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ORIGINAL ARTICLE

Riverine pollution influences the intraspecific variation in the gut microbiome of an invasive fish, *Cyprinus carpio* (Linn., 1758)

Meghali Bharti[1] · Shekhar Nagar[1,2] · Ram Krishan Negi[1]

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Abstract

Humans are significantly impacting riverine systems worldwide, prompting us to investigate the effects of water pollution on the gut microbiome of *Cyprinus carpio* (common carp). Using 16S rRNA gene sequencing, we compared the gut microbiomes of common carp from two sites along river Yamuna with different pollution levels. Water pollution significantly altered the fish gut microbiome structure and microbial composition. *Proteobacteria* dominated in both sampling sites, while *Bacteroidota* prevailed in polluted water samples, indicating sewage and fecal contamination. Less polluted samples exhibited *Verrucomicrobiae* and *Planctomycetes*, negatively correlated with pollution levels. The polluted site had higher prevalence of potentially pathogenic and heavy metal-resistant bacteria, as well as microbial communities associated with wastewater treatment systems. Functional prediction highlighted the significant role of the gut microbiome in digestion and metabolism, with active enzymes for breaking down various organic substances. Biosynthetic pathways for leucine, valine, and isoleucine were present in both sites, known to be involved fish immunity. The host maintained a stable and diverse bacterial consortium, while microbial diversity became more specialized due to human activities, adapting to anthropogenic stress and selection pressures.

Keywords Microbiome · Common carp · Anthropogenic activities · 16S rRNA gene

Introduction

Microorganisms form indispensable and multifaceted mutual associations with hosts, and these microbial communities are jointly known as microbiome (Ursell et al. 2012). Explicitly, the gut microbiome is of great interest as the intestine is a multifunctional organ system and harbors greater microbial diversity contrasted to other organs (Colston and Jackson 2016; Degregori et al. 2021). Microbial communities in the gastrointestinal tract (GI) of the host may influence development, growth, ecology, metabolism, reproduction, immune system, and evolution (Pérez et al. 2010). In spite of growing curiosity in vertebrate's gut microbiome, understanding of microbial diversity composition and evolutionary dynamics of fishes is comparatively limited (Ghanbari et al. 2015; Tarnecki et al. 2019). With a diversity of 34,600 species (FishBase 2021), presence of broad ecological varieties, and characteristic microbial profile within their GI tracts, fish can represent a relevant model organism to examine the association of microbial communities with their hosts (Nelson et al. 2016). Interaction of several factors such as quality of surrounding water, diet, host genetics, developmental stage, immune status, and other host-specific pressures influences gut microbiota in fishes (Llewellyn et al. 2014; Khurana et al. 2021). Intraspecific variation in the gut microbiome can provide an understanding of the adaptive potential of species challenged with environmental stressors (Des Roches et al. 2018; Walter et al. 2019; Xue et al. 2006).

While variation in physicochemical parameters of surrounding water may induce natural responses in the host, human-induced disturbances can have more drastic consequences and these effects can be further exaggerated in fragile ecosystems like rivers. Owing to the industrial revolution

✉ Ram Krishan Negi
negigurukul@gmail.com

Meghali Bharti
meghalibharti25@gmail.com

Shekhar Nagar
snagar@db.du.ac.in

[1] Fish Molecular Biology Laboratory, Department of Zoology, University of Delhi, North Campus, Delhi 110007, India

[2] Department of Zoology, Deshbandhu College, Kalkaji, New Delhi 110019, India

and anthropogenic activities, Yamuna has become one of the most polluted rivers in the world (CPCB 2006; Sharma et al. 2017, 2020). As the river flows through distinct regions of Delhi, various drains and untreated wastewater severely deteriorate its water quality (Dhillon et al. 2013; Said and Hussain 2019). The fish diversity of the river Yamuna from Dakpathar to Allahabad constitutes 143 fish species (10 orders, 29 families, and 73 genera) with Cyprinidae being the most abundant family followed by Schilbeidae, Bagridae, and Sisoridae (Sharma et al. 2017; Koushlesh et al. 2021). The rise in pollution load has led to declined fish catches, a remarkable increase in invasive fish populations, and shifts in the fish species composition (Sharma et al. 2017). Due to these dynamics, this river is facing a hard battle against pollution and can be used for understanding the influence of anthropogenic activities on the microflora that are sentinels of water quality. Pollution also has been studied to have profound effects on species' ecology as well as on their gut microbiomes (Degregori et al. 2021; Xia et al. 2014; Silbiger et al. 2018).

In spite of these facts, the impact of deteriorated water quality of river Yamuna on taxonomic and functional aspects of habitant fish's gut microbiome remains unexplored. The common carp (*Cyprinus carpio* Linnaeus, 1758) is a widespread freshwater fish that is included in the world's 100 worst invasive species (Courtenay Walter and Welcomme 1989; Dwivedi et al. 2016; GISD 2021). *C. carpio* serves as a keystone ecosystem engineer, as its feeding method involves agitating sediments at the bottom of the water body, thus causing uprooting macrophytes and modification of habitats for native fish and other aquatic species (FAO 2009). The microbial composition residing in the gut of *C. carpio* has formerly been explored by cultivation-based approaches (Sugita et al. 1990; Namba et al. 2007; Tsuchiya et al. 2008) but culture-independent methods such as 16S rRNA gene screening serve as a more reliable and detailed strategy to evaluate microbial community composition along with the functional potential in the GI tract of this fish (van Kessel et al. 2011; Eichmiller et al. 2016; Kakade et al. 2020). Being an aquatic bioindicator (Yeşilbudak and Erdem 2014), screening the intraspecies microbial diversity would lead the way for understanding the issues that resident fishes are facing in the polluted river Yamuna.

In this backdrop, our goal was to perform a comparative study of the microbial diversity and functional attributes of river Yamuna water and gut microbiome samples of *C. carpio* by using Illumina HiSeq 2500 platform for high-throughput sequencing of 16S rRNA gene. Samples were collected from two different geographical locations (~ 120 km away) of river Yamuna (i) anthropogenically impacted and highly polluted Wazirabad barrage, Delhi (site-α) (Bharti et al. 2022), and (ii) a comparatively less polluted site in Dabarkipar, Karnal, Haryana (site-β), to understand whether a core microbial consortia can be defined or are host host-specific selective pressures overwhelmed by the environmental conditions, in particular water pollution. Also, capturing the patterns of variation across the intraspecific gut microbial community composition would shed light on the consequences of changes in the environment.

Methodology

Collection of samples and amplicon sequencing

Water and fish samples were collected from the highly polluted site at (1) Wazirabad barrage, Delhi (site-α) (28° 40′ 5.53″ N, 77° 15′ 0.35″ E) (Supplementary1), (2) and a relatively less polluted site Dabarkipar, Karnal, Haryana (site-β); (29.6556° N, 77.1198° E), river Yamuna, India. Twenty samples of common carp from each site were collected by cast net of different mesh sizes and were brought to the laboratory. Length–weight (45.12 ± 3.21 cm; 620 ± 32.9 g (site-α) and 50.13 ± 2.11 cm; 700 ± 13.8 g (site-β)) estimation of all sampled fishes was performed. Physicochemical parameters of water (pH, temperature, total dissolved solids (TDS), dissolved oxygen (DO), electrical conductivity (EC), and chemical oxygen demand (COD)) were recorded on the spot by Orion 5-star Portable Multimeter (Thermo Fisher Scientific Inc. [NYSE: TMO], MA, USA) from both the sampling sites (Supplementary S1). Estimation of heavy metal traces of iron (Fe), zinc (Zn), cadmium (Cd), lead (Pb), nickel (Ni), and chromium (Cr) in the water samples was done by employing Atomic Absorption Spectroscopy (Sensa AAS Dual, GBC, Australia) with EPA's acid digestion procedure (EPA, 3050B). Aseptic dissection of 20 fish samples was performed followed by removal of gastrointestinal tract (GIT) and storage in sterile cryotubes at – 80 °C. Water samples were filtered using 0.22 µm filter (MF-Millipore TM), and filters were carried on with DNA extraction. Power Soil® DNA isolation kit (MO BIO) was used to extract metagenomic DNA from water sample (5L) and gut contents (700 mg). Amplicon sequencing was performed using the Illumina HiSeq 2500 sequencing platform (Novogene Co., Ltd., China) by PCR (341 F and 805R barcoded fusion primers) from V3–V4 region of 16S rRNA genes.

