.

4. Discussion

Gastrointestinal tract harbour highly abundant and diverse microbial community having significant nutritional and physiological interactions among them and with the host (Maynard et al., 2012; Xing et al., 2013). Moreover, a malfunctioning gut microbiota can be detrimental to the host subsistence and fitness (Zilber-Rosenberg and Rosenberg, 2008). Accordingly, this internal ecosystem is now considered as a "novel" trait under strong natural selection (Shin et al., 2011), and a burst of studies is now attempting to unravel its role within the host. Though fish are the most abundant and species-rich vertebrates with a large spectrum of dietary niches, they remain poorly studied especially in the wild context (Clements et al., 2014; Llewellyn et al., 2014). Awareness on the natural bacterial abundance in gut and gills which are the primary routes of entry for pathogens in fish; is an important aspect of their health evaluation (Tarnecki et al., 2016). For this, enumeration of gut bacteria was done as the first step. Results showed that the total viable bacterial loads in gut of L. *argentimaculatus* was found to be similar to that of the other marine fish species (Austin, 2006; Ringo et al., 2006; Smriga et al., 2010; Tarnecki et al., 2016). All these findings support the statement of Kim et al. (2007) that gut microflora in fish contribute less to the volume of gut materials, with an estimated value of 10^6–10^8 CFU/g compared to 10^{11} CFU/g reported in terrestrial mammals. However, within the same sample type, ZMA displayed higher density when compared with the other non-selective media (BHIA) ($p < 0.05$), signifying the suitability of ZMA to obtain baseline data on marine samples. As vibrios are the predominant bacteria in intestinal tract of many marine fish (Depaola et al., 1994; Matsunaga et al., 2011), specific enumeration was done in TCBS agar, in order to get information on the viable counts of presumptive vibrios (Bolinches et al., 1988). It was found that the TCBS count varied between 300 and 460/g of gut of wild L. *argentimaculatus*. In contrast, higher densities of vibrios in the order of 10^8/g of intestine have been reported in some fish species (Karunasagar et al., 1987; Depaola et al., 1994; Thampuran and Surendran, 1998). The decreased count may be attributed by the species under study or the area from where the fish are collected. However, no such studies are available in the wild L. *argentimaculatus* for comparison.

Out of total of 128 bacterial isolates selected for characterization, 11 distinct bacterial species belonging to 6 genera and two phyla were identified. It was observed that members of the phylum Proteobacteria (97.7%) absolutely dominated the cultivable gut bacterial communities of L. *argentimaculatus,* which is concurrent to the previous reports on some other fish species (Huber et al., 2004; Kim et al., 2007; Zhou et al., 2009; Feng et al., 2011). The genera wise abundance was in the order of

Table 4
Biodiversity indices of the isolated bacteria from individual red snappers.

Snapper ID	1	2	3	4	5	6	7	8	9	10
Shannon index (D)	1.36 ± 0.008	1.17 ± 0.008	1.92 ± 0.043	1.58 ± 0.014	1.41 ± 0.008	1.41 ± 0.022	1.86 ± 0.008	1.17 ± 0.008	1.32 ± 0.008	1.95 ± 0.287
Simpson index (H)	0.68 ± 0.00816	0.69 ± 0.1633	0.88 ± 0.00816	0.82 ± 0.01414	0.72 ± 0.01633	0.78 ± 0.00816	0.91 ± 0.01633	0.71 ± 0.00816	0.82 ± 0.01414	0.90 ± 0.07188
Pielou's evenness (J)	0.76 ± 0.00816	0.84 ± 0.02449	0.92 ± 0.02828	0.88 ± 0.00816	0.79 ± 0.02449	0.88 ± 0.01633	0.96 ± 0.01633	0.84 ± 0.01414	0.95 ± 0.02160	0.94 ± 0.00816

