ISSN: 2347-5129
IJFAS 2014; 2(2): 78-88

www.fisheriesjournal.com
Received: 18-09-2014
Accepted: 06-10-2014

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Gastrointestinal microbiota in *Oreochromis mossambicus* (Peters) and *Oreochromis niloticus* (Linnaeus): scanning electron microscopy and microbiological study

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Abstract
Gastrointestinal (GI) microbiota in two species of tilapia, *Oreochromis mossambicus* (Peters) and *Oreochromis niloticus* (Linnaeus) were evaluated in the present study. Scanning electron microscopy (SEM) observation detected rod or round shaped bacteria associated with mucous and firmly attached to the intestinal mucosa. Distinct yeast colonies were also noticed either in the intestinal fold (microvilli) or with mucosa. Microbiological examination in different regions of the GI tract revealed that heterotrophic bacterial populations were the maximum in the hindgut regions in both the species. Further, proteolytic, amylolytic and cellulolytic bacterial populations were also detected abundantly within the GI tracts. Among different regions of the GI tract, protease and cellulase-producing bacterial community were the maximum in the hindgut regions; however, amylolytic population was the highest in foregut regions in both the fish species studied. Finally, on verification of the extracellular enzyme-producing capacity and considering dominance of bacteria within fish gut, three bacteria isolates, viz., OmM2, OmM3 and OnM1 were identified by 16SrDNA partial sequence analysis. Nucleotide blast in the NCBI GenBank revealed that the isolates OmM2 and OmM3 were belonged to *Bacillus subtilis* and *Staphylococcus* sp., respectively. The isolate OnM1 was considered as an uncultured bacterium clone. Further studies should be carried out to appraise the role of these autochthonous enzyme-producing microorganisms *in vivo* to explore their potential in commercial aquaculture.

Keywords: Tilapia, gut bacteria, yeasts, extracellular enzymes, Bacillus subtilis

1. Introduction
The gut microbiota of marine and freshwater fish has been widely investigated during the last two decades [1]. Presently, it is a consensus view that dense microbial population occurs in the gastrointestinal (GI) tract of fish [2, 3], and the gut microbiota can be defined as either autochthonous (indigenous) or allochthonous (transient) depending upon its ability to adhere and colonize the mucus layer in the GI tract [4, 5, 6]. It has been opined by several authors that the microbial flora might play a significant role for the benefit of their host [7, 8]. Studies based on the Indian major carp, rohu (*Labeo rohita*) suggested that the gut microbiota might be beneficial in the nutrition of the fish [9, 10, 11]. Furthermore, attempts have also been made to use beneficial gut bacilli isolated from rohu as the probiotics for the fish [12, 13]. One of the major criteria for selecting a probiotic strain is its ability to adhere and colonize the digestive tract of the host [14, 15, 16]. In this respect, microbiological examination without use of electron microscopy observation might be speculative. Therefore, efforts have been made to demonstrate adherence of microorganisms in the GI tract of fish using scanning electron microscopy (SEM) and transmission electron microscopy [3, 5, 6]. However, this topic is undervalued and merits further investigations.
Tilapia, being the second most common farm-raised food fish in the world [17] are one of the most widely introduced fish that has clearly emerged as a promising group in aquaculture. Mossambic tilapia, *Oreochromis mossambicus* (Peters, 1852) was the first tilapia species to be taken up for large scale aquaculture, followed by *Oreochromis niloticus* (Linnaeus, 1758), *Oreochromis aureus* and *Tilapia rendalli*.

At present, *O. niloticus* contributes more than 80% of tilapia aquaculture production globally and its performance in the ponds and reservoirs of India is much better than *O. mossambicus*. The specific micro-ecological system in the digestive tract of every species consists of different species of bacteria and yeasts. Several studies indicated bacteria as the major microbial colonizers in the GI tract of fish [18, 19], although, yeasts have also been reported to colonize within the GI tract of some fish [20, 21, 22, 23, 24]. In fact, it is a common limitation that research publications asserting to consider the microflora of fish focus mainly only on the bacteria, typically the aerobic heterotrophic bacterial component, excluding the eukaryotes [2]. Previously, GI tract of the *Oreochromis* spp. have been evaluated in several studies in course of enumerating extracellular enzyme-producing [25, 26, 27, 28], or pathogenic bacteria [29, 30]. However, visual evidence on gut associated microbiota in *Oreochromis* spp. is wanted. Therefore, the present study was intended to detect adherent gut microbiota in the GI tract of *O. mossambicus* and *O. niloticus* by SEM. Presence of high bacterial load in the gills and intestine of *Oreochromis* spp. might be associated with high metabolic activity of the species having increased feeding rates [31]. In this context, contribution of the GI tract microbiota through supplementation of digestive enzymes to facilitate food utilization in these species may not be ruled out. Therefore, objective of the study was to enumerate potent exoenzyme producing gut microbiota from the *Oreochromis* spp. in view of their probable application as probiotics. Hence, the present study investigated the population level of heterotrophic, proteolytic, amylolytic and cellulolytic microbiota within the GI tract of *Oreochromis* spp. and identified few potent enzyme-producing gut bacteria by 16S rDNA sequences.

2. Materials and methods

2.1. Fish examined

Mossambic tilapia, *Oreochromis mossambicus* and *Oreochromis niloticus* used for the present study were collected from local catch at and around Burdwan (23°14′N, 87°39′E), West Bengal, India. During the sampling periods, the water temperature varied between 25 °C and 28 °C. The feeding habit, average weight, total length (LT) and gut length (LG) of the fish studied are presented in Table 1. Relative gut length is reported as the ratio of the gut length to the total length (LG/LT).

2.2. Post mortem examination

Live specimens of each, *O. mossambicus* (200 ± 14.32 g) and *O. niloticus* (125 ±10.88 g) were collected, transported in an oxygenated container to the Aquaculture Laboratory at Golapbag, Burdwan where the fish were acclimated for 10 days prior to the experiment. Prior to sampling for scanning electron microscopy (SEM) and isolation of the gut microbiota, the experimental fish were starved for 24 hours to detect the autochthonous intestinal microorganisms and to eliminate most of the allochthonous microorganisms associated with digesta. After starvation, five randomly sampled fish of each species were anaesthetized and sacrificed. The GI tracts were removed aseptically and cut into three regions; foregut, midgut and hindgut. The gut segments were opened by a longitudinal incision, transferred to sterile petri dishes and thoroughly washed 3 times with sterilized chilled 0.9% saline solution in order to remove the non-adherent (allochthonous) microorganisms. To analyze microbial community, gut segments from three specimens of each species were pooled together region-wise for each replicate, and there were three replicates for the study. Pooled samples were used to avoid erroneous conclusions due to individual variations in gut microbiota, as described elsewhere [3, 32].

