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Metagenomic Characterisation of Antimicrobial Resistance Gene Profiles in Shrimp Farm, Pond Microbiomes From the Southern Coastal Areas of Kerala, India

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Abstract

Aquaculture is the fastest growing animal protein production sector in worldwide and Asia contributing annually with about 90% of the global seafood production. *Penaeus monodon* commonly known as the giant tiger prawn is a marine crustacean that is widely reared for food. The global rise in antibiotic resistance vitalised by indiscriminate antibiotic usage presents a significant public health challenge. The detrimental impact of antibiotic resistance includes the persistence of antimicrobial residues in fish and the surroundings. It gradually leads to the accumulation of resistance genes with the consequent contamination of the environment and the human food chain. There is a scarcity of data regarding the antibiotic resistance of shrimp aquaculture farms and ponds in Kerala. In the present study the metagenomic analysis revealed the occurrence of AMR genes across the southern coastal areas of Kerala, India. The pooled shrimp gut, water and sediment samples were subjected to metagenomic analysis. The antimicrobial resistance (AMR) genes were determined by various databases like RGI (Resistance Gene Identifier) and the Comprehensive Antibiotic Resistance Database (CARD). In addition to this, KEGG and COG databases revealed the important biochemical pathways, cellular functions and specific protein ortholog of bacterial and archaea genomes of the studied locations. The outcomes imply

noticeable regional differences in presence of AMR genes in the shrimp gut, sediment and water samples of the different southern coastal areas of Kerala.

Keywords: Metagenomics, Antimicrobial resistance, KEGG and COG Analysis

1.Introduction

Aquaculture is the productive industrial activity that will play a crucial role in providing solutions to the millennium challenges[1].Fish and seafood consumption will increase by 27%,according to expectations for aquaculture for 2030, in which a doubling of fish production is expected[2].Aquaculture is a strategic sector for the years ahead, providing food required for a rapidly growing human population, taking into account the need to reduce impacts for health and for the environment during food production. These goals are designed based on the 2030 UN Agenda of Sustainable Development Goals (SDGs), which aims to provide people with food and protect the Planet from degradation [3]. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators [4].Aquaculture systems and farms have been designated as “genetic reactors” or “hotspots for AMR genes” where significant genetic exchange and recombination can occur, which can shape the evolution of future resistance profiles [5,6]. Once bacteria have acquired Antibiotic resistant genes(ARG), they may exist in the environment for a long time, even after the selection pressure ceases [7].The major obstacles for the sustainable development and productivity of aquaculture is the frequent outbreaks of microbial diseases. These diseases cause huge amount of economic loss every year and put a burden on the farm owner and divert them which in many cases limit the production[8].Shrimp farmers intentionally or unintentionally use different aqua drugs to prevent disease which could be the probable reason for the occurrence of disease causing antibiotic resistant bacteria (ARB), i.e., the emergence of

antibiotic resistance genes [9]. Mixing of antibiotics in the feed or adding in the rearing water is the common mode of administration of antibiotics in shrimp aquaculture, which add the antibiotic residue in the environment and subsequently increase the possibility of AMR [10]. ARGs is recognized as one of the topmost threats to food safety and global health by the World Health Organization[11]. Therefore, responsible use of antibiotics is central to reduce the emergence of AMR phenomenon in aquaculture. Microbial community inhabiting the intestine of the shrimp, *Penaeus monodon* and their surrounding environments (e.g., water and soil) is considered as a key contributing factor for the sustainable farming of shrimp [12]. Indiscriminate application of antibiotics in aquaculture is a growing concern due to the emergence of antibiotic resistant bacteria, more specifically the antibiotic resistance genes. There is a consensus on the role of mobile genetic elements (MGEs) in the spread of bacterial resistance. Many resistance genes are carried on mobile genetic elements such as plasmids, integrons and transposons[13]. The gut microbiota plays a fundamental role in the health, growth, and disease resistance of animals, making it a critical area of focus in aquaculture research. A review on global antibiotic resistance reported that by the year 2050, 10 million lives per year are at risk due to the rise of infection by antimicrobial-resistant strains[14]. According to the United States Department of Agriculture (USDA), ~97% of fish and shellfish consumed in the U.S. are imported from China, Ecuador, India, Indonesia, Thailand, and Vietnam [15,16]. In the large aquaculture operations, where farmed shrimps are cultivated as monoculture at high density, the prevalence of pathogens is one of the biggest challenges faced by the aquaculture industry [17].

Furthermore, several studies have indicated that shrimp is a carrier of antibiotic resistant bacteria[18]. The rapid expansion of aquaculture practices, stocking overload, poor water quality, and inadequate biosecurity measures have resulted in increased disease outbreaks throughout the globe in different shrimp

aquaculture systems[19].Elevated transfer rates of antibiotic resistance may potentially give rise to new antibiotic resistant bacteria through the exchange of resistant genes within the human gut. This could disrupt the balance of human intestinal microbiota, posing a potential health risk [20].Our main objective in this study was to analyse antimicrobial resistance in southern coastal areas of Kerala shrimp farms and pond using metagenomic analysis. In our study we have investigated the microbiome composition and the occurrence of ARGs from 3 different shrimp farming locations including Munroe Island, Anjuthengu and Neyyatinkara. The shrimp gut samples, sediment and water samples from each specific locations were subjected to the metagenome analysis. The Resistance Gene Identifier (RGI) software acts as the analytical interface for the Comprehensive Antibiotic Resistance Database (CARD), mapping the specific sample DNA sequences against CARD's curated reference sequences. RGI uses Antibiotic Resistance Ontology (ARO) to classify the location specific antimicrobial resistant genes providing perfect algorithmic matches to identify antibiotic resistant genes (ARGs).The KEGG and COG databases revealed the important biochemical pathways, cellular functions and specific protein ortholog of bacterial and archaeal genomes of the studied locations.

2.Materials and methods

2.1. Sample Collection

The shrimp gut, water and sediment samples were systematically collected from three distinct southern coastal areas of Kerala. The specific locations are Munroe Island, Anjuthengu and Neyyatinkara. From each locations, 75 harvested shrimp samples were collected from a water depth of 70 cm by using a sterile grab. Approximately 250ml of the pond water and 25g sediment samples were collected carefully from each locations. These samples were then precisely stored in distinct autoclaved amber glass bottles and clearly labelled. All samples

were placed in sterile plastic bags, securely sealed, and transported on ice (4 °C), and subsequently stored at −20 °C for further analysis. The shrimp guts were isolated with proper care. The collected shrimps from different locations were pooled to location specific shrimp gut samples individually. The specific sampling method were illustrated in table 1. The isolated pooled shrimp gut samples were immediately transferred to ethanol. Shrimp gut DNA extraction was done as per the manufacturer's recommendation. The environmental parameters of each farm and pond were depicted in table 2.

