Journal of Applied Microbiology, 2023, 134, 1–10
https://doi.org/10.1093/jambio/lxad208
Advance access publication date: 12 September 2023
Research Article

JOURNAL OF APPLIED MICROBIOLOGY
OXFORD UNIVERSITY PRESS

Diversity analysis of hilsa (*Tenualosa ilisha*) gut microbiota using culture-dependent and culture-independent approaches

Megha Chakraborty, Debarun Acharya, Tapan K. Dutta*

Department of Microbiology, Bose Institute, Kolkata 700091, West Bengal, India

*Corresponding author. Department of Microbiology, Bose Institute, EN-80, Sector V, Salt Lake, Kolkata 700091, West Bengal, India. E-mail: tapan@jcbose.ac.in

Abstract

Aims: The bacterial communities associated with the gastrointestinal (GI) tract are primarily involved in digestion, physiology, and the immune response against pathogenic bacteria for the overall development and health of the host. Hilsa shad (*Tenualosa ilisha*), a tropical anadromous fish, found predominantly in Bangladesh and India, has so far been poorly investigated for its gut bacterial communities. In this study, both culture-based and metagenomic approaches were used to detect intestinal isolates of hilsa, captured from both freshwater and seawater to investigate the community structure of intestinal microbiota.

Methods and results: Culture-dependent approach allowed to isolate a total of 23 distinct bacterial species comprising 16 Gram-negative, and 7 Gram-positive isolates, where Proteobacteria and Firmicutes were identified as the two most dominant phyla. While metagenomic approach explored a wide range of important GI bacteria, primarily dominated by Proteobacteria, Firmicutes, and Bacteroidetes, with Proteobacteria and Firmicutes, being the most abundant in freshwater and seawater samples, respectively.

Conclusions: A combination of these approaches provided the differential GI-associated bacterial diversity in freshwater and seawater hilsa with the prediction of overall functional potential.

Impact Statement

The study explored the diversity of gut microbiota in hilsa, one of the most preferred nutritious dietary fish, captured from freshwater and seawater habitats, which may encourage to comprehend the composition of the gut microbiome in relation to the migratory behavior and polyunsaturated fatty acid profile of anadromous fish in general.

Keywords: gut microbiome, next-generation sequencing, anadromous fish, hilsa, community analysis, 16S rRNA

Introduction

The native microflora of fish has been an area of research interest for a long time, mostly aiming to understand the microbial ecology of fish gastrointestinal (GI) tract that are beneficial to the host (Talwar et al. 2018, Yukgehnaish et al. 2020). GI tract microbiota and/or their synthesized enzymes play pivotal roles in digestion processes, supplying vitamins to enhance nutrition, preventing colonization of pathogens by competing for nutrients and adhesion sites, producing antimicrobial substances, and modulating the host immune system (Ray et al. 2012, Tarnecki et al. 2017). In comparison to other vertebrates, attempts are being made in recent times to gather knowledge on intestinal microbial communities of fish and the factors affecting them, particularly focusing on ecologically and economically important fish species. Nevertheless, colony morphology-based isolation is the traditional method of choice to identify and classify culturable bacterial species. Lately, it has been realized that large subpopulations of microorganisms remain uncultivable on traditional agar media (Schut et al. 1997), and to address the limitations, metagenome analysis evolved as the best alternative technique to accurately classify the associated microbial species in a particular habitat. Moreover, with the development of various next-generation sequencing (NGS) platforms and the advent of metagenomic studies based on 16S ribosomal DNA, the culture-independent approach has reached a greater height to catalogue the diversity of fish gut microbiome (Luna et al. 2022).

The present study focuses on hilsa shad (*Tenualosa ilisha*), which is an anadromous fish like salmon (Jonsson and Jonsson 2009) and American shad (Quinn and Adams 1996). It spends most of its life span in the sea and migrates to freshwater rivers for spawning, after which it again returns to the sea (Rahman et al. 2015). Specifically, hilsa migrates from the Bay of Bengal and the Persian Gulf region to various rivers and their tributaries, especially those of Bangladesh, India and Myanmar, besides Pakistan, Iran, Iraq, and Kuwait (Rahman 2006). Hilsa is a microphagus and it filter-feeds mostly on copepods, detritus, algae, mysis, molluscan larvae, diatoms, rotifers etc. (Karna et al. 2014).

Hilsa is popular in India, Bangladesh, and South Asia not only for its economic aspects but also for its delicious taste and nutritious values owing to the presence of a high level of omega-3 polyunsaturated fatty acid, apart from proteins, minerals, and fats, whose composition varies from 22% to 36% of the muscle weight (Ayyappan et al. 2006). Because of its high nutritional value, taste, and culinary properties, hilsa marks its unique position primarily in the popular Bengali cuisine,

Received: March 17, 2023. **Revised:** August 25, 2023. **Accepted:** September 8, 2023

being designated as the 'king of fish'. Directly or indirectly, the livelihood of a large number of people in this region depends on hilsa fishery (Sunny et al. 2018). Alone in Bangladesh, ∼0.5 million people directly depend on hilsa fishing for their livelihood while another 2 million people indirectly depend on hilsa fishery through the activities like transportation, marketing, processing etc. (Islam et al. 2017).

This study was aimed at exploring an underpinning account of the hilsa gut microbiota through culture-dependent and culture-independent approaches to understand the diversity of its gut microflora and functional attributes based on the hypothesis that anadromous fish like hilsa should have a core gut microbiota composition, with the subtle difference between freshwater and seawater groups.