2.3. Scanning electron microscopy (SEM) of gut

SEM was carried out in order to detect microorganisms associated to the intestine, the autochthonous microbiota, following Ghosh *et al* [3]. The gut segments were processed as follows; incised longitudinally to expose the mucosal surface, cut into small pieces and spread out on thin thermocol sheets with the mucosal surface uppermost. Thereafter, the segments were fixed in 2.5% glutaraldehyde in suitable buffer solution (cacodylate) for 30 minutes, repeatedly washed in heparinised saline [2 g heparin (10,000-15000 i.u.) and added 20 mL of 0.67% NaCl solution] for 5 to 7 minutes to remove mucous partially. After rinsing in phosphate buffer (pH 7.2) the tissues were again fixed in glutaraldehyde for 18 hours at 4 ^{0}C. Then the tissues were dehydrated in graded ethanol as follows: 50% (30 minutes), 70% (45 min), 90% (1 hour) and absolute ethanol (1 hour). Thereafter the tissues were given three consecutive changes (30 min each) in ethanol and amyl acetate solution in the ratio of 3:1, 2:2, and 1:3 respectively. Then the tissues were kept in pure amyl acetate for overnight. Critical point drying (CPD) was done (liquid nitrogen in vacuum medium). Then the tissues were coated with gold in IB ion coater and placed under scanning electron microscope (Hitachi S530) for observation and subsequent photography.

2.4. Isolation of autochthonous gut microbiota

Intestinal homogenates were made by adding a sterile 0.9% sodium chloride (NaCl) solution (10:1; volume: weight) as described elsewhere [33]. Serial dilutions (up to 10^{-7}) were made by mixing the homogenate solution with sterilized distilled water. Diluted samples of the different regions were spread onto Tryptic soya agar (TSA; Himedia Laboratories Pvt. Ltd., Mumbai, India) plates, peptone gelatin (PG) agar plates, starch (ST) agar plates and carboxymethylcellulose (CMC) agar plates to determine the heterotrophic, proteolytic, amylolytic and cellulolytic microbial populations, respectively [3]. The plates were inoculated with 100 µl of the diluted samples. Colonies were counted after 24 hours of incubation at 30 ^{0}C under aerobic conditions. Colonies having apparently different morphological appearance and coloration were isolated. Pure cultures were obtained by repeated streaking (7 times) on TSA plates and maintained at 4 ^{0}C in a refrigerator.

2.5. Detection of gut inhabiting yeasts

Gut inhabiting yeasts were detected after Das and Ghosh [23]. Intestinal homogenates were inoculated in Chloramphenicol added (0.025%, w/v) YPD (2% Glucose, 2% peptone and 1% yeast extract) broths and incubated at 30 ^{0}C for 5 days. Subsequently, 100 µl of the broth culture was plated onto YPD plates and kept under incubation at 30 ^{0}C for 5 days under aerobic conditions. Colonies having apparently different morphological appearance and coloration were isolated. Pure cultures were obtained by repeated streaking (7 times) on YPD plates and maintained at 4 ^{0}C in a refrigerator. For conformation, the smear from YPD broth culture was prepared on cover slip and heat fixed. Then, it was again fixed in 2.5% Glutaraldehyde for 4 hours and rinsed in buffer solution. Then the samples were dehydrated in graded ethanol as follows: 50% (30 minutes), 70% (45 min), 90% (1 hour) and absolute

ethanol (1 hour). The cover slips were air dried, coated with gold in IB ion coater and placed under scanning electron microscope (Hitachi S530) for observation and subsequent photography.

2.6. Screening of isolates by qualitative and quantitative assay for extra-cellular enzyme production

Following growth on respective media plates, appearance of halo zone by flooding the plates with 1% Lugol's iodine or 15% $HgCl_2$ indicated amylase and protease activities, respectively [34]. Cellulolytic activity was determined on CMC plates. Appearance of halo after flooding the plates with congo red dye prepared in 0.7% agarose indicated utilization of cellulose as sole carbon source and thereby capacity of cellulase production [35]. There were three replicates for each experimental set.

Quantitative assay for the production of amylase, protease and cellulase by the gut isolates containing both bacteria and yeasts were determined with the broth cultures in selective media using the methods described elsewhere [36, 37, 38]. A comprehensive description for measurement of these extracellular enzymes and quantitative enzyme assay has been mentioned elsewhere [26]. The protein concentration of the crude extract was determined using the method of Lowry *et al*. [39] taking bovine serum albumin as a standard and quantitative enzyme activities were expressed as units (U).

2.7. Identification of potent gut bacteria by 16S rDNA sequencing

Out of the 57 and 53 microbial strains isolated from the GI tracts of *O. mossambicus* and *O. niloticus*, respectively 2 isolates from *O. mossambicus* and 1 isolate from *O. niloticus* with high enzyme-producing ability were analyzed by 16S rDNA sequence analysis for identification as described in Das *et al*. [40]. The template DNA was obtained by extracting genomic DNA using Gen EluteTM Bacterial genomic DNA Kit (SIGMA-Aldrich) from a fresh colony grown on nutrient agar slant. Amplification of complementary sequence of 16SrDNA was performed by polymerase chain reaction (PCR) using universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR reactions were performed using PCR mix containing 200 µM of deoxynucleotides (dNTPs), 0.2 µM of each primer, 2.5mM $MgCl_2$, 1 × PCR buffer and 0.2 U of Taq DNA polymerase (Invitrogen). The following cycle was used for PCR reaction: initial denaturation at 95 ^{0}C for 3 minutes followed by 35 cycles of 95 ^{0}C for 1 minute, annealing 55 ^{0}C for 1 minute, extension at 72 ^{0}C for 2 minutes and a final extension at 72 ^{0}C for 3 minutes [41]. PCR products were sent to the commercial house for Sanger sequencing using automated DNA sequencer (Applied Biosystem Ltd.). The PCR amplicons were separated by electrophoresis in 1.0% agarose (Sigma) gel and visualized using gel documentation system (Bio-Rad). The obtained sequences were matched with the related sequences using BLAST search program of National Center for Biotechnology Information (NCBI) and bacterial identities were established.