2.2 Genomic DNA Isolation, Library Preparation, and Sequencing

Location specific genomic DNA was extracted from the pooled shrimp gut, water and sediment samples by QIAamp DNA microbiome Kit(QIAGEN), Nucleospin eDNA (QIAGEN) and the QIAGEN DNeasy Power Soil Pro kit respectively. The integrity and concentration of the extracted DNA were assessed using 1% agarose gel electrophoresis and Nanodrop TM (Thermo Scientific, Waltham ,MA, USA),respectively, and the samples were subsequently preserved at −20 °C for future analysis. A criterion for the DNA library construction was established, requiring an optical density (OD) absorbance between 1.8 and 2.0 at a 260/280nm purity ratio and a minimum DNA concentration of 1µg. The purified DNA from the respective samples were sent to GeneSpec Biotech firm, Kerala and Redcliff Labs ,Pune for library preparation and AMR prediction. The KAPA Hyper Plus DNA Library Prep Kit was employed to prepare the paired end sequencing library following the manufacturer's protocol. The DNA fragments were then purified using a minielute PCR purification kit. Post preparation of the libraries includes the segmentation of the DNA followed by quantification in conjunction with Hyper Ladder IV to ascertain the size of the DNA library. The libraries were pooled at equal molar concentrations for sequencing. An Illumina specific HiSeq model of

2X150 bp was utilized for sequencing and duplicate the samples were allocated over two lanes for comprehensive sequencing. The pre-processing of the raw reads is carried out using AdapterRemoval-v21 tool with quality threshold 20. The raw reads were processed to remove adapter sequences and low quality bases using fastp v.20.1 tool. Both the pre-processing tools were used sequentially in all the samples to ensure the highest possible data quality before downstream analysis. A summary statistics obtained through metagenomic analysis from the samples is mentioned in table 3.

2.3 Metagenome functional analysis

Data quality of the specific DNA samples were checked using in-house script. The data was checked for base quality distribution, %GC, and sequencing adapter contamination. The raw reads were processed to remove adapter sequences and low quality bases using fastp v.20.1 tool. The trimmed reads were processed to assembly using Megahit v1.2.9 tool. K-max and K-min parameters were used to assemble the reads. Shorter contigs were removed from the assembly and assembly quality were checked by mapping the trimmed reads on to the assembled contigs using Bowtie2 v2.4.4 tool. For obtaining the taxonomic classification of the samples, the contigs were processed to Kraken2 v2.1.2, which classifies the contigs using k-mer method against Kraken nucleotide database. Kraken2 results were visualized using Pavian package in R. Pavian gives the taxonomic classification in the form of sankey plots(Figure 1-3).The contigs were annotated and the eukaryotic and prokaryotic contigs were separated. The prokaryotic contigs KEGG and COG annotations were obtained by submitting the protein sequences of each sample to KAAS and Rpsblast respectively.

2.4 Bacterial Diversity Detection

The trimmed reads were processed to assembly using Megahit v1.2.9 tool. K-max and K-min parameters were used to assemble the reads. Shorter contigs were removed from the assembly and assembly quality were checked by mapping the trimmed reads on to the assembled contigs using Bowtie2 v2.4.4 tool. The contigs were processed to Kraken2 MEGARes 2.0, which classifies the contigs using k-mer method against Kraken database. Kraken2 results were visualized using Pavian package in R. Pavian gives the taxonomic classification in the form of sankey plots. Eukaryotic and Prokaryotic contigs were separated and Prokaryotic contigs were processed further for the annotation using Prokka v1.14.6 with the metagenome parameter. KEGG and COG annotations were obtained by submitting the protein sequences of each sample to KAAS and Rpsblast respectively. In the present study the shrimp gut, sediment and water sample analysis, the assembly file was annotated using PROKKA v1.14.6. To ascertain the presence of ARG within the respective samples the databases used were RGI - Resistance Gene Identifier (McMaster University) and Comprehensive Antibiotic Resistance Database (CARD). For the annotation of core orthologues, consensus sequences were subjected to BLAST analysis against the KOfam, a database of KEGG orthologues, employing KOfam KOALA. Following this, the egg NOG-mapper tool in conjunction with the EggNOG database, was utilized to systematically categorize all core orthologue sequences into clusters of orthologous groups of proteins (COGs). Metagenomes from the southern coastal areas of Kerala were analyzed using Clusters of Orthologous Groups (COG) mapping and KEGG Orthology (KO) mapping. Functional analysis revealed that a major proportion of the metagenome coded for housekeeping functions. According to COG mapping, the open reading frames (ORFs) were mainly associated with metabolism, cellular processes and signaling, and information storage and processing .

2.5 Statistical Analysis of samples

The complexity of biodiversity in Shrimp gut, water and sediment samples were analyzed by Alpha diversity. Alpha diversity was applied in analyzing the complexity of biodiversity using Shannon, Simpson, Radar plot, abundance-based coverage estimator (ACE) which were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Rather than manual calculation, these metrics were generated using the software such as the R vegan package and the QIIME 2 bioinformatic platform. These specialized tools process large abundance datasets to compute precise ecological diversity values. The Shannon index tracks community uncertainty and rare species, while the Simpson index highlights the influence of dominant species, providing a clear mathematical picture of our data's overall diversity. The species occurring once and species occurring twice were employed by the estimator to extrapolate the number of unobserved species [21]. Shannon Diversity Index expressed the quantifying richness (number of species present) and evenness (distribution of abundances among species) in a community [22]. It responded to small taxa and indicated how evenly individuals were distributed among species. Larger values indicated more diverse and evenly-balanced communities. Simpson Diversity Index was the probability of two randomly selected individuals in a sample belonging to the same species [23]. The Simpson index were more sensitive to dominant taxa and less sensitive to rare species compared to the Shannon index. The calculation of beta diversity distance were implemented by QIIME v1.9.1. Principal coordinate analyses (PCoA) plots were performed with internal scripts, based on Bray–Curtis distances and visualized using three-dimensional plots in EMPeror (a part of QIIME). The ASV abundance heatmap was directly designed from the abundance of unique amplicon sequences inferred from the raw sequencing data and showed the microbial composition of the samples with the top fifty most abundant species identified. Hierarchical clustering was performed on samples based on the Bray–Curtis dissimilarity Dada2 pipeline. For taxonomic

abundance analysis, raw abundance data were log-transformed ($\log_{10}[x+1e-9]$) and subsequently standardized using z-score normalization to allow cross-sample comparisons. Heatmaps were constructed based on z-scores to visualize the distribution patterns of key bacterial and fungal genera across environments.