Materials and methods

Sampling of hilsa

Three freshwater hilsa (*T. ilisha*) fish were collected from Ahiritola Ghat (22.5960°N, 88.3530°E; 28°C, pH = 7.29) of Hoogly River (a tributary of Ganges) in Kolkata, India and another three fish were collected from the seawater near Udaypur beach (24.5854°N, 73.7125°E; 26°C, pH = 7.8), Balasore, Bay of Bengal, India (Table S1). All these fish were obtained from local fishermen, and caught during the early morning hours. Immediately after collection, hilsa fish were packed in ice and taken to the laboratory for physical measurements and dissection. The fish were 28–36 cm in fork length and 700–1000 g in body weight. Each group of freshwater and seawater hilsa samples contained two females and one male fish.

Isolation of gut microbiota

At the outset, the number of incidental microorganisms was reduced by rubbing fish skin with 70% ethanol inside a biosafety cabinet (BSC) of biosafety level-2 (BSL-2) prior to dissection. Then, the fish were dissected ventrally with sterile scissors maintaining an aseptic condition. After dissection, 2–5 ml of the intestinal content of each fish was collected and homogenized with 0.89% physiological saline solution under germ-free conditions and refrigerated at 4°C. The homogenates of the intestinal content were then diluted with sterile 0.89% saline, and aliquots (100 µl) of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions were plated separately in triplicate onto freshly prepared Tryptone soya-agar and Zobell marine-agar plates containing 1,8% (w/v) of agar powder (HiMedia Laboratories Pvt. Ltd, India). The isolation plates were incubated in the dark for 1 week at 28°C. Isolates were obtained in pure culture after three successive transfers and loop-streaking on fresh agar media. The whole procedure was executed inside a BSL-2 cabinet to ensure germ-free conditions. Glycerol stocks of pure cultures ($n = 23$) were frozen and kept at −80°C for long-term storage.

Diversity analysis of culturable isolates

Genomic DNA of 23 cultivable isolates was extracted using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific Inc, USA). The extraction was done in triplicate for each sample to increase the amount of DNA for subsequent analysis. The PCR amplification of the partial 16S rRNA gene was performed from the purified genomic DNA as a template using bacterial universal primers 27F and 1492R (Hou et al. 2018). The amplified product was sequenced according to the manufacturer's specifications for Taq DNA polymerase-initiated cycle sequencing reactions using fluorescently labeled dideoxynucleotide terminators with an ABI PRISM 377 automated sequencer (Perkin-Elmer Applied Biosystems). Levels of 16S rRNA gene sequence similarity were determined using BLAST version 2.2.12 of the National Center for Biotechnology Information (Altschul et al. 1990). A phylogenetic tree was constructed using ClustalX version 2.1 (Larkin et al. 2007) and Tree Explorer version 2.12 (Ortega-Villaizan et al. 1999) based on the 16S rRNA gene sequences of the culturable isolates and that of representative type strain sequences.

Metagenomic DNA extraction and bacterial diversity analysis

In order to ensure the detection of noncultivable bacteria, metagenomic DNA was extracted directly from the intestinal samples of the collected hilsa using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany). The quality of DNA obtained from the gut samples was ascertained by measuring absorbance at 260 and 280 nm. DNA concentration was estimated using Qubit Fluorimeter (V.3.0). The V3–V4 hypervariable region of 16S rRNA gene was amplified using universal prokaryote primers, Pro341F (5′—CCTACGGGNBGCASCAG–3′) and Pro805R (5′—GACTACNVGGGTATCTAATCC–3′) (Takahashi et al. 2014). The amplified product was checked on 2% agarose gel and gel purification was done to rule out nonspecific amplification in subsequent experiments. The amplified product (5 ng) was used for library preparation using the NEBNext Ultra DNA library preparation kit. The quantification and quality estimation of the prepared library were done in Agilent 2200 TapeStation and sequencing was done using Illumina HiSeq 2500 platform.

For metagenomic analysis, the raw reads obtained from the Illumina sequencing platform were subjected to the FastQC program (version. 0.11.8) after demultiplexing to check the quality of the reads with default parameters. The base quality (Phred Score; Q), base composition, GC content, ambiguous bases (other than A, T, G, and C), and adapter dimers were thoroughly checked prior to bioinformatics analysis. After trimming primer sequences with in-house PERL script, high-quality paired-end reads were allowed to merge/stitch to get the V3–V4 amplicon consensus FASTA sequences. The reads were merged using the FLASH program (version 1.2.11) with a minimum overlap of 10 bp to overlap a maximum of 240 bp with zero mismatches. Only properly paired-end reads with Phred score quality ($Q > 20$) were considered for the generation of V3–V4 consensus sequences.

Bioinformatic analyses

The fastq files were processed by the QIIME bioinformatics pipeline [Version: 1.9.1] (Caporaso et al. 2010b). Chimeras were removed using the *de novo* chimera removal method UCHIME (version 11) implemented in the VSEARCH tool. QIIME1 program was used for the entire downstream analysis. The representative sequences from each clustered operational taxonomic unit (OTU) were picked and aligned against the SILVA (Quast et al. 2012) core set of sequences using the PyNAST (Caporaso et al. 2010a) program. Further, taxonomy classification was performed using an RDP classifier by