2.8 Statistical Analysis

Statistical analysis of the quantitative enzyme activity data was performed by the one-way analysis of variance (ANOVA), followed by Tukey's test according to Zar [42] using SPSS Version 10 software [43].

3. Results

3.1. The test fish

Data pertaining average weight, total length, relative gut length and feeding habit of the fish examined are presented in Table 1. The value of the relative gut length (RGL) was less in the Mossambic tilapia, *O. mossambicus* than the Nile tilapia, *O. niloticus*. However, the values were >1 in both cases indicating their herbivorous, or rather omnivorous feeding aptitude in both the species.

Table 1: Average weight, total length, relative gut length and feeding habit of the fish examined. Results are mean ± S.E. of the three observations.

Fish species	Body weight(g)	Total length (cm.) (LT)	Weight of the gut	Gut length (cm) (LG)	Relative gut length (LG/LT)	Feeding habit
Oreochromis mossambicus	200±14.32	22.86±2.43	4.362±0.61	100.5±9.84	4.39±0.73	Plankton, weeds, Omnivore
Oreochromis niloticus	125±10.88	19.91±2.21	3.125±0.55	90.1±8.34	4.53±0.52	Plankton, weeds, Omnivore

3.2. Association of microbiota in fish intestinal walls SEM study

Scanning electron microscopic (SEM) observation detected bacteria firmly attached to the mucosa (Figure 1A) in the intestine. The microbial colonies attached with the gut epithelium were associated with mucous (Figure 1F). SEM study revealed that the bacteria present in the GI tract were either rod shaped (bacilli) (Figures 1A, 1F) or round shaped (cocci) (Figure 1C). Along with colonization capacity the dividing stages of bacteria indicated their ability to propagate within the GI tract (Figure 1B). In addition, distinct yeast colonies were also noticed either in the intestinal fold (microvilli) (Figure 1E) or with mucosa (Figures 2A, B) in *O. mossambicus* and *O. niloticus*, respectively.

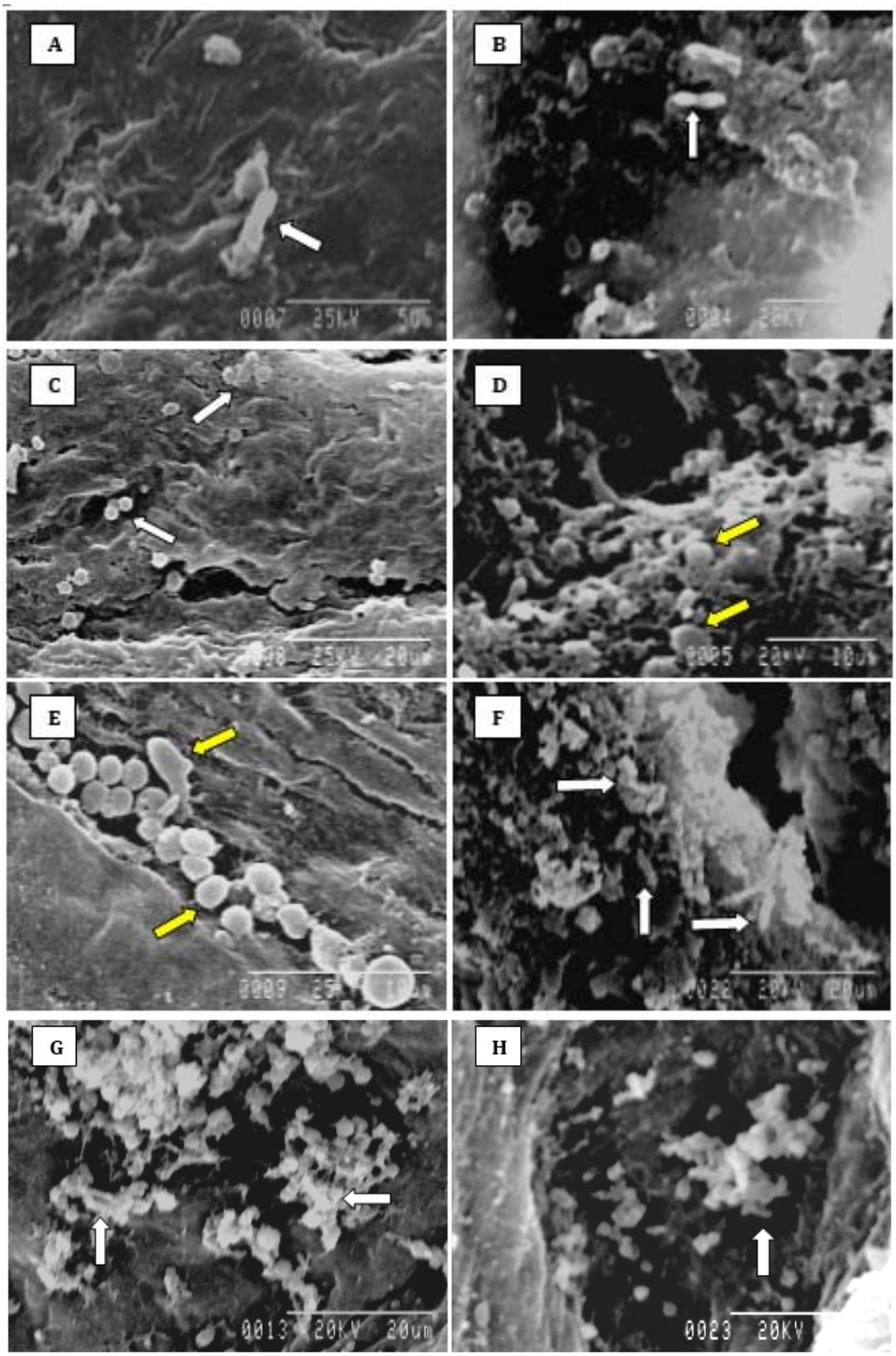

Fig 1: Microbiota associated with the gastro-intestinal tract of Mossambic tilapia, *Oreochromis mossambica* showing foregut (A, B), midgut (C, D, E) and hindgut (F, G, H) regions. White and yellow arrows indicate presence of bacteria and yeasts respectively.