3. Results

3.1. Sequencing Summary

The estimated size of the libraries of the samples varied between 2.2Gb, 3.8Gb, and 3.89Gb. The assembly statistics revealed that the largest contigs in the shrimp gut, sediment and water are 2,117,470bp, 1203245 bp and 883243 bp respectively.

Sample ID	Location of pond/Farm	Sample type	Number of sample pooled
1	Munroe Island	Shrimp gut	25
		Water	50ml
		Sediment	100gm
2	Anjuthengu	Shrimp gut	25
		Water	50ml
		Sediment	100gm
3	Neyyatinkara	Shrimp gut	25
		Water	50ml
		Sediment	100gm

Table 1: Characterization of shrimp farms and pond incorporated into the stud

Sample location	Temperature	pH	Salinity	Dissolved oxygen	Ammonia	Transparency	Nitrate/nitrite	Alkalinity	Aeration
Munroe Island	26- 32°C	8.2	20	35-4ppm	1.0 ppm	30cm	1.0pm	120	~350m ³
Neyyatinkara	27°C	8.5	20	35-4ppm	1.0 ppm	35cm	1.0pm	120	~450m ³
Anjuthengu	27°C	7.7	20	35-4ppm	1.0 ppm	37cm	1.0pm	120	~370m ³

Table 2: Environmental parameters of samples

Sample	Read of orientation	Library raw reads	Number of bases	Number of Scaffolds	L90	Largest contig	N50	N90	GC%
Water	R1	24,028,205	3,628.26	501447	36521	2117470bp	18211bp	536bp	48.39
	R1	24,028,205	3,628.26	501447	36521	2117470bp	18211bp	536bp	49.09
Shrimp gut	R1	14,316,917	2,161.85	22544	11506	1047319bp	1231bp	389bp	42.96
	R2	14,316,917	2,161.85	22544	11506	1047319bp	1231bp	389bp	47.27
Soil	R1	15,315,652	2312.66	45231bp	24447	739428bp	15083bp	512bp	55.23
	R2	15,315,652	2312.66	45231bp	24447	739428bp	15083bp	512bp	55.65

Table 3: Summary of statistics from metagenome assembly

3.2 Bacterial Diversity Analysis

Analysis of bacterial diversity conducted using Kraken2 and Pavian demonstrated that *Vibrio parahaemolyticus* (Vibrionaceae) were the most abundantly found species across the investigated gut samples of shrimp (Figure 1). Pseudomonades is a major phylum of gram-negative bacteria. The Bacillota phylum were Gram-positive bacteria. The Gram-negative bacteria were commonly known to be pathogenic to fish, more resistant than Gram-positive bacteria, and can cause significant morbidity and mortality worldwide[24]. Furthermore, Phylum Pseudomonadota is the most prominent Gram-negative bacterial fish pathogen[25]. The pathogens including toxins, adhesins, effectors, and enzymes promote colonization and infection of bacteria. Vibrionaceae and Shewanellaceae family were observed in the shrimp gut microbiome observed in the southern coastal areas like Munroe Island, Anjuthengu and Neyyatinkara. Proteobacteria plays an important role in nutrient cycling processes, such as nitrogen cycling, sulfide cycling, and mineralization of organic compounds in aquaculture ponds[26]. The water sample revealed the dominance of *Sinirhodobacter*. Meanwhile, in the sediment samples the dominance of *Sulfurospirillum* species were observed in respective locations. We observed the contribution of bacteria in the habitat by the Sankey diagram. The contributions of microbial genomes to individual metabolic and biogeochemical processes, with entire elemental cycles were described with Sankey diagram[27]. Freshwater bacteria are at the hub of biogeochemical cycles and control water quality in ponds [28]. In their natural habitats, microbial communities take the shape of intricate networks that share and compete for metabolites.