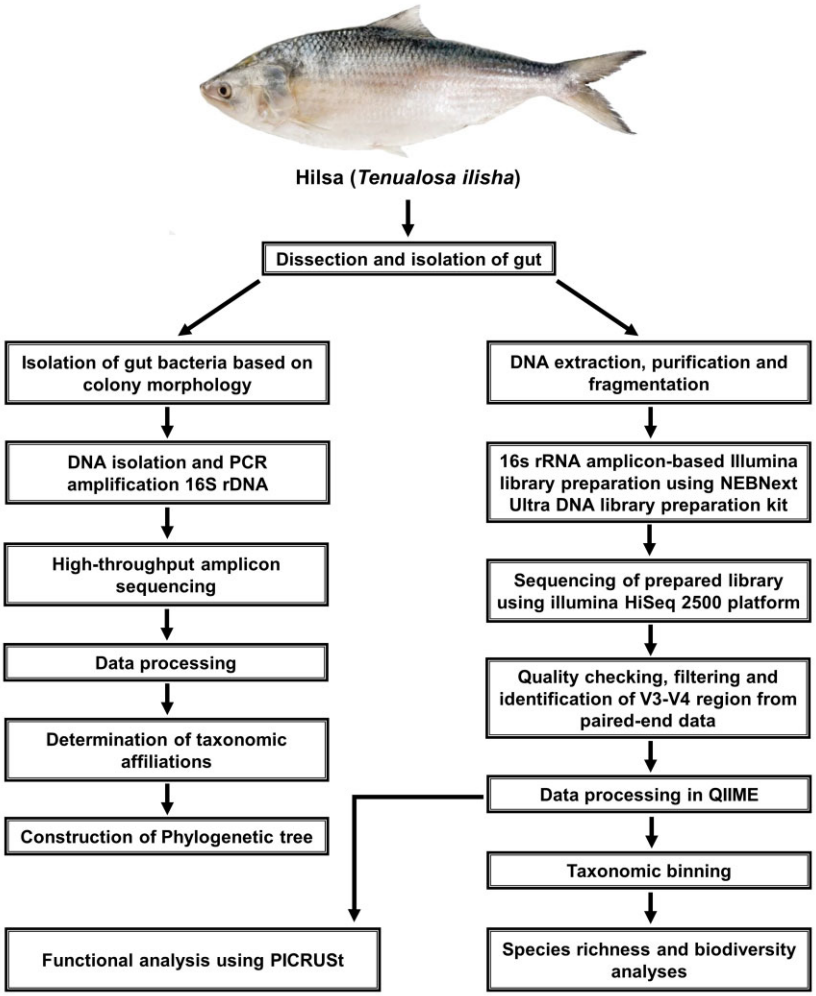

Figure 1. Schematic representation of the experimental designs in the study of Hilsa (*T. ilisha*), the Indian Shad.

mapping each representative sequence against the SILVA OTUs database (Wang et al. 2007). The OTU table was converted to text and graphical formats to depict the representation of microbial communities at specific taxonomic levels (Ramírez and Romero 2017). Further, the OTU table obtained from QIIME was used to predict KEGG functions (Kanehisa and Goto 2000) using the Galaxy server of PICRUSt (Douglas et al. 2018).

Alpha diversity analysis

The microbial diversity within the samples was calculated based on Shannon, Chao1 and observed species metrics. The Chao1 metric estimates the species richness while the Shannon metric is the measure to estimate observed OTU abundances, and accounts for both richness and evenness. Moreover, due to the nonlinearity of the Shannon index, the observed indices were converted into Shannon effective number of species, a linear expression, using the formula of Lou Jost (Jost 2007). The observed species metric is the count of unique OTUs identified in the sample. The metric calculation was performed using QIIME1 software.

An outline of the experimental design depicting a scheme for the isolation of the gut, diversity analysis of culturable and metagenomic bacterial population and their functional affiliation is presented in Fig. 1.

Results

Isolation and characterization of gut microbiota

The freshwater and seawater hilsa gut samples were screened for the presence of bacterial isolates. Initially, 55 culturable isolates were obtained from serially diluted gut content of hilsa using tryptone soya broth-agar and Zobell marine broth-agar plates. Further screening based on morphological features including pigmentation patterns, 23 isolates were found to be distinct from one another. All these isolates were obtained from the tryptone soya broth-agar plate, although seven of these isolates were also obtained from the Zobell marine broth-agar plate. The systematic position of these isolates was determined based on 16S rRNA sequencing followed by blast analysis and phylogenetic affiliation study. Table 1 represents the list of culturable isolates and their taxonomic affiliation, while Fig. S1 depicts their phylogenetic relationships. At the phylum level, Proteobacteria and Firmicutes were the most predominant among the culturable isolates, while at the genus level, *Staphylococcus* was the most dominant

Table 1. Identification of bacterial strains isolated from the gut of hilsa (*T. ilisha*) based on 16S rRNA genes.