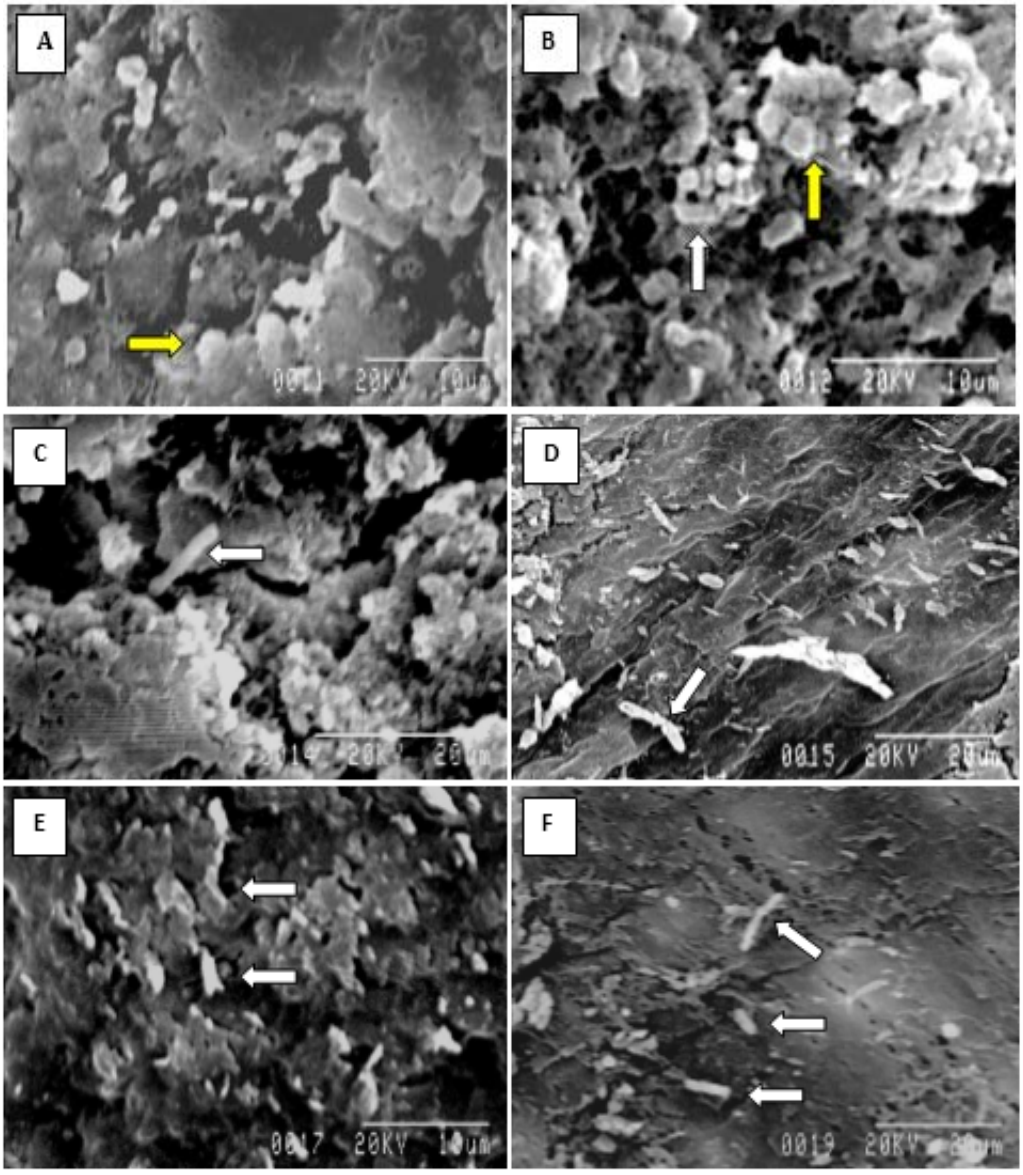

Fig 2: Microbiota associated with the gastro-intestinal tract of Nile tilapia, *Oreochromis niloticus* showing foregut (A, B), midgut (C, D) and hindgut (E, F) regions. White and yellow arrows indicate presence of bacteria and yeasts respectively.

3.3. Bacteria in the gastrointestinal tract: Microbiological study

Microbiological examination in different regions of the GI tract revealed presence of culturable aerobic or facultative anaerobic heterotrophic bacterial population on TSA plates irrespective of the fish species studied. Heterotrophic bacterial counts were highest in the hindgut regions in both the species. Further, proteolytic, amylolytic and cellulolytic bacterial populations were also detected abundantly within the GI tracts. Analysis of the enzyme-producing bacterial community in the different regions of the GI tract in *Oreochromis* spp. indicated higher population level in the hindgut and midgut regions than that present in the foregut regions (Table 2), except for the amylolytic bacterial population. Among different regions of the GI tract, amylolytic population dominated in the foregut and midgut regions, however, proteolytic population dominated in the hindgut regions in both the fish species studied (Table 2).

Table 2: Heterotrophic bacterial count in different regions of the gastrointestinal tract in two species of Tilapia.

Fish species		Bacterial populations (CFU g^{-1} digestive tract)			
		TSA plate ($\times 10^4$)	Proteolytic ($\times 10^3$)	Amylolytic ($\times 10^3$)	Cellulolytic ($\times 10^3$)
Oreochromis niloticus	Foregut	3.67 ± 0.67^a	1.33 ± 0.33^a	7.34 ± 0.88^b	2.33 ± 0.88^a
	Midgut	8.0 ± 0.58^b	$2..33\pm0.33^a$	2.67 ± 0.89^a	2.67 ± 0.33^a
	Hindgut	13.33 ± 0.33^c	7.33 ± 0.88^b	1.33 ± 0.33^a	5.0 ± 1.15^b
Oreochromis mossambicus	Foregut	4.31 ± 0.38^a	1.34 ± 0.08^a	6.91 ± 0.22^c	1.57 ± 0.84^a
	Midgut	6.66 ± 0.37^b	2.84 ± 0.67^a	4.34 ± 0.37^b	2.33 ± 0.33^a
	Hindgut	9.21 ± 0.51^c	6.06 ± 0.23^b	1.11 ± 0.38^a	4.85 ± 0.53^b

Values with same superscripts in the same row are not significantly different ($P<0.05$).