Data curation, OTU clustering and taxonomic analysis

Low-quality bases, adaptor, barcodes, primer sequences were removed employing cut adapt in QIIME 2.2019.7 (Bolyen et al. 2019), and paired-end reads were merged using FLASH (v1.2.7) (Magoč and Steven 2011). Further, downstream analysis was performed with the processed reads (> 70% bases; Phred score ≥ 30). QIIME 2.2019.7 was

employed for data analysis using de novo approach. Identification of operational taxonomy units (OTUs) (clustered at 97% similarity), taxonomic affiliation using a naïve Bayesian classifier (Wang et al. 2007), and SILVA 138 database (updated 2014) (Quast et al. 2013) using UCHIME algorithm (Edgar 2013) were performed. Phylum hierarchy was visualized using Circos v 0.63.10.

Statistical analysis

Alpha diversity metrics analysis was done using the rarefaction QIIME (2.2019.7) process with default parameters (Bolyen et al. 2019). For comparison of alpha diversity across water and gut microbiome samples, Shannon, Simpson, ACE, Chao1, and Good-coverage indices were calculated and visualized in R software (V.2.15.3) (R-core-team 2013). The significant differences in alpha diversity index between samples were checked with the Kruskal–Wallis test ($p < 0.05$). We compared beta diversity across the samples using the UniFrac distance metrics (Lozupone and Knight 2005). Consequently, Bray–Curtis distance-based principal coordinate analysis (PCoA) plot was generated to visualize the microbiome samples' beta diversity pattern. Likewise, NMDS (non-metric multidimensional scaling) was performed for visualization of variation in microbial community composition between samples. The PCoA and NMDS dimensionality reduction maps were generated in R software.

To perform OTU network-based analysis, network maps were constructed using QIIME and visualized using Cytoscape (v. 3.0.1) (Shannon et al. 2003). OTU table (97% sequence similarity) was converted to the Cytoscape format. The edges connecting nodes representing fish and water samples (circles) to species-level OTUs in a particular sample are colored according to the host–habitat type (edge-weighted spring embedded model in Cytoscape v. 3.0.1).

Functional prediction

We employed PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2) for prediction of the functional repertoire of microbial consortia in water and gut samples from site-α and site-β (Douglas et al. 2020). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and enzymes were deciphered.

Results

Physicochemical analysis of water samples

Physicochemical characteristics from both sampling sites are provided in Supplementary S1. The pH values pointed out neutral to alkaline nature of the examined water samples. Low DO (1.8 ± 0.8 mg/L) whereas elevated BOD (102.4 ± 1.4 mg/L), COD (266.4 ± 3.10 mg/L) and TDS (1050.7 ± 3.34 mg/L) values were observed in site-α water sample (Bharti et al. 2022). In site-β, DO, BOD, and COD values were 4.8 ± 0.7, 19.5 ± 1.8, and 103 ± 2.0, respectively. Also, in site-α, among the tested heavy metals, Fe (2.89 ± 0.07 mg/L), Cr (1.06 ± 0.04 mg/L), and Zn (2.25 ± 0.07 mg/L) were beyond their permissible limit, i.e., 2 mg/L, 0.05 mg/L, 0.01 mg/L (WHO 2011), respectively. In site-β, all the physicochemical parameters were within the permissible range for water samples. Overall, based on the above parameters, it could be inferred that these two sampling sites are physicochemically different.

Sequencing and diversity analysis

Illumina paired-end platform was used for amplicon sequencing to produce 250 bp paired-end raw reads (30,44,701) followed by merging to obtain clean tags. The SILVA database was used to quantify the chimeric sequences in clean tags, and these sequences were removed to obtain 1,346,070 effective tags which were then used for subsequent analysis (Table 1). After clustering at 97% sequence similarity, a total of 1318 OTUs were identified. Statistical indices of alpha diversity at clustering threshold 97% (number of reads chosen for normalization: cutoff = 19,448) are depicted in Table 1.

The rarefaction curve showed that each sample flattened with high sequence numbers, thus indicating reasonable sequencing depth (Fig. 1a). Alpha diversity calculated as Shannon and Simpson's indices showed fluctuations in the diversity across the gut and water samples from site-α and site-β (Table 1; Fig. 1b). Shannon values were in range from 2.3 to 5.4 (minimum in gut samples from site-β and highest in water samples from site-α). Collectively, all samples from site-α showed higher diversity as compared to site-β (Fig. 1b). Simpson's index (equitability) values were in range 0.5–0.9: maximum in site-α water samples and minimum in site-β water samples. Chao1 and ACE indices reflected the species richness and showed that fish gut from site-β had a lower microbial community richness compared to the fish gut samples from site-α (Table 1). From both the sites, water samples possessed greater microbial community richness as compared to the gut samples. Good's coverage values (≥ 0.9) signify that good sequencing coverage was accomplished. As Kruskal–Wallis test showed $p > 0.05$, and thus, no statistically significant relationship was found between the standard deviations of the alpha diversity values (Shannon/Simpson).

Non-metric multidimensional (NMDS) analysis was employed using weighted and unweighted UniFrac distance matrix (stress values: 5.9e–05; 0.019) and showed that gut

Table 1 Sequencing statistics and alpha diversity indices of samples collected from polluted and less polluted sites of river Yamuna

Sample	Effective tags	Average length (nt)	Q30	GC %	Alpha diversity indices				
					Shannon	Simpson	ACE	Chao1	Goods coverage
Cyprinus carpio (α-C1)	93,304	455	96.9	54.3	2.7	0.7	166.4	158.2	0.9
Cyprinus carpio (α-C2)	459,351	457	98.2	55.0	3.3	0.8	153	132.1	0.9
Water (α-W1)	25,118	443	97.1	50.6	4.9	0.9	485.3	473	0.9
Water (α-W2)	512,921	455	98.4	50.9	5.4	0.9	565.7	592	0.9
Cyprinus carpio (β-C1)	36,820	468	99.7	55.4	2.3	0.7	44	44	1.0
Cyprinus carpio (β-C2)	31,655	469	99.6	55.9	2.9	0.7	39	41	1.0
Water (β-W1)	92,580	426	95.7	52.9	3.3	0.5	745	693	0.9
Water (β-W2)	94,321	427	95.9	52.8	3.5	0.6	512	473	0.9