Vibrio (68%) > *Morganella* (11.7%) > *Schewanella* (9.4%) > *Photobacterium* (6.2%) > *Pantoea* (2.34%) > *Bacillus* (2.3%). Whereas, previously reported abundant genera in the gut of other marine fish species include *Vibrio* (Zhou et al., 2009; Smriga et al., 2010; Feng et al., 2011), *Pseudoalteromonas* (Verner-Jeffreys et al., 2003; Korsnes et al., 2006; Martin-Antonio et al., 2007; Ringo et al., 2008), *Cetobacterium* (Givens et al., 2015) and *Photobacterium* (Hovda et al., 2007; Ward et al., 2009; Zhou et al., 2009; Smriga et al., 2010). The high proportion of *Vibrio* spp. in gut of wild Mangroove red snapper in the present study is concordant with prior analyses that demonstrated the dominance of vibrios from Great Barrier Reef fishes (Sutton and Clements, 1988). Similar observations on the abundance of Vibrios in various coral reef fish guts was made using culture independent methods also (Smriga et al., 2010; Feng et al., 2011).

Interestingly, within the genus *Vibrio*, 6 different species were isolated, of which two could not be assigned to any known species. *V. alginolyticus* (37.5%) was found to be the most dominant species. The species wise richness in the present study was in the order of *V. alginolyticus* (37.5%) > *V. natriegens* (17.19%) > *M. morganii* (11.7%) > *S. haliotis* (9.38%) > *P. damselae* (6.25%) > *V. hepatarius* (4.69%), *V. parahaemolyticus* (3.12%), *B. megaterium/P. dispersa* (2.34%). Of these *V. alginolyticus*, *P. damselae*, *V. parahaemolyticus* are known fish pathogens and their presence in apparently healthy fish supports previous reports of these organisms as opportunistic pathogens in fish (Rivas et al., 2013; Chatterjee and Haldar, 2012). The other bacteria such as *M. morganii* (Kim et al., 2003; Ruzauskas et al., 2016), *V. natriegens* (Feng et al., 2011), *S. haliotis* (Kim et al., 2007), *V. hepatarius* (Thompson et al., 2003; Balcazar et al., 2006) and *B. megaterium* (Saha et al., 2006) are regarded as harmless bacteria in fish. Although *P. agglomerans* has been reported to be associated with terrestrial and aquatic animals (Walterson and Stavrinides, 2015), *P. dispersa* in gut of fish has not been described earlier. Similarly, *M. morganii* is reported as an endogenous bacterium in the skin and gills of Scombroid fish but rarely in their gut (Kim et al., 2003). The occurrence of this bacterium in the gut of snappers has not been described even with the metagenomics approach so far (Feng et al., 2011; Tarnecki et al., 2016). *M. morganii* the most prolific histamine producing bacteria, plays a major role in the accumulation of histamine during storage of fish, causing food borne chemical intoxication (Kim, 2001). Therefore, the presence of this bacterium in the gut of apparently healthy wild caught Mangroove red snapper points out the importance of immediate de-gutting prior to storage to prevent the food borne intoxication.

When the biodiversity indices of individual fish were estimated, Simpson richness was found to be ranged from 0.67 to 0.96 and Pielou's evenness varied from 0.75 to 0.97, signifying a moderate to high richness and comparatively higher evenness respectively. Shannon's index was found to be varied from 1.16 to 1.96 indicating a relatively low to moderate diversity supporting the observation of Holben et al. (2002) indicating the less diversity of fish gut microbiome than that of mammals. There was a statistically significant difference in biodiversity indices based on fish ($p < 0.0005$). Similarly, ordination analysis through non-MDS also revealed individual fish variations in the gut microbial community composition. These variations may be attributable to the transient environmental effects (Smith et al., 2015) or diet (Sullam et al., 2012), although these were not examined in this study due to the wild nature of host and similarity in environmental variables between sampling sites of the present study. However, host genetics are also known to play a role in shaping microbiota structure (Smith et al., 2015; Boutin et al., 2014) and as a result, high variability between individuals is not uncommon in fish microbiota studies (Fjellheim et al., 2012; Larsen et al., 2015).

The studies describing the endogenous digestive enzymes of fish have been conducted by several workers (Dhage, 1968; Kawai and Ikeda, 1972; Das and Tripathi, 1991). However, the information regarding the digestive enzyme producing intestinal bacteria, their abundance and significance in fish is scarce (Kar and Ghosh, 2008).