3.4. Screening of exoenzyme producing bacteria from the GI tract of Mozambique tilapia

Out of 57 total isolates, 15 randomly selected (more than 25%) intestinal isolates (5 each from 3 regions) from *O. mossambicus* were qualitatively assayed for extracellular amylase, cellulase, and protease production. The intensity of transparent zone (halo) produced by the isolates are presented in the Table 3. Among the tested isolates, 9 isolates (OmF1, OmF2 and OmF3 from foregut, OmM1, OmM2 and OmM3 from midgut, OmH1, OmH2 and OmH3 from hind gut) were selected on the basis of qualitative assay for the quantitative enzymatic assay.

The results of quantitative estimation of extracellular amylase, protease and cellulase production by the strains isolated from different parts of the GI tract in the Mozambique tilapia are depicted in Table 4. Amylase activity was found to be highest in OmH2, although the activity did not differ significantly ($P < 0.05$) from the strains OmM3 and OmM2. Protease activity was significantly ($P < 0.05$) higher in OmM3, followed by the strains Om M2 and Om H2. The strain Om M3 also exhibited the highest cellulase activity among the tested isolates.

3.5. Screening of exoenzyme producing bacteria from the GI tract of Nile Tilapia

Out of 53 total isolates, 15 randomly selected (more than 25%) intestinal isolates (5 each from 3 regions) from *O. nilotica* were qualitatively assayed for extracellular amylase, cellulase, and protease production. The intensity of transparent zone (halo) produced by the isolates are presented in the Table 5. In this case also, 9 isolates (OnF1, OnF2 and OnF3 from foregut, OnM1, OnM2 and OnM3 from midgut, OnH1, OnH2 and OnH3 form hindgut) were selected among the tested isolates on the basis of proficiency in the production of extracellular protease, amylase and cellulase as evident from the qualitative assay. These primarily selected isolates were further analysed by quantitative assay.

Table 3: Bacterial strains isolated from the different parts of gastro-intestinal tract of *Oreochromis mossambicus* and qualitative extracellular enzyme activity.

Bacterial strains	Enzyme activity *(appearance of halo, mm)*		
	Amylase	Protease	Cellulase
OmF1	11.67±0.33^{d}	15.33±0.34^{d}	10.33±0.34^{d}
OmF2	10.33±0.88^{c}	14.68±1.20^{d}	09.00±0.58^{c}
OmF3	08.33±0.68^{b}	17.00±0.58^{e}	12.58±0.67^{f}
OmF4	07.33±0.34^{a}	12.67±0.88^{c}	08.33±0.33^{c}
OmF5	08.67±0.33^{b}	10.33±0.33^{b}	06.00±1.15^{a}
OmM1	13.00±1.15^{e}	14.68±1.76^{d}	09.67±0.68^{c}
OmM2	14.67±0.88^{f}	12.00±0.58^{c}	13.33±0.88^{g}
OmM3	15.00±1.01^{g}	21.33±0.67^{f}	14.35±1.45^{h}
OmM4	10.58±0.66^{c}	10.67±0.33^{b}	07.00±0.58^{b}
OmM5	08.33±0.33^{b}	08.65±0.88^{a}	07.00±0.37^{b}
OmH1	13.00±0.58^{e}	22.00±1.53^{g}	11.11±0.89^{e}
OmH2	16.67±0.65^{h}	21.68±0.66^{f}	09.00±0.58^{c}
OmH3	11.68±0.67^{d}	22.67±0.66^{g}	10.67±0.68^{d}
OmH4	09.33±0.67^{b}	12.62±0.33^{c}	11.33±0.89^{e}
OmH5	07.67±0.66^{a}	15.57±1.23^{e}	06.67±0.34^{a}

Values with same superscripts in the same row are not significantly different (P<0.05).

Table 4: Profile of enzyme activity in the selected strains of *Oreochromis mossambicus*.

Bacterial strains	Enzyme activity		
	Amylase (U)[a]	Protease (U)[b]	Cellulase (U)[c]
OmF1	0.07±0.08^{b}	8.44±0.07^{b}	1.16±0.09^{b}
OmF2	0.07±0.09^{b}	9.22±0.03^{c}	1.13±0.21^{b}
OmF3	0.05±0.01^{a}	9.33±0.04^{c}	3.96±0.54^{c}
OmM1	0.08±0.09^{b}	7.8±0.02^{a}	0.43±0.31^{a}
OmM2	0.11±0.01^{c}	12.05±0.02^{f}	6.72±1.1^{d}
OmM3	0.13±0.01^{c}	12.58±0.06^{g}	8.20±1.18^{e}
OmH1	0.08±0.09^{b}	11.35±0.04^{e}	1.79±0.03^{b}
OmH2	0.13±0.09^{c}	11.99±0.06^{f}	0.36±0.08^{a}
OmH3	0.07±0.08^{b}	10.31±0.03^{d}	1.40±0.07^{b}

Values with same superscripts in the same row are not significantly different (P<0.05).

[a]mg maltose liberated per mg. of protein in culture filtrate/min.

[b]µg tyrosine liberated per mg. of protein in culture filtrate/min

[c]mg glucose liberated per mg. of protein in culture filtrate/min

Quantitative assay of the extracellular enzyme production by the gut bacterial isolates from the Nile tilapia revealed that amylase activity was highest in the strain OnM3 isolated from midgut, which was followed by the strain OnH2. Protease activity was significantly higher in OnH1 isolated from hindgut of *O. niloticus*. However, OnM1 exhibited best cellulase production capacity (Table 6).

Table 5: Bacterial strains isolated from the different part of gastro-intestinal tract of *Oreochromis niloticus* and qualitative extra cellular enzyme activity.