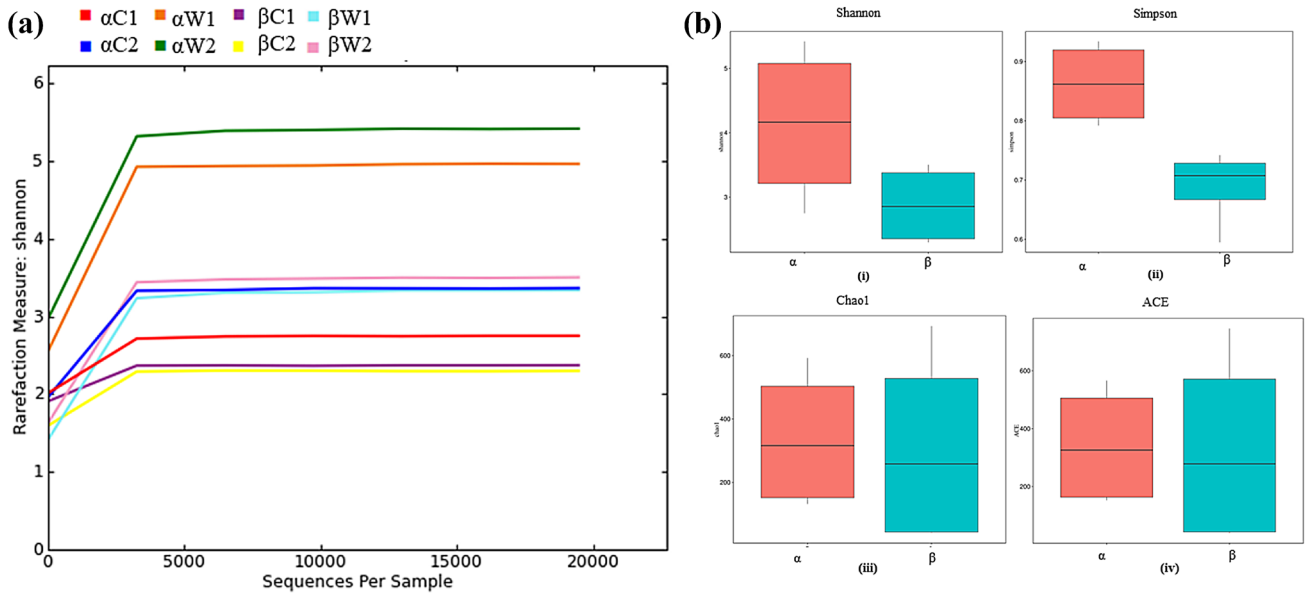

Fig. 1 Comparative rarefaction curves and alpha diversity of gut and water samples based on Shannon, Simpson, Chao 1, ACE indices. **a** Rarefaction curve indicated the sequencing depth of eight samples; *C. carpio* gut samples (αC1, αC2) and water samples (αW1, αW2) from polluted site-α; as well as *C. carpio* gut samples (βC1, βC2)and water samples (βW1, βW2) from the less polluted site-β; **b** Boxplots illustrated deviations in alpha diversity between the polluted (site-α) and less polluted (site-β). These deviations were assessed using four indices: (i) Shannon index, (ii) Simpson index, (iii) chao1, (iv) ACE index. Significance testing was conducted using the Kruskal–Wallis test ($p > 0.05$), revealing a relationship between the standard deviations of the alpha diversity values

and water samples from site-α clustered together, whereas samples from site-β tended to form a separate group indicating differences in bacterial composition between the two sites (Fig. 2a, b). The PCoA clustering employing weighted UniFrac distances showed concordant results where microbiome samples of gut and water from site-α and site-β separated along PC1 with 90.52% of total variation, whereas along PC2, variation was 8.09% and overall total variation was 98.61% (Fig. 2c). The ordinated beta diversity analysis supports the hypothesis that water and gut microbiomes from site-α differ from the microbiomes from site-β and implies that different environmental conditions and water quality has a huge influence on the bacterial community composition of the samples. The results implied the presence of different community dynamics in these geographically and physicochemically heterogenic ecosystems.

Taxonomic profiles

Multiple differences were observed in the relative abundance proportions of various taxa from phylum to genus level while comparing site-α and site-β samples. *Proteobacteria* was omnipresent in all the examined samples. In the area designed as site-α, *Proteobacteria* (97.7%) and

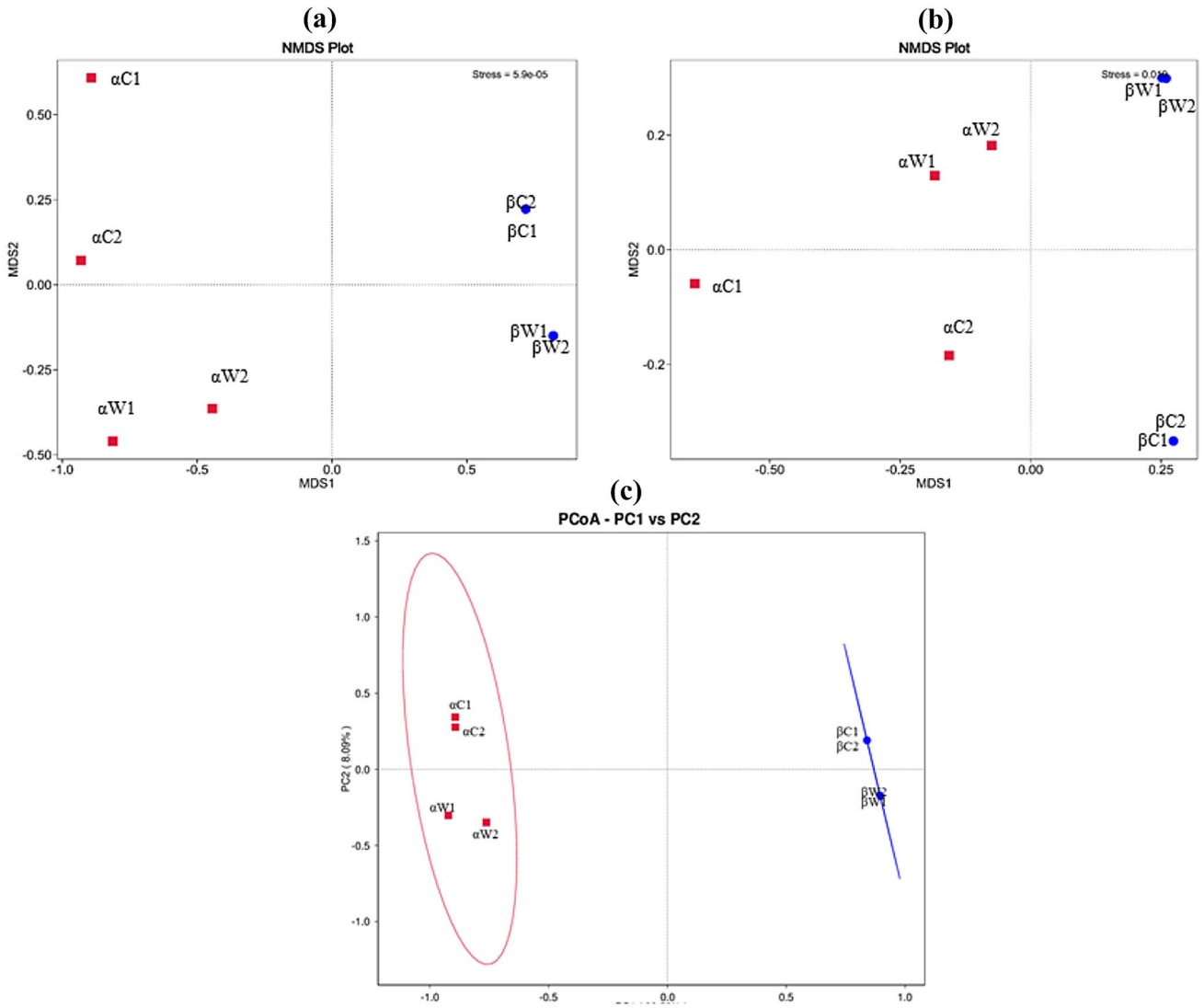

Fig. 2 The microbial community differences among gut samples of fish and water samples were visualized using non-metric multidimensional plot (NMDS) and principal coordinate analysis (PCoA). The stress values for the NMDS plot were 5.9e–05; 0.019 indicating a good representation of the data. The dots on the plot were color-coded, with red representing samples from polluted site (site-α) and blue representing samples from less polluted site (site-β). The pairwise distances between these samples were determined using both **a** weighted and **b** unweighted UniFrac algorithms; **c** Principal coordinate analysis (PCoA) was performed using the weighted UniFrac algorithm. In the NMDS plot and PCoA, the gut and water samples from the polluted site (site-α) clustered together, while the samples from less polluted site (site-β) formed a separate group. This clustering indicates significant differences in bacterial composition between the two sites, and this analysis was conducted using R (v-3.7) software