Isolates (strain name)	Isolation source	Closest relative (obtained from BLAST search)	Affiliation (Phylum/Class)	Identity (%)	Accession no.
MC1	FW, SW	*Pseudomonas* sp.	Proteobacteria/Gammaproteobacteria	99.71%	OP103979
MC2	SW	*Staphylococcus cohnii*	Firmicutes/Bacilli	99.65%	OP103980
MC3	FW, SW	*Pseudomonas weihenstephanensis*	Proteobacteria/Gammaproteobacteria	99.72%	OP103981
MC4	SW	*Staphylococcus nepalensis*	Firmicutes/Bacilli	100%	OP103982
MC5	SW	*C. maltaromaticum*	Firmicutes/Bacilli	99.78%	OP103983
MC6	SW	*Shewanella baltica*	Proteobacteria/Gammaproteobacteria	99.72%	OP103984
MC7	FW, SW	*Pseudomonas lundensis*	Proteobacteria/Gammaproteobacteria	99.79%	OP103985
MC8	FW	*Bacillus safensis*	Firmicutes/Bacilli	100%	OP103986
MC9	FW	*Morganella morganii*	Proteobacteria/Gammaproteobacteria	100%	OP103987
MC10	SW	*Morganella psychrotolerans*	Proteobacteria/Gammaproteobacteria	99.53%	OP103988
MC11	FW	*Rhodococcus qingshengii*	Actinobacteria/Actinimycetia	99.81%	OP103989
MC12	FW	*Staphylococcus epidermidis*	Firmicutes/Bacilli	99.15%	OP103990
MC13	FW	*Staphylococcus equorum*	Firmicutes/Bacilli	99.63%	OP103991
MC14	FW, SW	*Acinetobacter junii*	Proteobacteria/Gammaproteobacteria	99.06%	OP103992
MC15	FW, SW	*Lactococcus lactis*	Firmicutes/Bacilli	100%	OP103993
MC16	FW, SW	*Serratia marcescens*	Proteobacteria/Gammaproteobacteria	100%	OP103994
MC17	FW, SW	*Paracoccus* sp.	Proteobacteria/alphaproteobacteria	99%	OP103995
MC18	FW	*Bacillus megaterium*	Firmicutes/Bacilli	99%	OP103996
MC19	SW	*Shewanella* sp	Proteobacteria/Gammaproteobacteria	100%	OP103997
MC20	FW, SW	*Lactococcus garvieae*	Firmicutes/Bacilli	99%	OP103998
MC21	FW	*Bacillus* sp.	Firmicutes/Bacilli	98%	OP103999
MC22	FW, SW	*Aeromonas* sp.	Proteobacteria/Gammaproteobacteria	100%	OP104000
MC23	SW	*Shewanella putrefaciens*	Proteobacteria/Gammaproteobacteria	99%	OP104001

FW, Freshwater; SW, Seawater

followed by *Bacillus*, *Pseudomonas*, and *Shewanella*. When compared between marine and freshwater samples, *Bacillus* and *Rhodococcus* were found to be restricted in freshwater and *Carnobacterium maltaromaticum* and *Shewanella* in marine samples only, while *Acinetobacter*, *Aeromonas*, *Lactococcus*, *Morganella*, *Paracoccus*, *Pseudomonas*, *Serratia*, and *Staphylococcus* were found in both freshwater and seawater groups (Table 1).

Assessment of gut microbial diversity

The NGS of the V3–V4 regions of the 16S rRNA of metagenomic samples provided a total of 530 410 paired-end reads with an average of 88401.67 ± 9491.37. Following quality filtering and chimera removal, the preprocessed reads from all the samples were pooled and clustered into OTUs based on their sequence similarity using the Uclust program (similarity cutoff = 0.97) available in QIIME1 software. From 187 706 preprocessed consensus sequences obtained from freshwater hilsa samples, a total of 705 OTUs were selected for subsequent analysis after the removal of OTUs of <2 reads, which is an effective method of removing false-positive OTUs (Callahan et al. 2019). On the other hand, a total of 682 OTUs were identified from 153 758 preprocessed consensus sequences from seawater hilsa samples after the removal of OTUs with <2 reads. Out of the above, 532 and 519 OTUs were respectively detected at the genus level in the gut microbial communities of freshwater hilsa ($n = 3$) and seawater hilsa ($n = 3$). We obtained a total of 48 genera, of which 20 genera (41.67%) were shared between freshwater and seawater samples, while 18 (37.50%) and 10 (20.83%) genera were found unique in freshwater and seawater samples, respectively (Fig. 2).

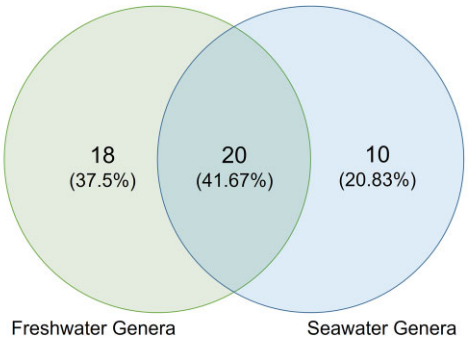

Figure 2. Number of bacterial genera in freshwater and seawater hilsa gut microbiota. The percentage of shared and unique bacterial genera for freshwater and seawater groups are represented.

In the metagenomic study of the freshwater (FW) and Seawater (SW) hilsa, the predominant phyla detected were Proteobacteria (FW, 52.07%; SW, 37.45%; $Z = 5.47$; $P < 1.00 \times 10^{-5}$), Firmicutes (FW, 36.52%; SW, 43.32%; $Z = -2.59$; $P = 9.60 \times 10^{-3}$) and Bacteroidetes (FW, 11.21%; SW, 14.03%; $Z = -1.58$; $P = 1.14 \times 10^{-1}$) (Fig. 3a). Actinobacteria and Verrucomicrobia were also found in these samples, but their abundance was quite low (<0.1%). The phylum Fusobacteria was restricted to seawater group only, representing 5.19% of total OTUs in the group (Fig. 3a). The SDs range from $0.00 \leq SD_{freshwater} \leq 23.68$ and $0.00 \leq SD_{seawater} \leq 25.42$ at the phylum level.