Bacterial strains	Enzyme activity *(appearance of halo, mm)*		
	Amylase	Protease	Cellulase
OnF1	11.00±1.53^{d}	26.00±0.58^{g}	12.33±0.33^{e}
OnF2	15.67±1.20^{g}	17.00±1.53^{d}	10.34±1.40^{c}
OnF3	07.67±0.33^{a}	20.67±0.88^{e}	12.33±0.88^{e}
OnF4	10.00±1.00^{c}	15.33±0.33^{c}	09.00±0.58^{c}
OnF5	08.00±1.53^{b}	10.68±0.34^{a}	11.67±0.89^{d}
OnM1	18.00±1.15^{h}	29.67±1.20^{h}	13.33±0.85^{f}
OnM2	08.67±0.88^{b}	18.33±0.33^{d}	10.31.±0.67^{c}
OnM3	08.33±1.20^{b}	19.67±1.20^{e}	07.33±0.88^{a}
OnM4	07.66±0.33^{a}	12.68±0.88^{b}	08.35±0.89^{b}
OnM5	09.66±0.34^{c}	11.00±0.58^{a}	12.31±0.87^{e}
OnH1	11.00±0.58^{d}	30.66±1.76^{h}	7.63±0.43^{a}
OnH2	13.33±0.33^{f}	24.33±0.33^{f}	8.69±0.24^{b}
OnH3	11.67±0.33^{e}	20.67±0.33^{e}	10.32±0.36^{c}
OnH4	10.33±0.88^{c}	12.58±0.34^{b}	08.34±0.28^{b}
OnH5	12.67±0.89^{e}	15.69±0.33^{c}	06.66±0.33^{a}

Values with same superscripts in the same row are not significantly different (P<0.05).

Table 6: Profile of exoenzyme activity by the selected strains isolated from *Oreochromis niloticus.*

Bacterial strains	Enzyme activity		
	Amylase (U)[a]	Protease (U)[b]	Cellulase (U)[c]
OnF1	0.07±0.01[a]	14.86±0.13[e]	6.33±1.11[f]
OnF2	0.11±0.01[c]	11.58±0.02[a]	1.32±0.28[c]
OnF3	0.05±0.01[a]	13.40±0.04[b]	6.29±1.37[f]
OnM1	0.11±0.06[c]	15.63±0.44[e]	7.10±1.22[g]
OnM2	0.06±0.01[a]	11.83±0.06[a]	4.42±0.94[e]
OnM3	0.28±0.21[e]	13.10±0.06[b]	0.81±0.05[b]
OnH1	0.08±0.02[b]	18.25±0.07[f]	0.10±0.03[a]
OnH2	0.13±0.01[d]	14.41±0.09[d]	1.59±0.07[c]
OnH3	0.06±0.02[a]	13.95±0.04[d]	2.34±0.05[d]

Values with same superscripts in the same row are not significantly different (P<0.05).
[a]mg maltose liberated per mg. of protein in culture filtrate/min.
[b]µg tyrosine liberated per mg. of protein in culture filtrate/min.
[c]mg glucose liberated per mg. of protein in culture filtrate/min

Table 7: Profile of exoenzyme activity by the gut inhabiting yeasts isolated from *Oreochromis* spp.

Yeasts strains*	Enzyme activity		
	Amylase (U)[a]	Protease (U)[b]	Cellulase (U)[c]
OmY1	0.112±0.04	15.22±2.27	5.057±0.88
OmY2	0.115±0.06	14.67±1.82	4.371±0.68
OnY1	0.220±0.11	15.31±1.27	3.854±0.55
OnY2	0.117±0.05	18.07±1.93	7.344±0.27

Values with same superscripts in the same row are not significantly different (P<0.05).
*OmY1, OmY2: Isolated from *O. mossambica*; OnY1, OnY2: Isolated from *O. nilotica*
[a]mg maltose liberated per mg. of protein in culture filtrate/min.
[b]µg tyrosine liberated per mg. of protein in culture filtrate/min.
[c]mg glucose liberated per mg. of protein in culture filtrate/min.

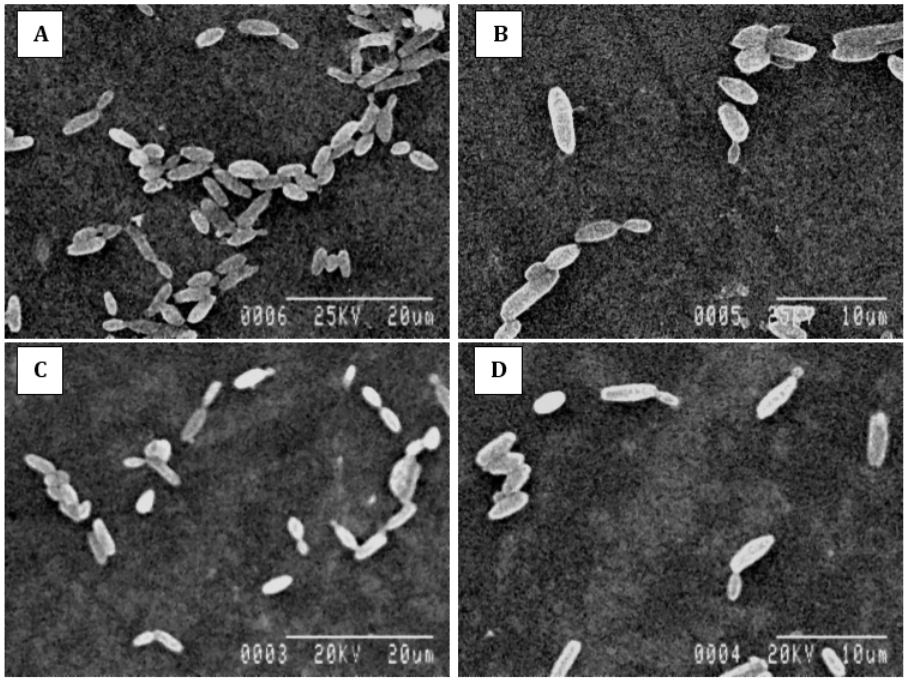

Fig 3: Scanning electron micrograph of few yeast isolates from GI tract of *O. mossambica* (A, B) and *O. nilotica* (C, D).

3.6. Yeasts isolated from gastrointestinal tract: Microbiological and SEM study

Microbiological study of the GI tract extract revealed presence of culturable heterotrophic yeasts on YPD culture media in both the fish species studied. SEM study of the pure culture from the isolates on YPD culture media confirmed the presence of yeasts within the GI tract (Figure 3). Further, quantitative assay of exo-enzyme activity of few selected yeast isolates revealed that gut inhabiting yeasts were efficient in production of protease, amylase and cellulase (Table 7). The isolate OnY2 isolated from Nile tilapia exhibited best protease and cellulase producing ability among the tested yeast isolates; however, amylase producing ability was highest in OnY1 isolated from the same species.