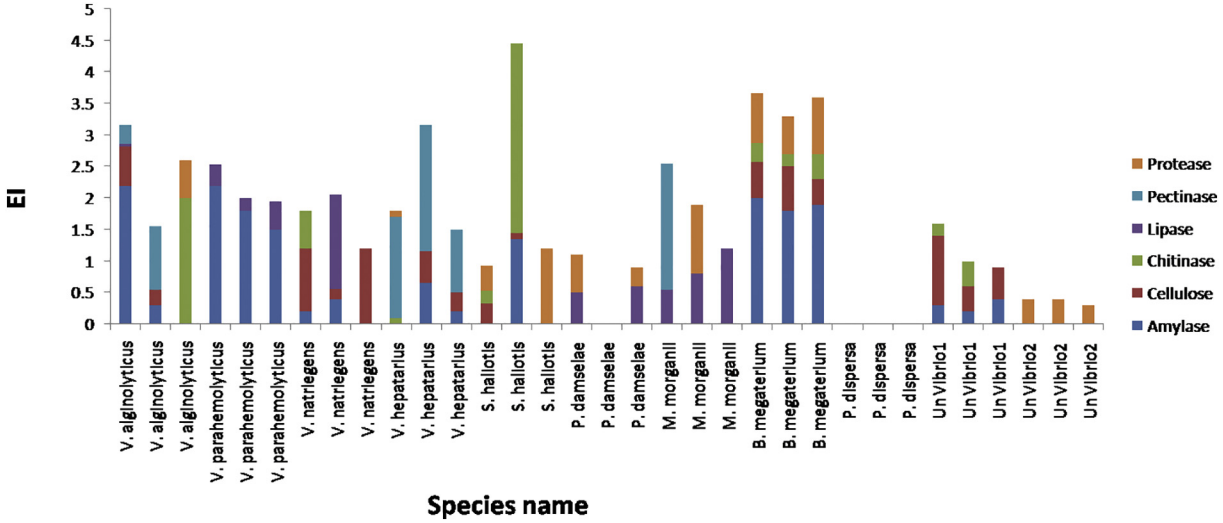

Fig. 6. Digestive enzyme production of representative gut isolates. Isolate ID was given in brackets of each species.