At the genus level, *Pseudomonas* (FW, 44.65%; SW, 19.40%; $Z = 8.76$; $P < 1.00 \times 10^{-5}$), *Peptostreptococcus*

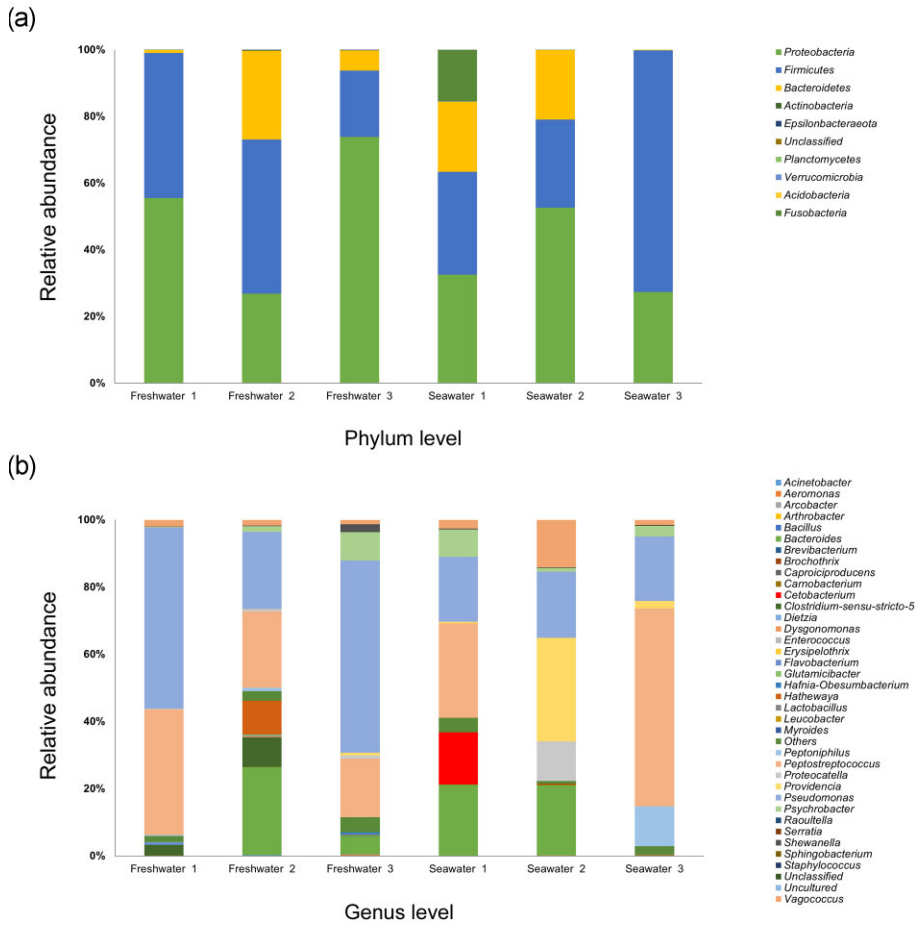

Figure 3. Relative abundance of OTUs at the (a) phylum and (b) genus levels of freshwater and seawater hilsa (*T. ilisha*).

(FW, 25.86%; SW, 29.01%; Z= −1.14; $P = 2.54 \times 10^{-1}$), *Bacteroides* (FW, 10.53%; SW, 14.01%; Z= −1.72; $P = 8.54 \times 10^{-2}$), *Psychrobacter* (FW, 3.45%; SW, 4.15%; Z= −0.59; $P = 5.55 \times 10^{-1}$), and *Vagococcus* (FW, 1.66%; SW, 6.08%; Z= −3.73; $P = 2.00 \times 10^{-4}$) were found to be dominant in both freshwater and seawater samples (Fig. 3b). However, *Clostridium* (FW, 4.00%; SW, 0.00%; Z = 4.60; $P < 1.00 \times 10^{-5}$) and *Hathewaya* (FW, 3.39%; SW, 0.00%; Z = 4.23; $P < 1.00 \times 10^{-5}$) were found to be restricted to freshwater group, while *Cetobacterium* (FW, 0.00%; SW, 5.18%; Z= −5.32; $P < 1.00 \times 10^{-5}$), *Proteocatella* (FW, 0.56%; SW, 3.90%; Z= −3.68; $P = 2.40 \times 10^{-4}$), and *Peptoniphilus* (FW, 0.43%; SW, 3.93%; Z= −3.90; $P = 1.00 \times 10^{-3}$) were detected in seawater group only (Fig. 3b). The SD ranges from $0.00 \leq SD_{freshwater} \leq 18.82$ and $0.00 \leq SD_{seawater} \leq 29.40$ at the genus level. The species richness was observed to be higher in the freshwater group, as suggested by the observed species and Chao1 (Fig. 4a and b).

The microbial species diversity of the samples was measured using the Shannon effective number, which showed a little higher value in the seawater group, suggesting higher species diversity despite having a lower species richness (Fig. 4c), as attributed to the distribution of relative abundance of species. The rarefaction curve of observed species against sequences per sample revealed fair coverage of species for freshwater and seawater samples (Fig. S2).

Functional analysis of hilsa gut microbiota

To assess functional attributes of the metagenomic bacterial population, the OTUs obtained from QIIME were used to predict KEGG functions using the Galaxy server of PICRUSt and the mean Nearest Sequenced Taxon Index (NSTI) of 0.07051 ± 0.006 was obtained. The evaluated NSTI values as observed in this study, signify that the inferences drawn in PICRUSt analysis correlate well with the pertinent metagenomic data, as described in earlier studies (Koo et al. 2017). The most abundant functional properties include transporter activity, DNA repair, purine and pyrimidine metabolism, amino acid metabolism, and peptidases (Fig. 5). Broadly, functional analysis of both the freshwater and seawater samples showed similar profiles; however, differences were noticed in functional abundances. The freshwater group showed relatively higher appraisals for ABC transporters, two-component system, bacterial motility proteins, secretion system, DNA repair and recombination proteins, purine metabolism, arginine and proline metabolism, other ion-coupled transporters, and transcription factors, among others. While the seawater group displayed comparatively higher estimates for shigellosis, carotenoid biosynthesis, D-arginine and D-ornithine