3.7. Identification by 16SrRNA partial sequence analysis

Finally, on verification of the extracellular enzyme-producing capacity and considering dominance of bacteria within fish gut three bacteria isolates, viz., OmM2, OmM3 and OnM1 were selected for 16SrDNA partial sequence analysis to interpret their identity for probable future use. Nucleotide homology analysis of the 16SrDNA partial sequences by nucleotide blast in the NCBI GenBank revealed that the isolate OmM2 belonged to *Bacillus subtilis* cluster. The isolate OmM2 showed 100% similarity with *Bacillus subtilis* strain PRL2 16SrDNA gene (Accession no. JN544151). The isolate OmM3 showed 100% similarity with the partial sequence of *Staphylococcus* sp. 16SrRNA gene (Accession no. JF799910). However, PCR amplification of the 16SrDNA gene fragment

yielded a short sequence (199 bases) for the isolate OnM1 and therefore, could not lead to reach a valid conclusion. Nucleotide homology analysis of the sequence showed 98% similarity with the partial 16SrDNA gene sequence of an uncultured bacterium clone (Accession no. JF178760) and thereby the isolate OnM1 was considered as an unknown bacterium. The closest relatives (with GenBank Accession No.) of the complementary 16SrDNA sequences from the selected isolates and are presented in Table 8.

Table 8: Identification of *Oreochromis* spp. gut bacterial isolates with partial sequence of 16SrDNA sequences referenced to accession numbers in NCBI GenBank.

Isolates	Closest relative (GenBank accession No.)	Similarity (%)
OmM2	*Bacillus subtilis* (JN544151)	100
OmM3	*Staphylococcus* sp. (JF799910)	100
OnM1	Uncultured bacterium clone (JF178760)	98

4. Discussion

It has only been during the last decade that there has been an improved understanding of the importance of commensal intestinal microbiota in the fish intestine. Fish are continuously exposed to the microorganisms present in the aquatic environment. Being rich in nutrient the digestive tract of fish confers a favourable growth environment for the microorganisms [44]. Relation between host and microorganisms for fish has so far been possible to demonstrate in only few cases [45]. These are mostly herbivore fish colonized by bacteria or other microorganisms facilitating the digestion of polysaccharides [46]. The function that colonizing microorganisms play within the fish GI tract is still unclear. Attention has been paid to identify autochthonous fish gut microbiota in order to gain information on their activities [47]. Electron microscopy examinations of the gut have been suggested as an important tool for investigating the microbial ecology of fish and determining the presence of autochthonous or allochthonous microbiota [5, 48, 49]. Numerous reports have been published during the last three decades describing bacteria attached to mucosa and microvilli of the intestine [5, 6]. Previously, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) evaluations of fish gut have demonstrated rod-shaped bacteria associated between the microvilli in common wolfish, *Anarhichas lupus* [50] and rohu, *Labeo rohita* [3]; and coccoid and rod-shaped bacteria associated with the surface of gut enterocytes of Arctic charr (*Salvelinus alpinus* L.) [48]. The present study is the first one demonstrating adherent bacteria and yeasts on the gut enterocyte surfaces and microvilli of *Oreochromis* spp. A micrograph demonstrating the dividing bacterium may also suggest that these intestinal bacteria also propagate within the intestine (Figure 1B). Microbial population of intestinal bacteria in *Oreochromis* spp. seem to be represented by the rod shaped, bacilli or round shaped, cocci, and this finding was confirmed by both SEM study and identification through 16SrDNA sequence analysis. Previous reports have also documented the presence of bacilli within the digestive tract of Mozambique tilapia (*O. mossambica*) [27, 28]. In the present study, 16SrDNA sequence analysis revealed that one of the two potent exo-enzyme producer isolates from *O. mossambica* belonged to bacilli (*Bacillus subtilis*), however, another one was a cocci (*Staphylococcus* sp.).

In the present study, population levels of heterotrophic, proteolytic, amylolytic and cellulolytic bacteria were evaluated in three different regions of the GI tract of *O. mossambica* and *O. nilotica*. As the fish were starved for 24 hours and their digestive tracts thoroughly washed with sterilized chilled 0.9% saline prior isolation of bacteria, it may be suggested that these bacteria belong to the autochthonus microbiota as indicated previously by Ghosh *et al*. [3]. This hypothesis was confirmed by SEM investigations. Several investigators have reported the presence of enzyme producing bacteria in the digestive tract of fresh water teleosts [9, 10, 11, 26, 44, 51, 52] including *Oreochromis* spp. [25, 26, 27, 28, 44]. The present study reports potent enzyme producing bacteria in the digestive tract of *Oreochromis* spp., of which two isolates (OmM2 and OmM3) were most closely related to *B. subtilis* and *Staphylococcus* sp., respectively. Moreover, in the present study the bacterial population level in the different regions of the GI tract of *Oreochromis* spp. showed higher numbers of heterotrophic microbial populations in midgut and hindgut regions compared to the foregut region, except for the amylolytic bacterial population. Previously, proteolytic, cellulolytic and amylolytic bacteria in the gut of *Oreochromis* spp. have been documented by Bairagi *et al*. [26] and Mandal *et al*. [44]. Bairagi *et al.* [26] observed higher densities of amylolytic strains in herbivorous grass carp, *Ctenopharyngodon idella*, detritivorous common carp, *Cyprinus carpio* and omnivorous tilapia, *O. mossambicus*, however, they did not address distribution of exoenzyme producing microbial populations at different regions of the GI tract. Presence of higher proteolytic and cellulolytic population level in the hindgut and midgut regions detected in the present study are in agreement with the results of Mondal *et al*. [44] and Ghosh *et al*. [3] who reported higher heterotrophic population levels in the hindgut than that detected in the foregut of fish. In the present investigation, amylolytic population dominated in the foregut and midgut regions, however, proteolytic population dominated in the hindgut regions in both the fish species studied. Colonization of amylolytic and cellulolytic bacteria at such high rate in the proximal part of the GI tract may be due to the fact that *Oreochromis* spp., though omnivorous, mostly prefer plant materials as their food. It was further revealed that the cellulolytic bacterial population was highest in the hindgut region compared to the foregut and midgut regions. This may indicate the possibility of the fermentative degradation of the plant material in this part of the GI tract in assistance with the highly colonized cellulolytic bacteria. The potential beneficial effects of those bacteria isolated in the present study are worth to investigate in further investigations.