55 Archaea

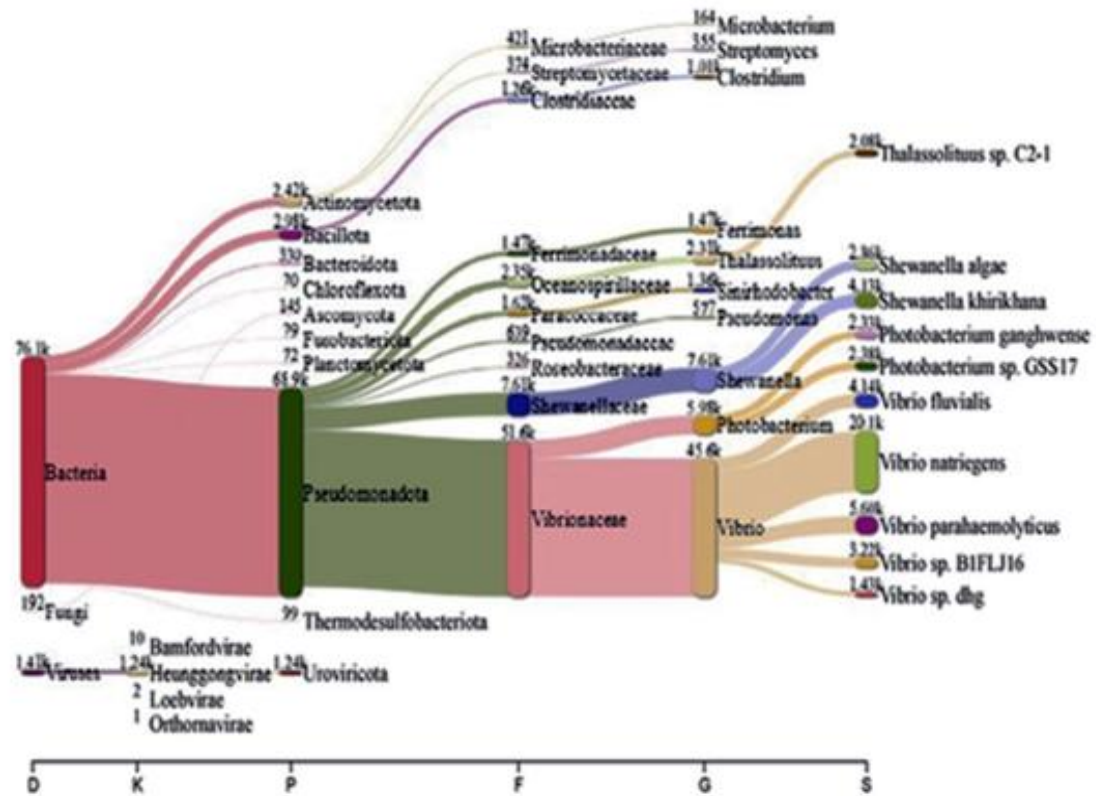


Figure 1: Sankey diagram of shrimp gut samples from different farm sites. The number above each node is the number of k-mer hits. A rank code, indicating domain (D), phylum (P), family (F), or genus (G) was used. The Sankey plot reveals the dominance of the taxon *Vibrio parahaemolyticus*.

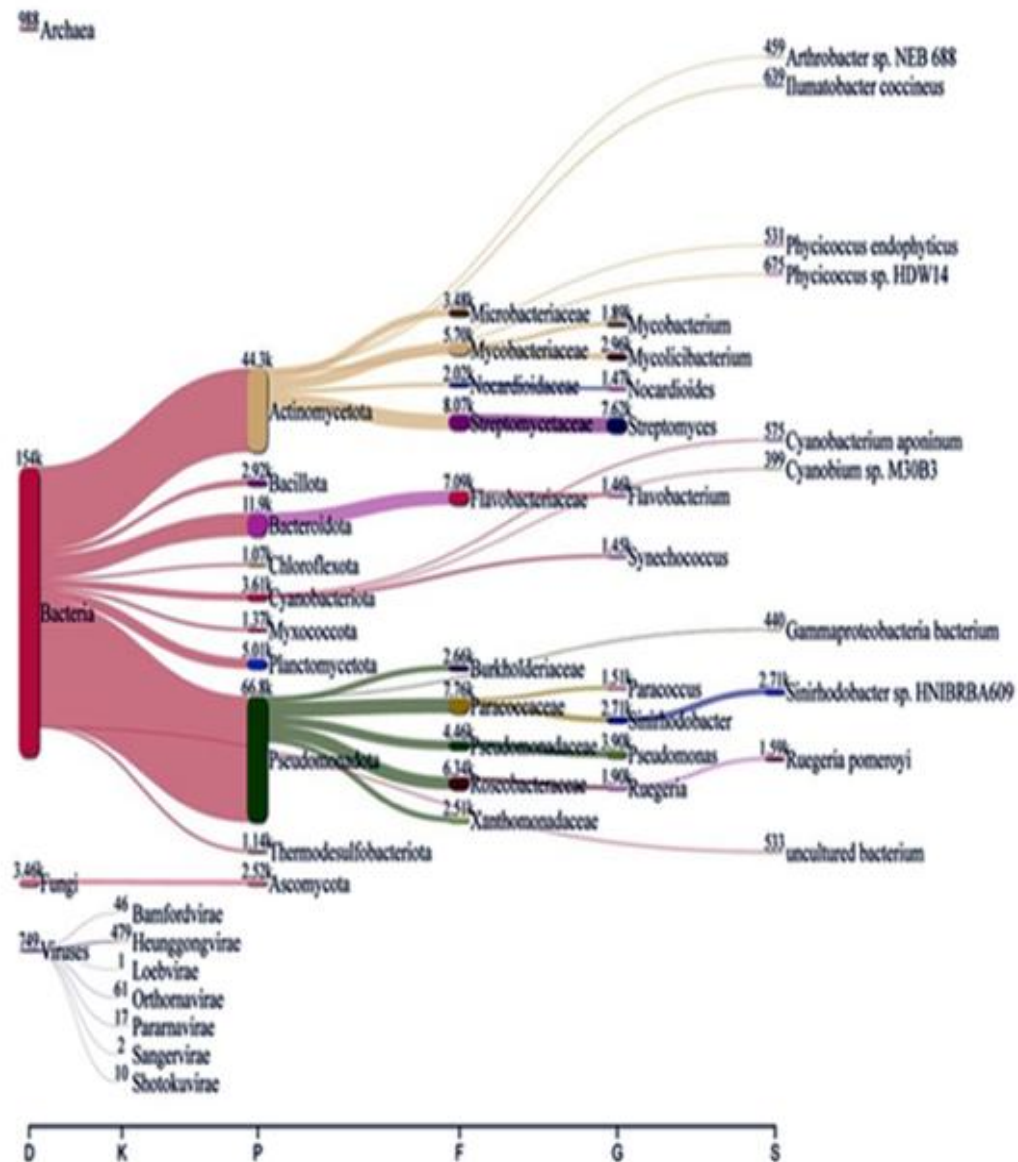


Figure2: Sankey diagram of soil sample from different farm sites. The number above each node is the number of k-mer hits. A rank code, indicating domain (D), phylum (P), family (F), or genus (G) was used. The Sankey plot reveals the dominance of the taxon *Sinirhodobacter* sp. HNIBRA609.