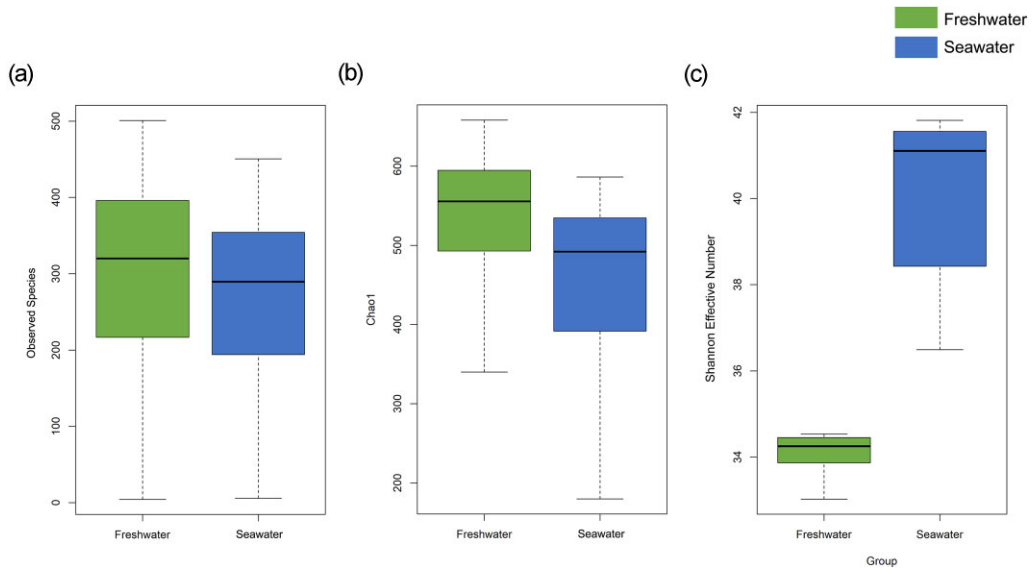

Figure 4. Alpha diversity measures of freshwater and seawater hilsa (*T. ilisha)* gut microbiota. (a) Observed species, (b) Chao1, and (c) Shannon effective number.

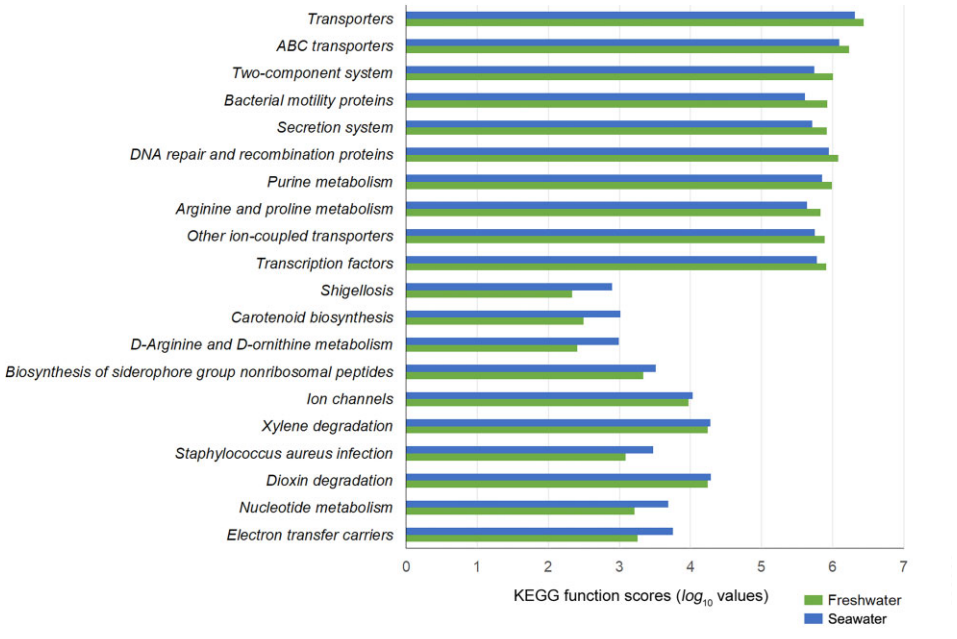

Figure 5. Enriched KEGG functions from freshwater and seawater hilsa gut microbiota. The top ten differential KEGG function scores (*log* values) both for freshwater and seawater samples are presented.

metabolism, biosynthesis of siderophore group nonribosomal peptides, ion channel, xylene and dioxin degradations, *Staphylococcus aureus* infection, nucleotide metabolism and electron transfer carriers. A detailed metabolic profile, associated with the KEGG pathways of gut microbiota of the freshwater and seawater hilsa is illustrated in Table S2.