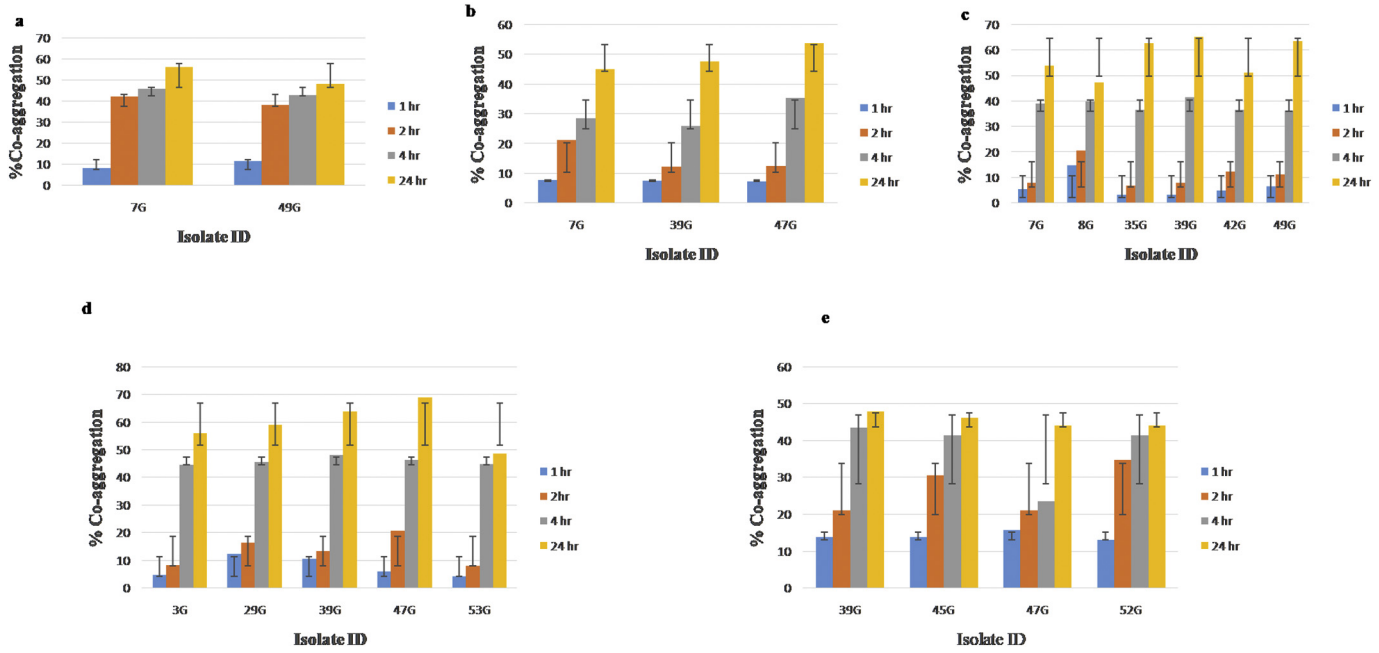

Fig. 7. Percentage of co-aggregation for strains showing positive results. a: Co-aggregation against *V. vulnificus*; b: Against *V. anguillarum*; c: Against *A. hydrophila*; d: Against *V. harveyii*; e: Against *V. parahaemolyticus*.

There are some studies indicating the correlation of gut microbial composition with the feeding habits of fish (Kar and Ghosh, 2008; Sullam et al., 2012; Liu et al., 2016). Therefore, specific enumeration of different digestive enzyme producers was done to find out their significance in the nutrition of L. *argentimaculatus*. Results revealed that chitinolytic, proteolytic, cellulolytic and amylolytic strains were statistically significantly higher in abundance than lipolytic and pectinolytic microorganisms ($p < 0.05$). Simultaneously, the enzyme production potential of various gut microbiota was individually found out on substrate amended agar plates (Nair et al., 2012). It was reported that the isolates showing EI value ≥1 can be considered as potential enzymatic producers (Fungaro and Maccheroni Jr., 2002). The highest EI values obtained for various enzymes were 2.2, 1.2, 1.5, 2, 3 and 1.2 for amylase, cellulase, lipase, pectinase, chitinase and protease respectively indicating the presence of potential producers of all the tested enzymes in the gut of L. *argentimaculatus*. Bacteria having the highest EI for chitinase, protease, amylase, cellulase, pectinase and lipase were found to be belonging to species *V. alginolyticus/S. haliotis*, *M. morganii/S. haliotis, V. alginolyticus, V. natriegens, M. morganii* and *V. natriegens/M. morganii* respectively, the four bacterial species occupying the first four positions in terms of abundance. This showed that all the gut bacterial species occupying higher position in terms of abundance have contribution towards digestive enzyme production which again pointed out the relevance of exogenous enzyme contribution of gut isolates towards the to the whole digestive capacity of the fish. In short, the results on the abundance and enzyme production potential suggests an omnivorous feeding aptitude of this mangrove red snapper. This has been further supported by the value of short relative gut length as recognized in other carnivorous/omnivorous fish (Hugueny and Pouilly, 1999; Drewe et al., 2004).

The higher abundance of chitinolytic bacteria in the digestive tract and the excellent chitinase producing capacity (EI-3 and 2) by two gut bacterial strains may indicate the preference of this Mangroove Red Snapper towards crustaceans as food. The abundance of proteolytic

(highest EI-1.2 and 1.1) and lipolytic bacteria (highest EI-1.5 and 1.2) in the gut also seems to support their feeding preference towards animal matter. Sugita et al. (1999) hypothesized that *Aeromonas* sp. and *Vibrio* sp. contributes towards chitin digestion in freshwater and marine fish respectively. In contrast, the highest chitinase producer in the present study belonged to *S. haliotis* followed by *V. alginolyticus*. Similar reports on the contribution of *Schewanella* towards chitin digestion in fish are not available which might be due to the scarcity of such studies. In the present study, the potential lipase producer belonged to *V. natriegens* (EI-1.5) and *M. morganii* (EI-1.2); whereas, the potential protease producer belonged to *S. halitosis* (EI-1.2) and *M. morganii* (EI-1.1). Although Bairagi et al. (2002) detected lipolytic and proteolytic bacteria in the gut of freshwater teleosts, the authors did not identify the species.