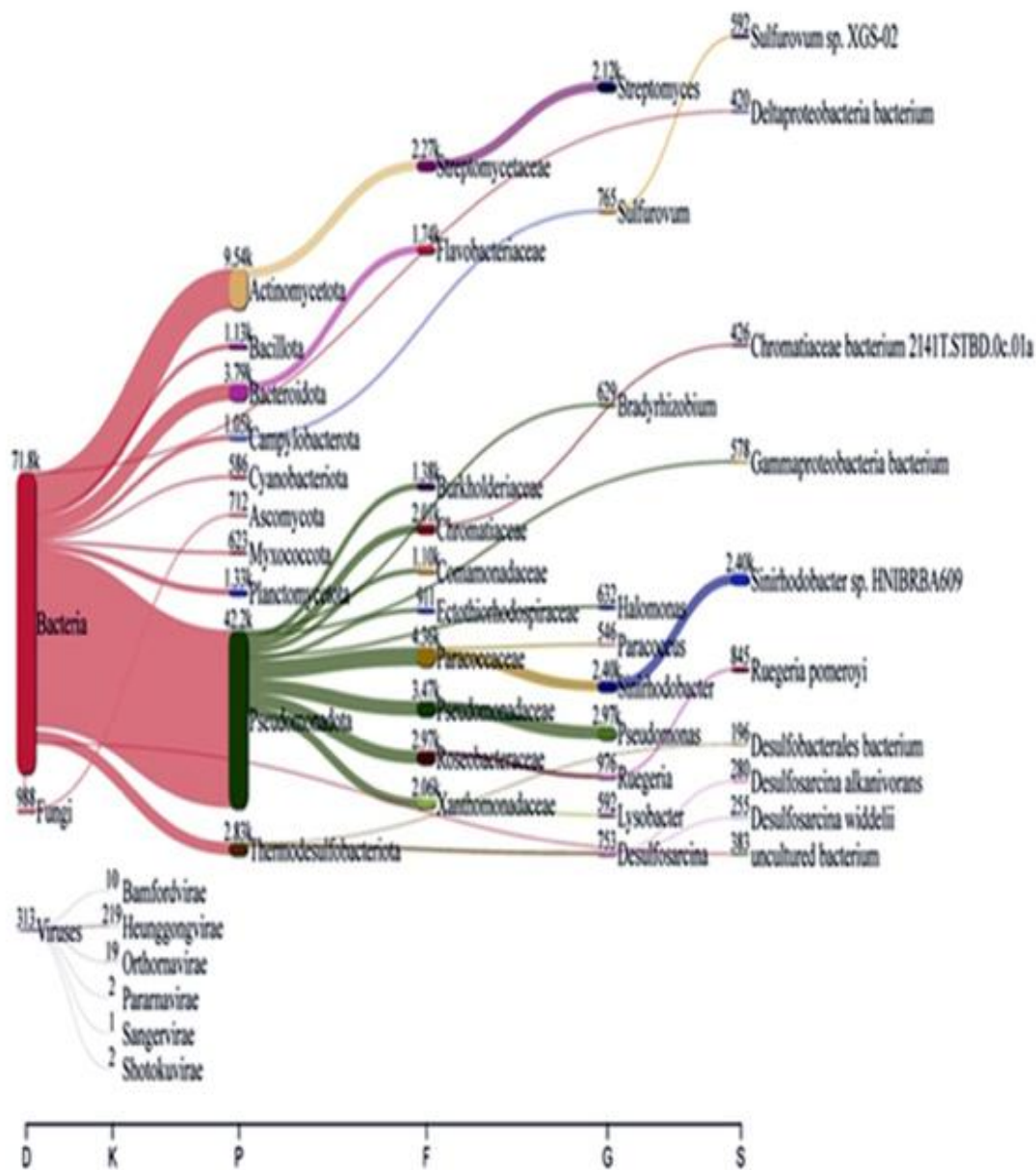


Figure 3: Sankey diagram of water sample from different farm sites. The number above each node is the number of k-mer hits. A rank code, indicating domain (D), phylum (P), family (F), or genus (G) was used. The Sankey plot reveals the dominance of the taxon streptomyces.

3.3 AMR Gene abundance

Geographical variability in ARG profiles were studied in the present study. This exploration depicted the geographical distribution patterns of antimicrobial resistance (AMR) genes across three discrete southern coastal areas of Kerala- Munroe Island, Neyyatinkara and Anjuthengu. The Open reading frame (ORF) prediction of the samples were done using Prodigal, the protein coding gene prediction analyser (v2.6.3).The assembled scaffolds from the denovo assembly is taken for the ORF prediction. The obtained ORFs are filtered using in house scripts. ORFs of length below 200 bp being filtered. Predicted ORF sequence was used to predict antimicrobial resistance genes (ARG) using RGI (Resistance Gene Identifier) database. The RGI uses the reference data from the Comprehensive Antibiotic Resistance Database (CARD),a curated collection of reference sequences and SNPs organized by the Antibiotic Resistance Ontology(ARO) to detect AMR genes. The specific RGI database version 6.0.x and CARD V3.2.x is used in AMR gene detection. .The RGI in the CARD applies stringent thresholds for AMR gene identification based on BLAST bit scores.The ORF length distribution of AMR genes in specific sites can be observed in table 2.We identified the diverse categories of AMR notably the antibiotics like Norfloxacin, Sulfamethoxazole, Ciprofloxacin, Sulfadimidin, Trimethoprim and Sulfadiazin .The outcome provides critical insights into the regional dissipation and the prevalence of AMR determinants. Norfloxacin resistance determinant such as qnrS, qnrS8 and qnrB were predominantly detected in shrimp gut samples, sediment samples and water samples from the southern coastal area of Kerala like Munroe Island. Munroe Island also showed the prevalence of Sulfamethoxazole resistant genes like sul1,sul1and sul2 and sul2 were detected in shrimp gut samples, sediment samples and water samples respectively. A novel Trimethoprim resistance marker particularly dfrA12 were prevalent in Shrimp gut samples, sediment samples were resistant with dfrA1 and

water samples with *dfrA* gene from the southern coastal area of Kerala like Anjuthengu. The prevalence of sulfadiazine resistant genes *sul1* and *sul2* were observed both in shrimp gut samples and sediment samples and the water samples were prevalent with both *sul1* and *sul2* genes from the spotted locations of southern coastal area of Anjuthengu, Kerala. *qnrA*, *qnrS* and *qnrS2* Ciprofloxacin predominant resistant genes were identified in shrimp gut samples, sediment samples and water samples from Neyyatinkara. Sulfadimidin resistant genes *sul1* in shrimp gut samples, *sul2* in sediment samples and *sul1* and *sul2* genes in water samples were specifically identified in the coastal area of Neyyatinkara, Kerala. The list of ARG found in the present study across the samples were depicted in table 4- 5.