Discussion

The gut microbiota, being an innate component of the host, has been luring the attention of the scientific community in recent years. The microbiota not only helps the host to acquire nutrients and the calories present within various complex dietary components but also prevents the invasion and colonization of harmful pathogenic microorganisms (Pickard et al. 2017). So far, there are only a few studies that identified culturable bacteria from the gut content of "fresh" hilsa. These studies identified Gram-negative species, such as *Pseudomonas*, *Achromobacter*, Enterobacteriaceae, *Flavobacterium*, *Aeromonas*, *Salmonella*, *Proteus*, *Vibrio*, *Psychrobacter*, *Planococcus,* and *Halobacterium* (Radwan et al. 2007) and Gram-positive species like *Micrococcus*, *Bacillus*, *Streptococcus*, *Staphylococcus*, *Listeria,* and a

few Coryneform bacteria (Rao et al. 2012, Rohomania et al. 2015, Akter et al. 2016, Hossain et al. 2018). On the other hand, in "iced" hilsa, *Micrococcus* appeared as the dominant species followed by *Staphylococcus*, *Pseudomonas*, *Bacillus*, and *Achromobacter*, in addition to coliform bacteria, like *Klebsiella*, *Enterobacter*, and *Escherichia coli* (Hoq et al. 1991). Nevertheless, more emphasis was given to the assessment of pathogenic bacteria in marketed hilsa, used for human consumption where *Aeromonas*, *Salmonella*, *Vibrio*, *Proteus*, and *S. epidermidis* were reported (Jha et al. 2010, Rohomania et al. 2015, Hossain et al. 2018). Apart from bacterial species, 26 zooplankton species comprised of copepods, protozoans, cladocerans, rotifers, chaetognaths, and ostracods were also identified from the gut content of hilsa (Akter et al. 2016).

Hilsa is phylogenetically closer to the American shad, both belonging to the order Clupeiformes (Rao et al. 2012). However, it is more distant in relation to salmon (*Salmo salar*), the most widely studied fish from the perspective of intestinal microbiota. Although a detailed account of the gut microbiota of salmon is available in several kinds of literature, to the best of our knowledge, there is no report on the microbial diversity of American shad. 16S rRNA gene sequence analysis identified 146 microbial isolates in Atlantic salmon, distributed among 31 phylotypes, where the majority of the species belong to the genera *Psychrobacter*, *Arthrobacter*, *Bacillus*, and Arctic seawater bacterium (Ringø et al. 2008). Our metagenomic analyses of hilsa revealed the presence of Proteobacteria as the predominant phylum followed by Firmicutes, Bacteroidetes, and Actinobacteria in freshwater while Firmicutes followed by Proteobacteria, Bacteroidetes, and Actinobacteria in seawater hilsa gut samples (Fig. 3a) reflecting sampling location between fresh and saline environments, confirming a salinity-linked turnover. A shift in gut community composition between marine and freshwater environments was also reported for whitefish and Arctic char, a more typically anadromous fish (Element et al. 2020). A previous report suggested Proteobacteria as the predominant phylum in fish gut microbiota during fasting and Firmicutes during feeding (Luan et al. 2023), which is consistent with hilsa migration pattern where they inhabit seawater for living and feeding and migrate to freshwater to spawn. The only previous report on the distribution of bacterial communities of the intestine in fresh hilsa also revealed the presence of Proteobacteria as the dominant phylum (Foysal et al. 2019). However, in gut metagenomic analysis, Proteobacteria and Bacteroidota were observed as the dominant phyla in the anadromous Atlantic salmon (Weththasinghe et al. 2022). Nevertheless, Proteobacteria and Firmicutes, in general, are the dominant phyla in most fish, including grass carps (*Ctenopharyngodon idella*) (Gong et al. 2019), turbots (*Scophthalmus maximus*) (Jiang et al. 2019), gibel carp (*Carassius auratus gibelio*) (Wu et al. 2013), zebrafish (*Danio rerio*) (Roeselers et al. 2011), and many other fish species (Sullam et al. 2012) irrespective of their migratory habits, which is also well reflected in both the culture-dependent and culture-independent investigations of the present study. Moreover, there is no report of gut microbiota from clupeidae fish, except for very few studies in hilsa, as already mentioned. Interestingly, Fusobacteria was found to be restricted in the seawater group only, represented by the only genus *Cetobacterium* in our study. According to a previous report, an increase in abundance of Fusobacteria was also observed during seawater transition in the anadromous fish Atlantic salmon (Jaramillo-Torres et al. 2019). *Cetobacterium* is an obligatory gut microbe involved in vitamin B_{12} (cobalamin) production to improve fish health (Tsuchiya et al. 2008, Gallet et al. 2022).

Among the prominent classes of bacteria identified through NGS approaches, Clostridia, Bacilli, Gammaproteobacteria, and Bacteroidia are the most abundant classes apart from Actinobacteria, Campylobacteria, Verrucomicrobiae, Erysipelotrichia, Alphaproteobacteria, Planctomycetacia, Fusobacteria, and Thermoleophilia. Similar results were also obtained in a study with *Coilia ectenes*, which, like hilsa, also belongs to the order Clupeiformes (Duan et al. 2017). The intestinal bacterial diversity of freshwater hilsa is found to be higher than seawater, both in terms of species richness and relative abundance (Fig. 4). A similar trend was observed in Atlantic salmon, where the intestinal microbial diversity reduces after freshwater to seawater transition (Dehler et al. 2017). The functional analysis using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) revealed the association of the hilsa gut microbiota mainly with transporter activity, signal transduction (two-component) and secretion systems, bacterial motility, regulatory transcription factors, and metabolism of various biomolecules, including nucleotides and amino acids (Fig. 5).