Actinobacteria (1.2%) were detected in the gut samples (Bharti et al. 2022), while in site-β, we observed the dominance of *Proteobacteria* (98.5%), *Fusobacteria* (1.04%) and *Firmicutes* (0.3%) in the fish gut (Fig. 3). Water samples pointed out a remarkable difference showing *Bacteroidota* (60.5%) as a major phylum followed by *Proteobacteria* (31.1%), *Actinobacteria* (3.6%), and *Cyanobacteria* (2.3%) in site-α, whereas *Proteobacteria* (85.8%), *Planctomycetes* (12.03%), *Bacteroidota* (4.4%), *Verrucomicrobiota* (3.7%), *Actinobacteria* (2.5%), and *Firmicutes* (1.8%) were enriched in the water samples from site-β.

At the class rank level, *Gammaproteobacteria* was highly frequent in all the samples. In common carp from site-α, besides *Gammaproteobacteria* (97.4%), *Actinobacteria* (1.27%) was also found. In site-β, *Gammaproteobacteria,* (98.5%), *Fusobacteria* (1.04%), and *Bacilli* (0.2%) dominated the fish gut. *Bacteroidia* (60.5%) was highest followed by *Gammaproteobacteria* (24.2%), *Alphaproteobacteria* (6.9%), and *Actinobacteria* (2.3%) in polluted water, while in the less polluted water samples, *Gammaproteobacteria* (81.1%), *Alphaproteobacteria*

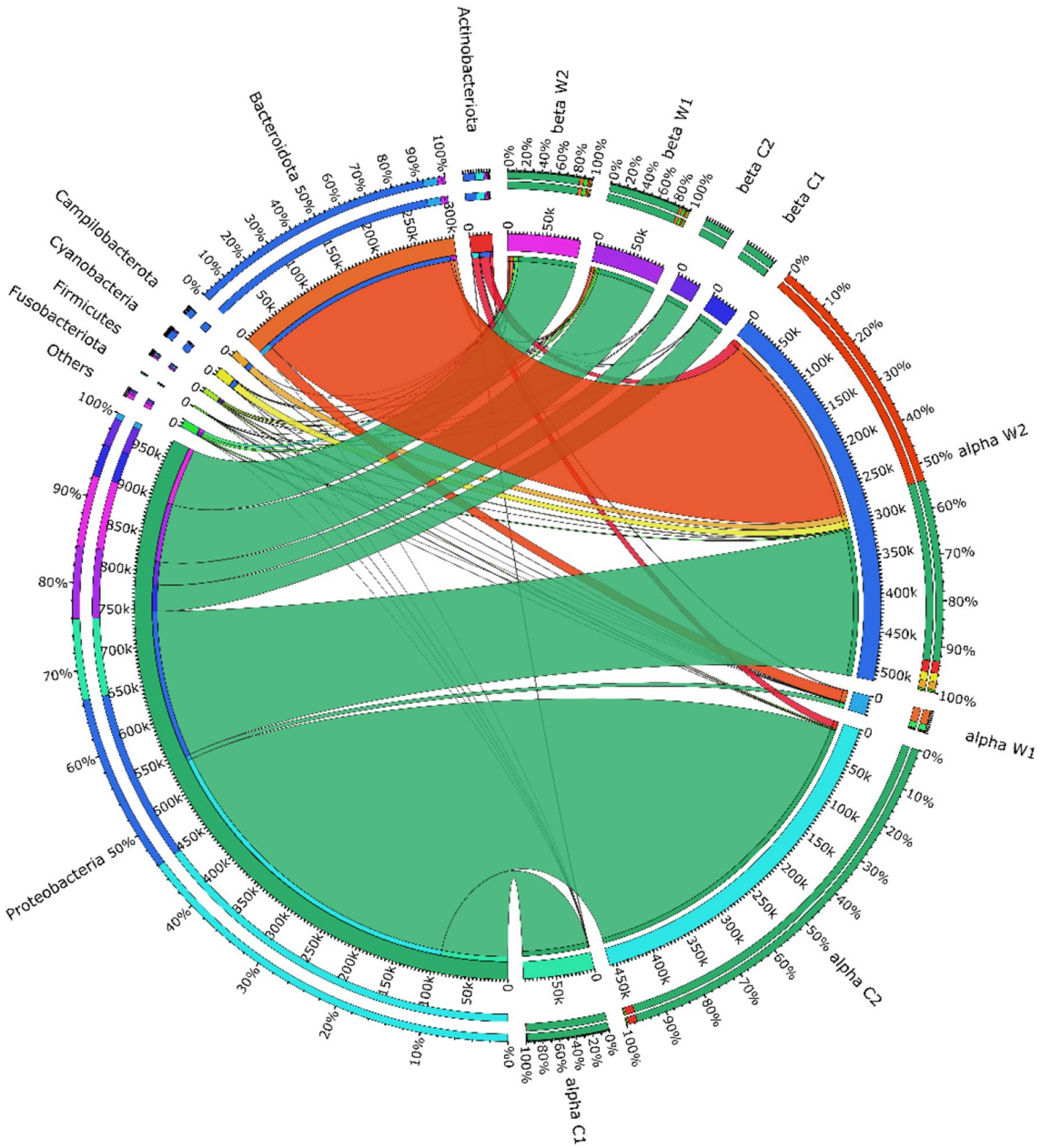

Fig. 3 Microbial community's abundance at the phylum level was assessed using Circos (v0.63-10). The analysis involved mapping eight samples based on their abundances, which were depicted in the outer circle, while the inner circle represents the synteny with the respective phyla. In the gut samples from site-α, *Proteobacteria* (97.7%) and *Actinobacteria* (1.2%) were enriched, whereas site-β exhibited dominance of *Proteobacteria* (98.5%), *Fusobacteria* (1.04%), and *Firmicutes* (0.3%). In the water samples, *Bacteroides* (60.5%); *Proteobacteria* (31.1%), *Actinobacteria* (3.6%), and *Cyanobacteria* (2.3%) in site-α. For site-β, the water samples were enriched in *Proteobacteria* (85.8%), *Planctomycetes* (12.03%), *Bacteroidota* (4.4%), and *Verrucomicrobiota* (3.7%)

(4.6%), *Bacteroidia* (4.4%), *Verrucomicrobia* (3.7%), and *Actinobacteria* (2.4%) were enriched (Fig. 4).