A considerable population of amylolytic, pectinolytic and cellulolytic bacteria was also detected in the present study with the highest EI value as 2.2, 2.0 and 1.2 respectively. Earlier studies have shown that the endogenous as well as microbial amylase activities in the intestine of carnivorous fish species are much less intense than in herbivorous species (Sarbahi, 1951; Dhage, 1968; Bairagi et al., 2002). Further, the reports had suggested that the cellulase activity in the digestive tract of herbivorous fish might be as a result of gut microbial population (Saha and Ray, 1998; Ghosh et al., 2002). Bairagi et al. (2002) could not detect cellulolytic and amylolytic bacteria in the gastrointestinal tract of carnivorous catfish and murrels. Although being reported as a carnivore species higher densities of amylolytic, cellulolytic and pectinolytic bacteria in the digestive tracts is thus surprising. However, similar findings had been reported by Kar and Ghosh (2008) on the abundance of cellulolytic and amylolytic bacteria in gut of murrels, a supposed carnivorous fresh water fish species. Stickney (1975) suggested that omnivorous and carnivorous fish may pick up cellulolytic bacteria from invertebrates that harbour these bacteria. This may explain the occurrence of both cellulolytic and amylolytic bacteria in the digestive tract of a supposed carnivore fish species, the mangrove red snapper. Surprisingly, no reports are available related to the pectinase producing gut microbiota of fish. It is stated that all the components of plant cell wall including pectin are indigestible or negligibly digestible in carnivorous and omnivorous fish (Guillaume et al., 1999). Thus, the abundant colonization of amylolytic, pectinolytic and cellulolytic bacteria may also suggest that contribution of amylase, pectinase and cellulase enzymes serves as the basis for the symbiotic (mutual) relationship between the gut bacterial flora and this fish species.

Previous studies in some fish species have shown that the interactions of intestinal microflora with pathogen can hinder the successful establishment of pathogen in gut (Sugita et al., 1997; Sugita et al., 1999). Therefore, to ascertain the probable beneficial roles in disease resistance, the gut isolates were individually tested for *in-vitro* antagonistic and co-aggregation property against 7 indicator aquatic pathogens. Results showed that, none of the isolates except two had antagonistic activity. The isolates 42G and 45 G (*V. natriegens* and *V. parahaemolyticus* respectively) had weak inhibition against the growth of *V. anguillarum* (only 2 mm). Contrary to these results, many of the gut isolates demonstrated commendable co-aggregation towards pathogens, and co-aggregation percentage increased with incubation time. Reports have shown that co-aggregation between native gut bacteria and pathogen is an important defence mechanism against colonization of pathogens (Spencer and Chesson, 1994; Rickard et al., 2003). Interestingly, none of the isolates showed co-aggregation with *V. alginolyticus*, which was the most dominant species in present study. Similarly, the highest percentage of co-aggregation observed for *V. harveyi*, *A. hydrophila*, *V. anguillarum* and *V. vulnificus*; might be the possible reason for the absence of these bacteria as normal inhabitant in the gut. Altogether, the results indicated that co-aggregation of native gut flora correlates well with the gut microbial diversity and constitute a key role in preventing the colonization of pathogens. Nevertheless, the ability of co-aggregation was found to be strain-specific rather than a species-specific property analogous to the observation made by other authors (Balakrishna, 2013; Campana et al., 2017).

5. Conclusions

The study revealed a moderate rich, higher even and less diverse gut microbiome than mammals for wild healthy L. *argentimaculatus* with 11 distinct bacterial species including two proposed novel *Vibrio* Sp. There was a statistically significant difference in the gut microbial community composition between fish. Results also implied that the gut microbes had considerable role in host nutrition through supplementation of digestive enzymes and in disease resistance by preventing the colonization of pathogens. Therefore, the imbalances in the native structure of gut microbes can be detrimental to the host subsistence and aquaculture production. In sum, this is the first study providing an insight towards the gut microbial ecology and role of gut microbes towards nutritional physiology and disease resistance of this high value marine fish. The basic knowledge obtained through this study will help to determine how successfully these microbes can be manipulated and engineered for sustainable aquaculture systems. The investigation also demonstrated some potential digestive enzyme-producing isolates having probiotic applications in commercial aquaculture.

Conflicts of interest

None.

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