The migration of hilsa from the salty marine environment (30–35 ppt) to the low-saline brackish water or zero-saline freshwater environments changes the osmotic balance of the cells. Nevertheless, increased PUFA is necessary to reorganize the composition of vital membranes to maintain homeostasis. The change in the fatty acid composition of hilsa toward PUFA might be possibly a physiological mechanism to counter the changes in the salinity of water during migration. A decrease in lipid content during the course of upward migration of Atlantic salmon was reported (Jonsson et al. 1997). Further, a decrease in lipid by 30%–40% was reported during the period of re-entry of Arctic charr from sea to freshwater (Jøsrgensen et al. 1997). Hilsa accumulates energy reserves during their growth phase in the form of lipids, which are metabolized to provide the necessary energy for anadromous migration and spawning. One of the studies revealed that the saturated fatty acid content in Godavari hilsa (24.98%) was lower than that obtained from brackishwater (36.76%) and seawater (36.03%). In parallel, an increase in PUFA was observed during the anadromous migration of hilsa (Rao et al. 2012). Thus, the role of enzymes involved in lipid and fatty acid metabolism (Table S2) may be linked to the migration of anadromous fish including hilsa based on the utilization of plasma-free fatty acids as one of the sources of energy for long-distance migration (Anttila et al. 2010). The presence of *Erysipelotrichaceae* detected through the NGS study (Fig. 3b) also hints at its role in lipid metabolism (Sylvain and Derome 2017).

In addition, *Psychrobacter maritimus* may have a role as probiotics to improve the overall growth of the host, apart from their involvement in cellular and innate immunity, and digestive enzyme secretion (Makled et al. 2020). In the present study, apart from various other functional attributes, the PICRUSt result of gut metagenome data also spotted the existence of enzymes of carbohydrate metabolic pathways and that for the metabolism of terpenoids and polyketides (Table S2), corroborating the presence of digestive enzymes and biosynthesis of various essential bioactive molecules, respectively.

Nevertheless, fisheries and aquaculture are important sources of food, nutrition, income, and livelihood for millions of people around the world. The evolving fisheries sector in India has currently become one of the important socio-economic attributes and emerged as the third largest producer of fish and aquaculture products in the world accounting for 16% and 5% of total global inland and marine fish production, respectively. In 2020, India's total marine and inland fish landing stood at 5.7 million tonnes, which includes 1.8 million tonnes from the inland sector and the rest 3.9 million tonnes from the marine sector with an average annual growth of 10.34%. On the other hand, the fish landing in Bangladesh stood at 1.92 million tonnes (1.25 million tonnes from the inland sector and 0.67 million tonnes from the marine sector). The contribution of the fisheries sector to the Indian economy accounts for nearly 30 billion US$ with an export value of around 7.2 billion US$ (https://dof.gov.in/sites/default/files/2023-01/HandbookFisheriesStatistics19012023.pdf, https://www.fao.org/3/cc0461en/cc0461en.pdf).

Hilsa is primarily available in countries bordering the Bay of Bengal and Arabian Sea and normally inhabits rivers, estuaries, and coastal waters. Among the countries, hilsa is primarily distributed in the rivers Padma, and Meghna in Bangladesh; the Ganga, Hooghly, Godavari, Krishna, Cauveri, Brahmaputra, Narmada, and Tapti in India; the Irrawaddy in Myanmar; the Indus and Jhelum in Pakistan and the Shatt-al-Arab, Tigris and Euphrates in Iraq (Suresh et al. 2017). Global hilsa shad production was mainly contributed by Bangladesh followed by India and Myanmar. According to the Fisheries Department, in Bangladesh, a record high of 565 kilotonnes of hilsa were harvested in the fiscal year 2020–21. The figures were 550, 533, 517, and 496 kilotonnes in the preceding four fiscal years, respectively. While Hilsa shad production in Bangladesh holds an economic value of USD 1.3 billion per year providing livelihoods for 3.0 million fishermen and people in the value chain and distribution (Bandara and Wijewardene 2023). In India, according to a 2016 estimate, the annual average production of hilsa is 40 kilotonnes, of which, the Ganga, Hooghly, and Brahmaputra along with their tributaries contribute about 70% of the total Hilsa production (Das et al. 2019).

The importance of hilsa shad in this region has driven a large number of scientific studies on various themes apart from identifying the knowledge gaps and future research directions. Overall, a strong positive relationship is reflected between the world hilsa shad production and the number of articles published between 2000 and 2020 signifying the magnitude of its demand (Bandara and Wijewardene 2023). Nonetheless, the contribution of hilsa is quite significant to the fisheries and aquaculture in India and Bangladesh. The present study on the bacterial diversity analysis of freshwater and seawater hilsa and their functional analyses is a small encouraging step in understanding the possible role of gut bacteria in its migratory behavior, the fatty acid composition and establishing the long-craved aquaculture of this species as a part of future endeavor.

Conclusion

The present study reveals the community structure of the gut microbiota of fresh hilsa through culture-based and culture-independent approaches, expanding the current state of knowledge of gut-associated bacteria in one of the fish of rare delicacy that contains numerous health-beneficial essential nutrients including omega-3 polyunsaturated fatty acid.

Funding

The authors thank the University Grants Commission (UGC), Government of India, for fellowship (760/CSIR-UGC NET/June 2017) to M.C. and Bose Institute, Kolkata, an autonomous institute of the Department of Science and Technology, Government of India, for financial support.

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest:

The authors declare no competing interests.

Author contributions

Megha Chakraborty (Conceptualization, Investigation, Writing – original draft), Debarun Acharya (Formal analysis, Investigation, Writing – review & editing), and Tapan K. Dutta (Conceptualization, Supervision, Writing – review & editing)

Data availability

The datasets generated and/or analysed during the current study are available in the NCBI repository with the 16S rRNA accession numbers (OP103979 to OP104001) (https://www.ncbi.nlm.nih.gov/nuccore/OP103979 and so on for other accession numbers) and BioProject accession number PRJNA868876 (https://www.ncbi.nlm.nih.gov/bioproject/868876) for raw FASTQ files.

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Received: March 17, 2023. **Revised:** August 25, 2023. **Accepted:** September 8, 2023