At genus level, *Aeromonas* (36.6%), *Escherichia-Shigella* (35.9%) and, *Vibrio* (6.1%) were abundantly found in the fish gut in site-α, whereas in site-β, *Aeromonas* (31.9%), *Enterobacter* (20.1%), *Plesiomonas* (2.6%), *Cetobacterium* (1.3%), and *Kosakonia* (0.8%) harbored the fish gut. In water samples from site-α, *Flavobacterium* (58.6%), *Limnohabitans* (10.1%), *Pseudorhodobacter* (4.6%), and *Polynucleobacter* (2.5%) were enriched, while *Limnohabitans* (5.9%), *Luteolibacte*r (2.5%), *Flavobacterium* (1.1%), *Pseudorhodobacter* (0.9), *Rhodobacter* (0.6%), *Novosphingobium* (0.5%), *Dechloromonas* (0.03), *Dechlorobacter* (0.01%), *Azoarcus* (0.01%), Candidatus "*Nitrospira*" (0.01%), and

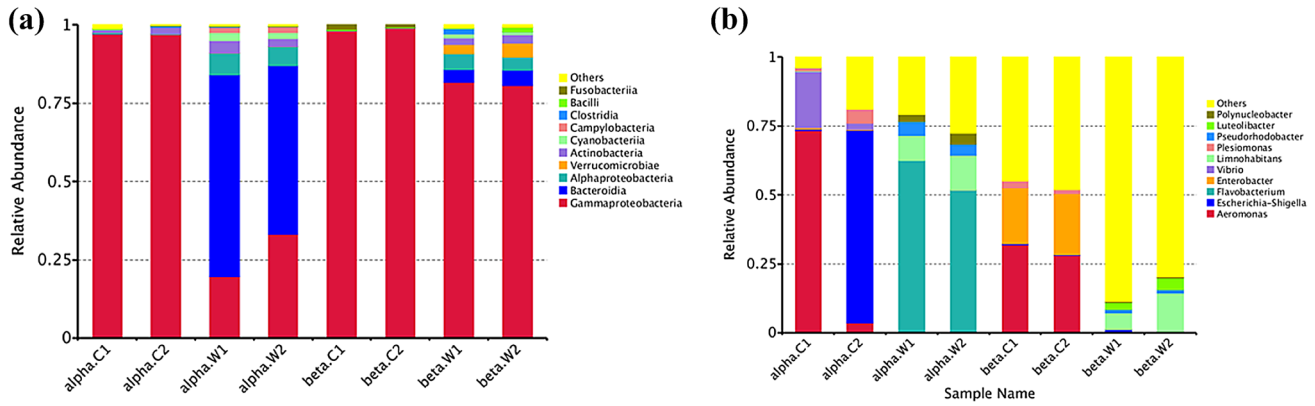

Fig. 4 A multi-stacked histogram plot was generated to illustrate the relative abundances of microbial diversity up to the class and genus level. **a** The plot depicted the distribution of the most abundant class distribution in gut samples, where *Gammaproteobacteria* accounted for 97.4% in site-α and 98.5% in site-β. Water samples in site-α exhibited the highest abundance of *Bacteroidia* (60.5%), followed by *Gammaproteobacteria* (24.2%) and *Alphaproteobacteria* (6.9%). In site-β water samples, *Gammaproteobacteria* (81.1%), *Alphaproteobacteria* (4.6%), and *Bacteroidia* (4.4%) were the enriched classes; **b** Multi-stacked histogram plot representing relative abundance at genus level. In the fish gut of site-α, the most abundant genera were *Aeromonas* (36.6%), *Escherichia-Shigella* (35.9%), and *Vibrio* (6.1%). In site-β, the dominant genera in the fish gut were *Aeromonas* (31.9%), *Enterobacter* (20.1%), and *Plesiomonas* (2.6%). Water samples from site-α showed an enrichment of *Flavobacterium* (58.6%), *Limnohabitans* (10.1%), and *Pseudorhodobacter* (4.6%). On the other hand, water samples from site-β comprised *Limnohabitans* (5.9%) *Luteolibacte*r (2.5%), and *Flavobacterium* (1.1%)

Sulphuritalea (0.01%) were detected in water samples from site-β (Fig. 4). The results showed that the microbial composition was different in site-α and site-β samples.

Upon data analysis, we found a repertoire of potentially pathogenic microbes, viz. *Aeromonas veronii* (0.3% C; 0.1% W), *Escherichia coli* (0.3% C; 0.01% W), *Vibrio cholerae* (0.9% C; 0.01% W), *Acinetobacter junii* (0.03% C; 0.1% W), *Streptococcus iniae* (0.2% C; 0.1% W), *Shewanella putrefaciens* (0.03% C; 0.8% W), *Pseudomonas aeruginosa* (0.08% C), and *Staphylococcus aureus* (0.03% C) in gut and water samples from site-α. These potentially pathogenic genera were also detected from site-β but with very low abundance (Table 2). We calculated the pathogenic OTUs from both the sites, and as a result, 68 pathogenic OTUs from site-α and 25 OTUs from site-β were detected.

An interesting observation from site-α host microbiome was the presence of potentially probiotic microbes such as *Bacillus velezensis* (0.5%), *Lactobacillus plantarum* (0.7%), *Enterococcus faecalis* (0.3%), *Bifidobacterium longum* (0.8%), *Lactococcus lactis* (0.3%), and *Leuconostoc falkenbergense* (0.1%), while site-β gut microbiome showed the presence of *Cetobacterium somerae* (1.3%), and *Blautia glucerasea* (0.01%) were found.

Heavy metal and organic matter tolerants

Physicochemical parameters of water samples from both sites revealed that heavy metals Cr, Fe, and Zn were exceeding the permissible limit in site-α as compared to site-β, and therefore, we attempted to explore heavy metal microbial tolerants. *Leucobacter chromiireducens* (1.05% C; 0.08% W), *Pseudomonas aeruginosa* (0.08%), and *Pseudomonas fluorescens* (0.2%) were found in gut samples from site-α, and opportunistic pathogen *Pseudomonas aeruginosa* (0.0005%) was detected from water samples from site-β. Furthermore, microbial communities involved in organic matter decomposition were also found which is in line with the high pollution load in river Yamuna. In site-α, *Methanothrix harundinacea* (0.25%) was detected in gut samples and *Dechloromonas agitata* (0.65%), Candidatus "*Nitrospira defluvii*" (0.5%), *Thauera humireducens* (0.12%), and *Zoogloea ramigera* (0.5%) were found in water samples. In water samples from site-β, only *Zoogloea oryzae* (0.02%), Candidatus "*Nitrospira defluvii*" (0.01%) were detected (Tables 2, 3). From site-α gut samples, a novel bacterium strain MB25^{T}, designated as *Sporosarcina cyprini* sp. nov., was isolated (Bharti et al. 2022). This bacterium demonstrated moderate tolerance to Cr^{+6} (20 mgL^{-1}) and Cd^{+2} (20 mgL^{-1}) which is line with the heavy metal pollution status of site-α.