Microbial isolates detected in the present study represented their ability for extracellular amylase, protease and cellulase production. Intensity of protease and cellulase activities were comparatively better than amylase production in the strains isolated from *Oreochromis* spp. Sugita *et al*. [25] determined the amylase-producing ability of the intestinal microflora in cultured tilapia (*O. niloticus*) along with some other fishes. Bairagi *et al.* [26] quantified the proteolytic activity in the bacterial strains isolated from nine freshwater teleosts and recorded the highest activity in the bacterial strain TP3A isolated from the gut of omnivorous tilapia, *O. mossambicus*. They however, did not attempt for identification of the described potential strain. Cellulase production by gut bacteria from tilapia has also been reported [27]. Later, Ray *et al*. [28] investigated the optimum environmental and nutritional conditions required to enhance cellulase production by the bacterial strain *B. circulans* TP3, originally isolated from the

gut of Mozambique tilapia. While evaluating enzyme producing bacteria in different freshwater teleosts, Bairagi *et al*. [26] and Mandal *et al*. [44] also detected cellulolytic bacteria in the GI tract of *O. mossambica* and *O. nilotica* respectively. The authors of these studies also addressed cellulase producing ability of the gut isolates. The result of the present investigation is in harmony with these previous reports. Quantitative assay of extracellular enzyme production showed highest value for all the studied enzymes in OmM3 among the strains isolated from *O. mossambicus*, while in *O. niloticus* the strains OnM1, OnM3 and OnH1 exhibited best cellulase, amylase and protease production, respectively. On the basis of exoenzyme producing abilities, finally three isolates (OmM2, OmM3 and OnM1) were selected for 16SrDNA sequence analysis to disclose their identity in view of likely future use of these microorganisms to explore their enzyme producing ability.

In the present study, one autochthonous isolate (OmM2) from the midgut of *O. mossambicus* has shown similarity to *Bacillus subtilis* strain PRL2 (Accession no. JN544151) described by Younas and Faisal (2011, unpublished data, National Center for Biotechnology Information, NCBI). *B. subtilis* has previously been reported in the intestinal tract of flathead grey mullet (*Mugil caphalus* L.) [53], Atlantic salmon (*Salmo salar* L.) [54], bata (*Labeo bata*) [52] and rohu (*L. rohita*) [3]. It has been indicated that gut microbiota in freshwater teleosts were fairly dominated by *Bacillus* spp. [1]. *Bacillus* spp. hold added interest in probiotics as they can be kept in the spore form and therefore stored for long time [55]. One isolate (OmM3) from the midgut of *O. mossambicus* showed high similarity to *Staphylococcus* sp. strain CIFRI H-TSB-6-HA (Accession no. JF799910) described by Behera *et al*. (2011, unpublished data, NCBI). In present study, isolation of *Streptococcus* sp., which is a facultative pathogen, may be of importance. Although, the bacterial presence in tilapia intestine probably had little effect on fish disease prevalence as opined by Al-Harbi and Uddin [31]. Ringø *et al.* [32] suggested that *Staphylococcus* along with other isolated microorganisms (*Agrobacterium*, *Pseudomonas*, *Brevibacterium*, *Microbacterium*) might contribute to nutritional processes in Arctic charr. While another isolate (OnM1) that yielded a short sequence during PCR amplification of the 16SrRNA fragment was most closely related to an uncultured bacterium clone (Accession no. JF178760) (Kong *et al*., 2010; unpublished data, NCBI) and considered as an unknown bacterium. It is worth to notice that isolates OmM2 and OmM3 isolated from midgut regions displayed high enzymatic activity.

Unlike the bacteria, evidence on yeasts as normal microbial symbiont in fish gut is scanty [20, 21, 56]. Yeasts are ubiquitous microorganisms that can grow in various environments where organic substrates are available [21]. Therefore, presence of yeasts within fish gut may not be surprising. Regarding colonization of yeast in fish gut most studies in fresh water fish were conducted in rainbow trout. Andlid *et al*. [56] demonstrated the ability of yeast to colonize the intestine of rainbow trout and turbot. The affinity of yeast for fish intestine was further established by the same authors [20, 57]. However, efforts to characterize yeasts as fish gut microbiota with the view to evaluate their possible effects on fish health and metabolism are still in the infancy. This investigation confirms the existence of yeasts within GI tracts of *Oreochromis* spp. through visual evidence by SEM study. Microbiological examination also detected presence of yeasts and their efficiency in extracellular amylase, protease and cellulase production has been established. The present study is the first one reporting yeasts in the GI tract of both, *O. mossambica* and *O. nilotica*. However, identity of the isolated gut inhabiting yeasts has not been addressed in the present study. An appraisal of their role along with their identity should therefore be given priority in future studies.

5. Conclusions

The results of the present study provide evidence that autochthonous bacteria and yeasts exist in the GI tract of *O. mossambicus* and *O. niloticus*, and further verify the existence of the enzyme-producing microbiota within the micro-environment of gut. The present study is the first one using electron microscopy to demonstrate gut microbiota of *Oreochromis* spp. and it may be suggested that these autochthonous microorganisms might have a beneficial potential that has to be evaluated in future investigations. Presence of high bacterial load in the gills and intestine of *Oreochromis* spp. have been assumed to have correlation with high metabolic activity of the species associated with increased feeding rates [58]. Therefore, probable benefit of using these autochthonous microorganisms to aggravate metabolic efficiency in the studied species or other freshwater teleosts may not be ruled out. Furthermore, enzymes produced by the fish gut-microbiota might have a significant role in digestion, especially for substrates such as cellulose, which few animals can digest, and also for other substrates [59]. The use of such beneficial bacteria or yeasts as probiotics has a long tradition in animal husbandry [60]. These beneficial microorganisms could be introduced in commercial aquaculture by incorporating them into formulated fish diets, or in the form of microbial biofilm to achieve colonization in the fish GI tract at a higher degree [12, 61, 62, 63]. Although, only enzyme producing ability may lead to designate a gut microorganism as probiotics, the antimicrobial potential of the beneficial gut bacteria isolated from *Oreochromis* spp. might be given high priority in future studies to explore complete potential of these autochthonous microorganisms in aquaculture.

6. Acknowledgements

The authors are grateful to the Head, Department of Zoology, The University of Burdwan, West Bengal, India; The Department of Science and Technology (FIST programme), New Delhi, India and The University Grants Commission (UGC-SAP-DRS programme), New Delhi, India for providing research facilities.

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