Sample ID	Total genes	Genes > 200bp	Genes > 1000bp	Genes<1000 bp
Shrimp Gut	800907	669131	78622	199690
Sediment	108137	92427	19533	32701
Water	59660	51856	1098	18048

Table 4: Summary of ORF length distribution of AMR genes

Sample location	ARO	Gene/Family	Drug Class	Resistance mechanism	Detection criterion	Identity/Coverage
Munroe Island	3004667	/Norfloxacin	Fluoroquinolone	Antibiotic efflux	CARD	53.9
	3000329	/Sulfamethoxazole	Sulfonamide	Sulfonamide inhibition	CARD	61.76
Neyyatinkara	3007010	/Ciprofloxacin	Fluoroquinolone	Antibiotic efflux	CARD	68.94
	3000325	/Sulfadimidin	Sulfonamide	Antibiotic target alteration	CARD	28.93
Anjuthengu	3000188	Trimethoprim	Antifolatene	Antibiotic efflux	CARD	38.66
	3000410	Sulfadiazin	Sulfonamide	Antibiotic target replacement	CARD	100

Table 5 : List of ARG found in the present study across the samples

This study highlighted distinct regional disparities in the distribution of AMR gene. The outcomes provide critical insights into the regional distribution of diverse categories of AMR genes, and the existence of AMR determinants.

3.4 KEGG Pathway Analysis

In the present study KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis revealed a various range of biological processes across all three distinct locations of the southern coastal areas of Kerala. The KEGG ortholog of annotated protein from specific samples were depicted in fig 4-6. The antimicrobial resistance pathways were significantly represented especially in Munroe Island and Neyyatinkara. The core metabolic pathways, including energy and carbohydrate metabolism, were uniformly present across all locations. There

was a noticeable diversity in amino acid metabolism pathways, enhancing the metabolic adaptability of organisms in different environments. We also found unclassified pathways, possibly linked with genetic information processing and the metabolism process.

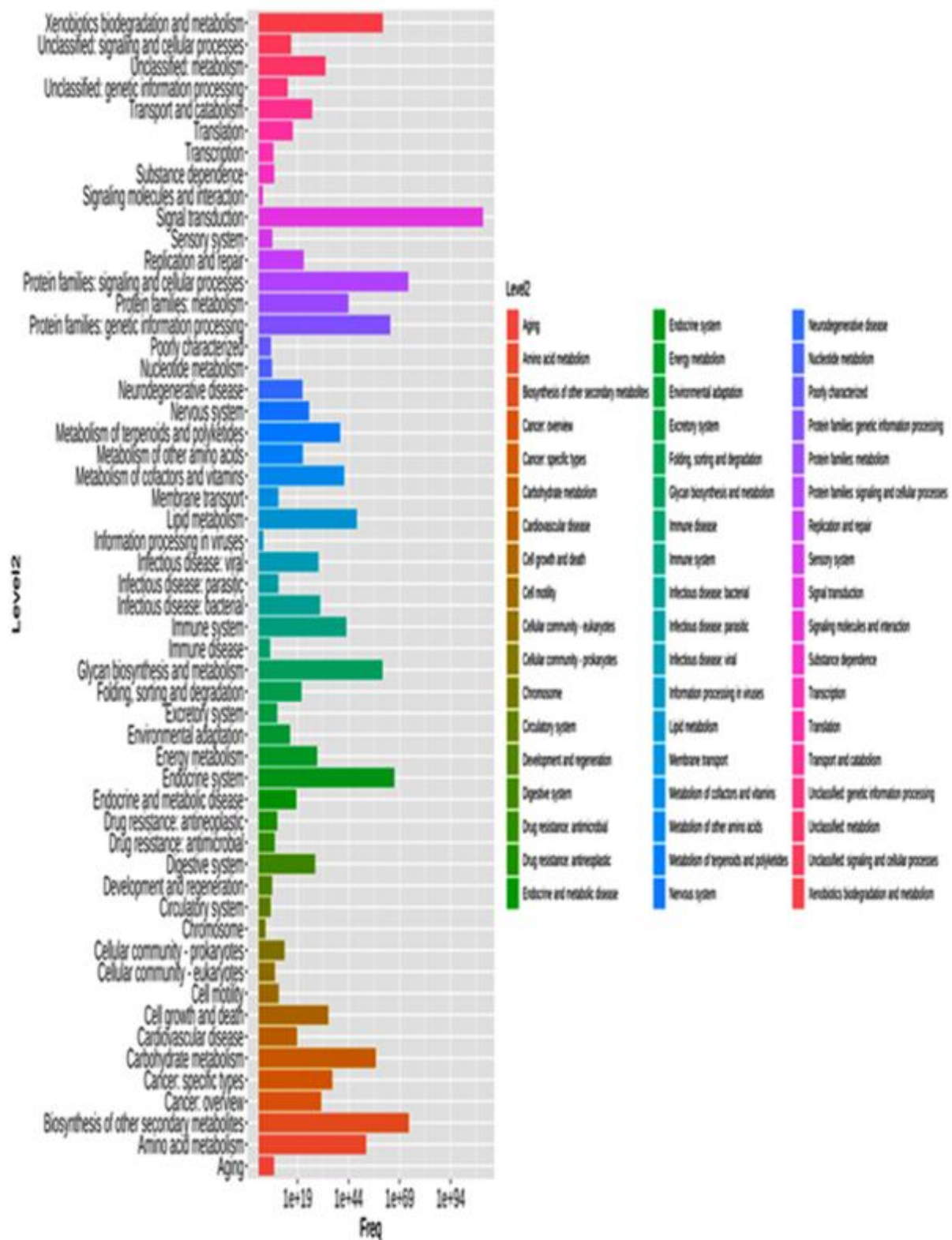


Fig 6: KEGG ortholog of annotated protein from Water

3.5 COG Analysis

The Cluster of Orthologous Groups (COG) analysis conducted across the three locations revealed varied distributions of functional categories. The categories related to the energy production and conversion, amino acid transport and metabolism, carbohydrate transport and metabolism were prevalent. The COG ortholog of annotated protein from specific samples were illustrated in fig 7-9. The significant representation of cell cycle control, cell division, chromosome partitioning and replication, recombination and repair highlights the active cellular processes occurring in these environments. The variability in defense mechanisms, Signal transduction mechanisms and inorganic ion transport and metabolism across locations were found.