Also, OTU network-based approach was employed to examine core and unique gut microbial communities clusters with the habitats together at the phyla level (Fig. 5). In the results, an expanded node represented a host–habitat sample and the OTUs were interconnected to the host and habitat in which they were residing. In accordance with the specific microbial compositional differences observed in the OTU network-based analysis, the OTU nodes of the host within the same habitat were more likely to connect with each other rather than to those from different habitats (Fig. 5). Here, we calculated unique OTUs in all the samples alpha-C1 ($n=30$); alpha-C2 ($n=12$); alpha-W1 ($n=2$); alpha-W2

Table 2 Comparative description of microbial diversity along with their biotechnological potential from polluted and less polluted sites of river Yamuna, India

Characteristics	Species	Gut microbiome samples from polluted site	Gut microbiome samples from less polluted site	Water microbiome samples from polluted site	Water microbiome samples from less polluted site	References
Pathogens	*Aeromonas sp.*	+	+	+	+	Kim et al. (2021)
	Escherichia sp.	+	+	+	+	Del Rio-Rodriguez et al. (1997)
	Vibrio cholerae	+	ND	+	+	Senderovich et al. (2010)
	Acinetobacter junii	+	+	+	+	Malick et al. (2020)
	Streptococcus iniae	+	ND	+	ND	Agnew and Barnes (2007)
	Shewanella putrefaciens	+	ND	+	ND	Allameh et al. (2014)
	Pseudomonas aeruginosa	+	ND	ND	ND	Thomas et al. (2014)
	Staphylococcus aureus	+	ND	ND	ND	Agnew and Barnes (2007)
	Flavobacterium sp.	ND	ND	+	+	Cai et al. (2013)
Probiotic potential	*Bacillus velezensis*	+	ND	ND	ND	Khalid et al. (2021)
	Lactobacillus plantarum	+	ND	ND	ND	Cebeci and Gürakan (2003)
	Enterococcus faecalis	+	ND	ND	ND	Allameh et al. (2014)
	Bifidobacterium longum	+	ND	ND	ND	Vijayaram and Kannan (2018)
	Lactococcus lactis	+	ND	ND	ND	Arriba et al. (2021)
	Leuconostoc falkenbergense	+	ND	ND	ND	Arriba et al. (2021)
	Cetobacterium somerae	ND	+	ND	ND	Kim et al. (2021)
	Blautia glucerasea	ND	+	ND	ND	Uyar GÖ and Yildiran (2019)
Bioremediation	*Leucobacter chromiireducens*	+	ND	+	ND	Tahri et al. (2016)
	Pseudomonas aeruginosa	+	ND	ND	ND	Jaber and Al-Mayahi (2020)
	Pseudomonas fluorescens	+	ND	ND	ND	Sharma et al. (2006)
	Methanothrix harundinacea	+	ND	ND	ND	Wagner et al. (2002)
	Dechloromonas agitata	ND	ND	+	ND	Wagner et al. (2002)
	Nitrospira defluvii	ND	ND	+	+	Silyn-Roberts and Lewis (2001)
	Thauera humireducens	ND	ND	+	ND	Wagner et al. (2002)
	Zoogloea ramigera	ND	ND	+	ND	Cydzik-Kwiatkowska and Zielińska (2016)
	Zoogloea oryzae	ND	ND	ND	+	Cydzik-Kwiatkowska and Zielińska (2016)

Table 3 Variation in microbial composition in fish gut and water samples from polluted and less polluted site

Species	Polluted site (%)	Less polluted site (%)
Gut samples		
Aeromonas sp.	36.60	31.9
Vibrio cholerae	6.0	0.06
Plesiomonas shigelloides	3.0	2.0
Cetobacterium sp.	0.09	1.04
Pseudomonas aeruginosa	0.08	0.005
Bifidobacterium sp.	0.06	0.005
Enterobacter sp.	0.36	20.1
Water samples		
Flavobacterium sasangense	6.27	0.01
Pseudorhodobacter sp.	4.5	1.2
Curvibacter sp.	1.3	1.7
Acidovorax sp.	0.96	0.09
Hydrogenophaga sp.	0.6	0.2
Polynucleobacter cosmopolitanus	0.6	0.2
Nitrospira defluvii	0.5	0.01
Acinetobacter junii	0.15	0.03
Shewanella sp.	0.06	0.005
Arcobacter cryaerophilus	0.04	0.01
Zoogloea sp.	0.04	0.02
Caulobacter sp.	0.03	0.005
Luteolibacter sp.	0.01	0.03

($n = 132$); and beta-W1 ($n = 228$); beta-W2 ($n = 95$), while in the case of beta-C1, C2 samples, all the OTUs were sharing homology with different samples. The shared OTUs among the gut samples from both the sites were $n = 106$, whereas among water microbiome samples were $n = 96$. Shared OTUs between all the samples from site alpha were 320 and from all the samples from site beta were 250. The results suggested that microbial diversity in fish from polluted site was higher than that from less polluted site clearly evident with the results of alpha diversity. Also, less number of shared OTUs between gut samples of both the sites suggested environment-driven variation was minimal while intraspecific microbiota was perpetuated.

Functional prediction

To understand variations in functional repertoire of the intestinal and surrounding water microflora as a consequence of anthropogenic activities and pollution, metagenomes' functional potential between the two groups were predicted by PICRUSt2. About 422 pathways were enriched among which 35 were found to be completed as shown in the heatmap (Supplementary S2, Fig. 6a). A maximum number of predicted functions were allocated to metabolic pathways in which biosynthesis pathways for L-isoleucine, cis-vaccenate, stearate, and palmitoleate, *bifidobacterium* shunt, and ectoine were present in the fish gut from site-α and menaquinol pathways were present in gut samples from both sites. Pathways for aerobic respiration, L-isoleucine biosynthesis, L-isoleucine biosynthesis, L-valine biosynthesis, and fatty acid and beta-oxidation were enriched in water samples from both sites.

PICRUSt2 also showed the incidence of enzymes involved in digestion and metabolism in the gut samples (Supplementary S3, Fig. 6b). Enzymes active against cellulose, hemicellulose, lignin, debris, and organic matter (cellulase (EC 3.2.1.4), lysophospholipase (EC 3.1.1.5), lysozyme (EC 3.2.1.17), chitinase (EC 3.2.1.14)) were found to be active in fish gut from both the sites as the principal nutritional constituent of this invasive fish includes phytoplankton, plant debris, and detritus. Commonly, peptidyl-prolyl isomerase (EC 5.2.1.8) and fumarate reductase (EC 1.3.5.4) were enriched in all gut samples. Nitrate reductase (EC 1.7.99.4) was more predominant in site-β samples and was also detected in gut samples from site-α. The presence of lignin-degrading enzymes such as peroxiredoxin (EC 1.11.1.15) and glutathione peroxidase (EC 1.11.1.9) in the water samples from site-α indicated the prevalence of debris and organic matter at the region; besides this enzyme chitinase (EC 3.2.1.14) was also present in the site-α samples.

Discussion

Microbial community structure of carps along with the function potential has been examined to be strongly influenced by the environment (Kakade et al. 2020; Jing et al. 2021). In present study, water contamination's influence on the microbiome dynamics of common carp was well attained by contrasting metagenomics analysis at two geographically unrelated and physicochemically different sites of river Yamuna. Low concentration of DO and elevated levels of BOD are pointers of worsening quality of the river water and are consistent with the anthropogenic activities and pollution at site-α of river Yamuna. In addition, high species diversity was observed in samples from polluted site as contamination can supplement surplus bacteria load and hence can lead to inflated alpha diversity (Minich et al. 2019). Beta diversity analysis revealed water contamination enlarged the differences in the composition of microbiome within the same species as the microbiome samples from two sites formed distinct clusters.

Taxonomic profiling showed that among all the classified sequence reads, phylum *Proteobacteria* predominated the fish gut irrespective of the site and could be signified as the core microbiome. The similar trend of preeminence of *Proteobacteria* in the fish gut has earlier been

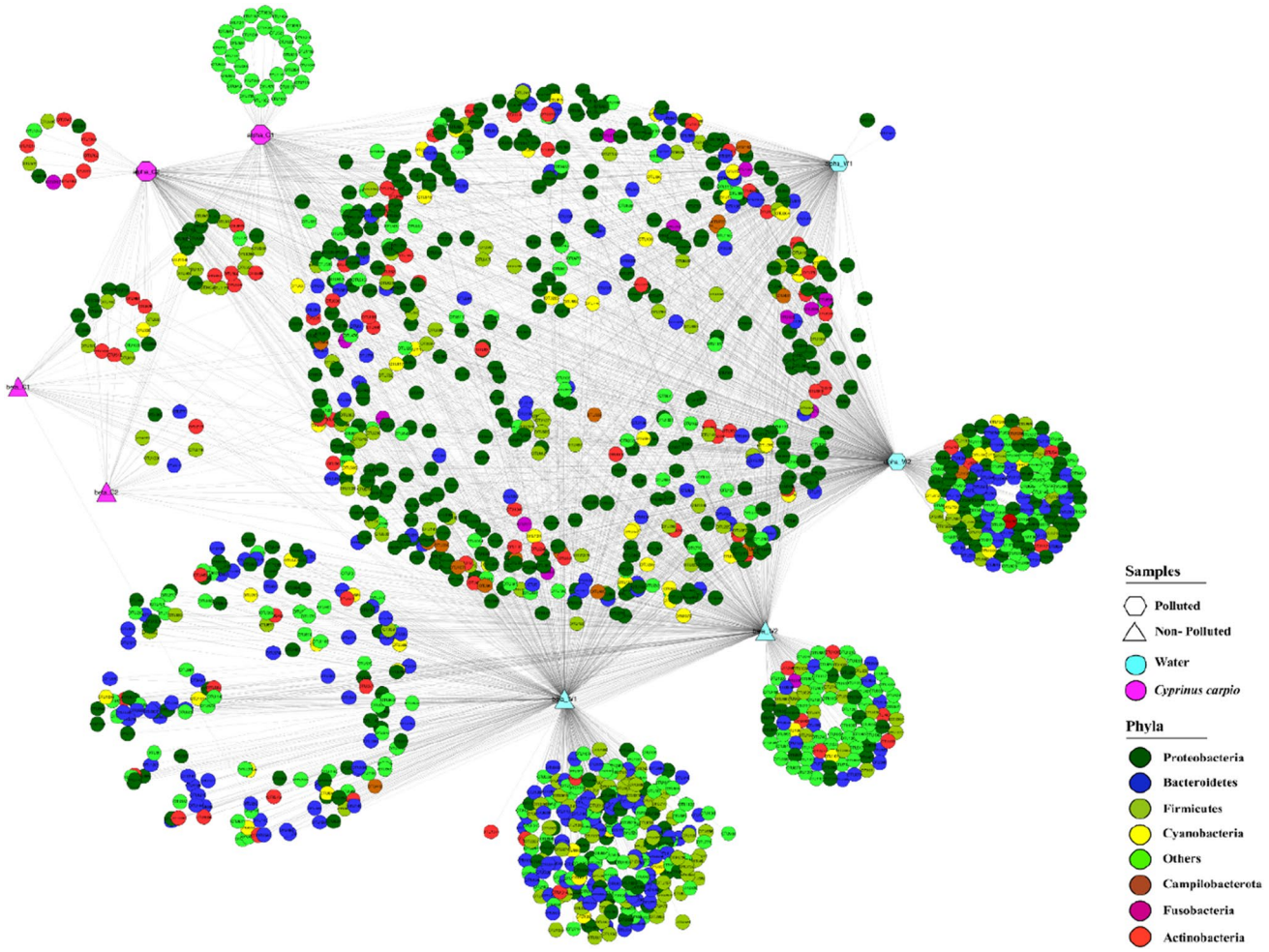

Fig. 5 An OTU network-based analysis was conducted to explore the shared and unique microbial communities present in fish gut and water samples from polluted and less polluted sites. The analysis involved connecting nodes representing fish and water samples (circles) to species-level OTUs, with color-coded edges indicating the host–habitat type. The edge-weighted spring embedded model in Cytoscape (v-3.0.1) was utilized for this purpose. Notably, OTU nodes belonging to the same habitat displayed a higher likelihood of being interconnected. In the gut samples, 106 shared OTUs were found to be shared, while in the water samples, 96 OTUs were shared. Furthermore, there were 320 shared OTUs among all the samples from site-α (polluted site) and 250 shared OTUs among all the samples from site-β (less polluted site). Within the gut samples, *Proteobacteria* exhibited the highest abundance, with 268,744 and 33,741 OTUs in site-α and site-β, respectively. In contrast, *Bacteroidota* (OTUs = 145,888) and *Proteobacteria* (OTUs = 80,173) were dominant taxa in water samples from site-α and site-β, respectively

supported by several research studies (Talwar et al. 2018; Liu et al. 2016; Tyagi et al. 2019; Johny et al. 2021). The occurrence of alike bacterial phylum irrespective of the geographical site specifies its role in vital functions such as digestion, nutrient absorption, and generation of the immune response. At genus level, *Aeromonas* and *Plesiomonas,* members of *Gammaproteobacteria,* were ubiquitous in all examined gut samples. Numerous studies have suggested that *Aeromonas* is a predominant genus in the gut of fishes feeding on detritus of plant origin and in omnivorous freshwater fishes as cellulose degrading species (Khurana et al. 2020, 2021). Likewise, *Plesiomonas* has been previously found in the gut microbiome of several fish species (Zhang et al. 2020, 2021). *Cetobacterium somerae,* a member of phylum *Fusobacteria,* deserves special mention as its presence might reflect combination of a fermentative metabolism and vitamin production in the GI tract. Several studies have demonstrated the role of *Cetobacterium* in many freshwater fish to satisfy their vitamin B_{12} dietary needs and can prevent the growth of harmful pathogens (Khurana et al. 2021; Sugita et al. 1990; Kim et al. 2021). The fact that carps do not have a dietary vitamin B_{12} requirement is well explained by the incidence of *Cetobacterium* in their gut (van Kessel et al. 2011). Another member with similar role is *Enterobacter* that has been earlier reported to be involved in production

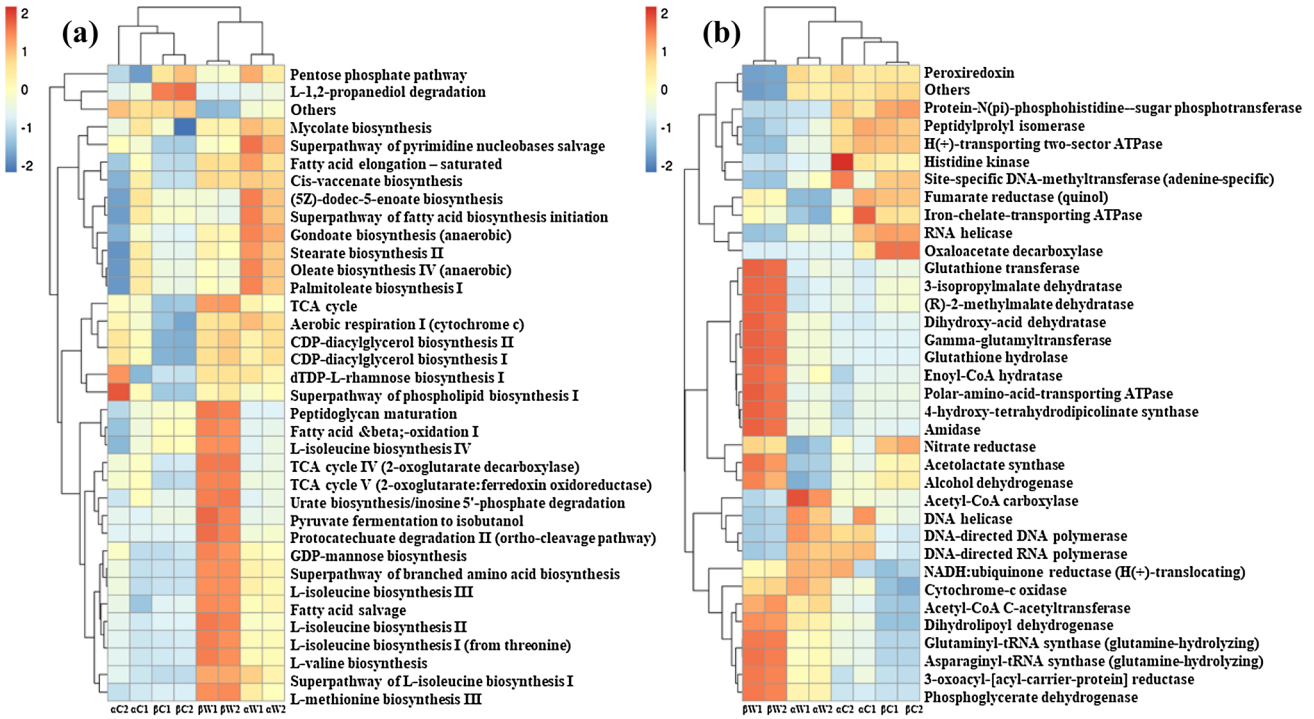

Fig. 6 Abundances of UniRef90 families and predicted metabolic pathways were represented using PICRUSt2 (Douglas et al. 2020). **a** Functional pathway prediction, **b** predicted enzymes of microbial communities in microbiome samples. A total of 422 pathways were enriched, with 35 complete pathways depicted in the heatmap. Gut samples from site-α exhibited pathways for L-isoleucine, cis-vaccenate, stearate, and menaquinone and stress protectant ectoine biosynthetic pathway. In the water samples from both sites, enriched pathways included L-isoleucine biosynthesis, L-isoleucine biosynthesis, L-valine biosynthesis involved in immunological function; The fish gut samples from both sites displayed the presence of cellulose, lysophospholipase, lysozyme, and chitinase enzymes, involved in the digestion of organic matter. The samples from site-β exhibited a higher predominance of nitrate reductase. In the water sample from site-α, the presence of lignin-degrading enzymes, such as peroxiredoxin and glutathione peroxidase, indicated the prevalence of debris and organic matter at the polluted site

of cellulose, amylase, and protease (Ray et al. 2012) stipulating its role in digestion.

In the water samples, from the site with better water quality, *Proteobacteria* was the most abundant that is consistent with the past studies of river Yamuna (Mittal et al. 2019; Rajeev et al. 2021). Another phylum within our amplicon sequences from the less polluted water samples was the *Verrucomicrobiae* that has been negatively correlated with pollution levels (Berg et al. 2012) and is involved in aerobic/obligate anaerobic fermentative metabolism. Additionally, occurrence of *Planctomycetes*, a bioindicator phylum, in site-β water samples and its absence in site-α indicate decent water quality in site-β, since its richness seems to decrease with increased pollution (Chen et al. 2019). Contrastingly, in the polluted site, a remarkable difference was observed with *Bacteroidota* being the predominant phyla followed by *Proteobacteria* that is distinct from previous metagenomics studies on River Yamuna (Mittal et al. 2019; Rajeev et al. 2021). This could be due to the fact that the phylum *Bacteroidota* is influenced with physicochemical parameters such as temperature and TDS and seasonal variation TDS (Kaevska et al. 2016; Zhang et al. 2012) and its prevalence in a polluted water sample indicates poor water quality, fecal contamination, and organic pollution (Tani et al. 2002; Ahmed et al. 2016). Also, the incidence of members of *Actinobacteria* in all samples from polluted site may suggest the increased susceptibility of the fish toward pathogens as *Actinobacteria* are known as antibiotic factories and are able to produce secondary metabolites against pathogenic microbes (Jami et al. 2015).

Furthermore, the prevalence of fish disease-relevant pathogens (*A. veronii*, *E. coli*, *V. cholera*, *A. junii S. putrefaciens, P. aeruginosa*, *S. iniae, S. aureus*) in polluted site indicated that deteriorating surroundings facilitated the invasion of pathogenic bacteria in fish gut eco-environment. Moreover, in the GI tract of fishes from polluted environment, probiotic members (*B. velezensis*, *L. plantarum*, *E. faecalis*, *L. lactis,* and *L. falkenbergense*) could be serving as a defense mechanism against above-mentioned fish pathogens by the production of active metabolites and bacteriocins (Meidong et al. 2017; Khurana et al. 2021). Hence, the incidence of both potentially pathogenic and probiotic strains in fish gut

from the polluted region suggests symbiotic relationship and reflects the affinity of the host for the microflora that contributes to the maintenance of immune function in stressed environments.

Chromium degrading *L. chromiireducens* and *P. aeruginosa* could be correlated with the high concentration of chromium in the polluted site (Joshi-Tope and Francis 1995; Austin and Allen-Austin 1985; Zhu et al. 2008; Bakiyaraj et al. 2014; Jaber and Al-Mayahi 2020). Past studies have explained the metabolic strategies underlying the possible mechanisms of bacterial resistance to heavy metals, involving direct ion export or reduction to a lower toxic/soluble (Silver and Phung 1996). *Nitrospira* species are anaerobic bacteria, involved in nitrogen cycling, and thus, their presence could offer new solutions for the removal of nitrogen from polluted water (Silyn-Roberts and Lewis 2001). A denitrifying member *Zoogloea sp.* was enriched in polluted site as compared to the other site. It is known for its role in poly-B-hydroxybutyrate production, metal biosorption, and bioremediation and is generally enriched in organically polluted waters (Wagner et al. 2002; Sağ and Kutsal 2000).

Functional prediction emphasized the significance of gut microbial community as contributors to the digestion and metabolism. Biosynthetic pathway for leucine, valine, isoleucine found in gut samples of both sites has been reported to have a role in immunity in fishes by enhancing lymphocyte proliferation in response to environmental pollutants (Tarnecki et al. 2019). Incidence of menaquinone pathway suggests role of gut microbiome in vitamin K2 production as fish receive its menaquinones from gut microflora, and it is essential for posttranslational modification of certain proteins required for blood coagulation (Krossøy et al. 2011). In gut samples from polluted site, ectoine biosynthesis constructed via an evolutionarily conserved biosynthetic pathway and is a stress protectant (Richter et al. 2019), while *Bifidobacterium* shunt (Bif Shunt) pathway has a unique feature for energy harvesting and its presence depicts an evolutionary adaptation confers a fitness advantage during natural colonization (Sanders et al. 2018). Thus, this study reflects that anthropogenic stress can modulate the fish gut microbiomes by influencing not only the microbial diversity, but also their potential function as well. On the one side, the host retains the stable multifunctional bacterial consortia, while on the other side, microbial diversity becomes specialized due to anthropogenic stress and selection pressure.

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Author contributions RKN proposed the idea. MB wrote the manuscript. MB and SN performed the analysis. RKN and SN critically reviewed the manuscript and improved it. All authors read and approved the final manuscript.

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Data availability The raw sequencing read data have been deposited in the National Centre for Biotechnology Information (NCBI) under the project Accession Number PRJNA809116.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval No special permission was required for this study.

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