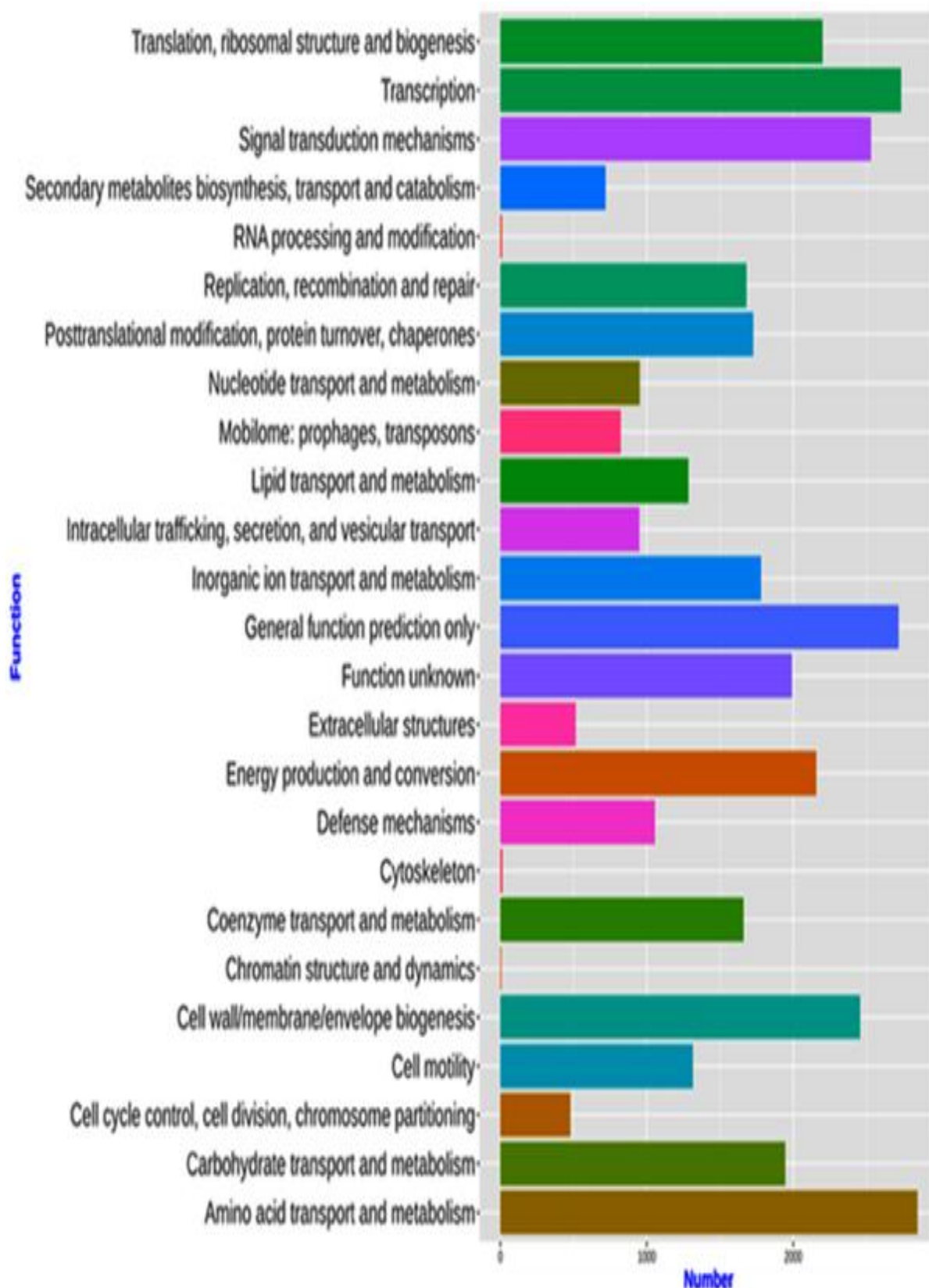


Fig 7 : COG functional category distribution of shrimp gut

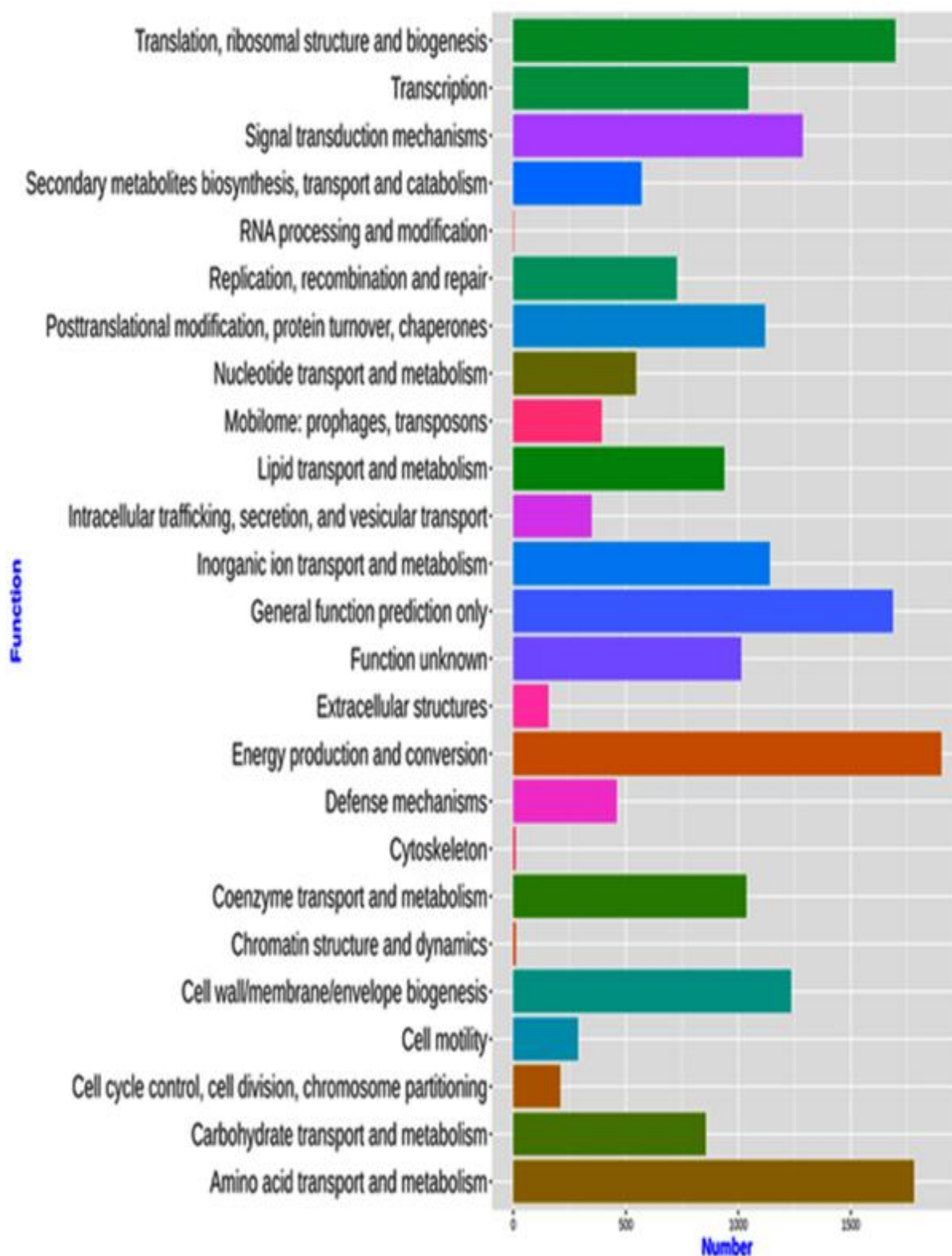


Fig 8 : COG functional category distribution of sediment samples

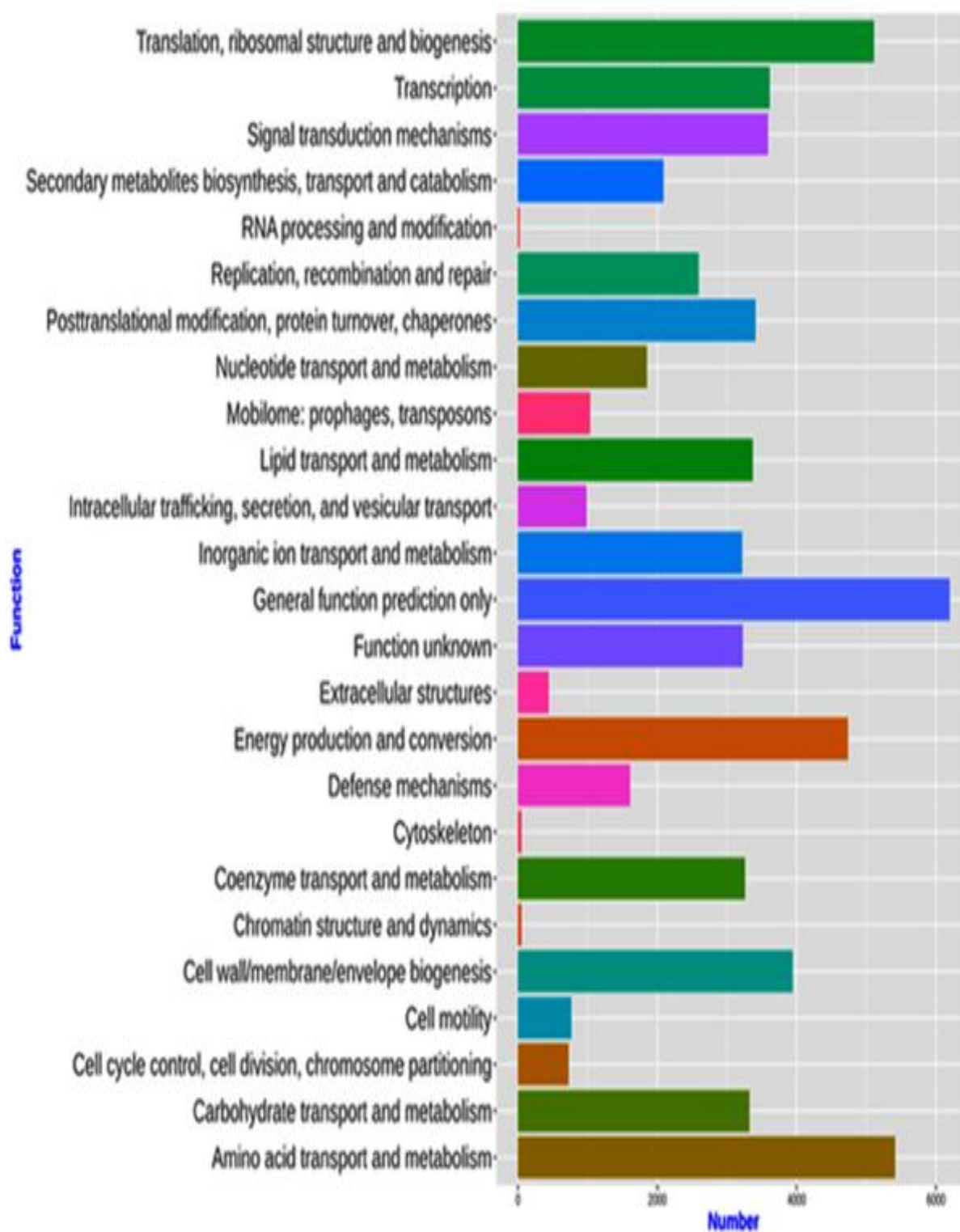


Fig 9 : COG functional category distribution of water samples

3.6 Statistical Analysis

There was no significant difference in the alpha diversity index of shrimp gut bacteria between the two groups of samples. The alpha diversity indices highlight notable differences in species richness and diversity among bacterial, archaeal, and viral communities in aquaculture pond sediment, shrimp gut, and water fig 10. For bacterial communities, water samples exhibit the highest species richness, followed by shrimp gut and sediment having the lowest fig 11. Both sediment and water show high diversity and minimal species dominance (Shannon and Simpson indices), while shrimp gut shows moderate diversity and higher dominance by fewer species fig 12. Archaeal communities follow a similar pattern, with high species richness in sediment and water, and lower richness and diversity in shrimp gut in heat map. Viral communities also show the highest species richness and diversity in water, followed by sediment, and the least in shrimp gut. This suggests that water supports the most diverse microbial communities, with sediment being moderately diverse, and shrimp gut being less diverse and more dominated by specific species. Beta diversity analysis showed the significant differences in microbial community composition among the different sediment samples based on the Bray-Curtis index. It showed significant differences in microbial community composition among the different shrimp farms and pond sediment samples, which were strongly influenced by water salinity. Sediment samples showed dissimilarity ≥ 0.851 suggesting a quite different microbial composition. Meanwhile, pond water and shrimp gut showed a < 0.717 , indicating greater similarity in their microbial communities. These data suggest variations in microbial composition and structure in three specified sampling locations were depicted in fig 13.

Alpha Diversity Comparison

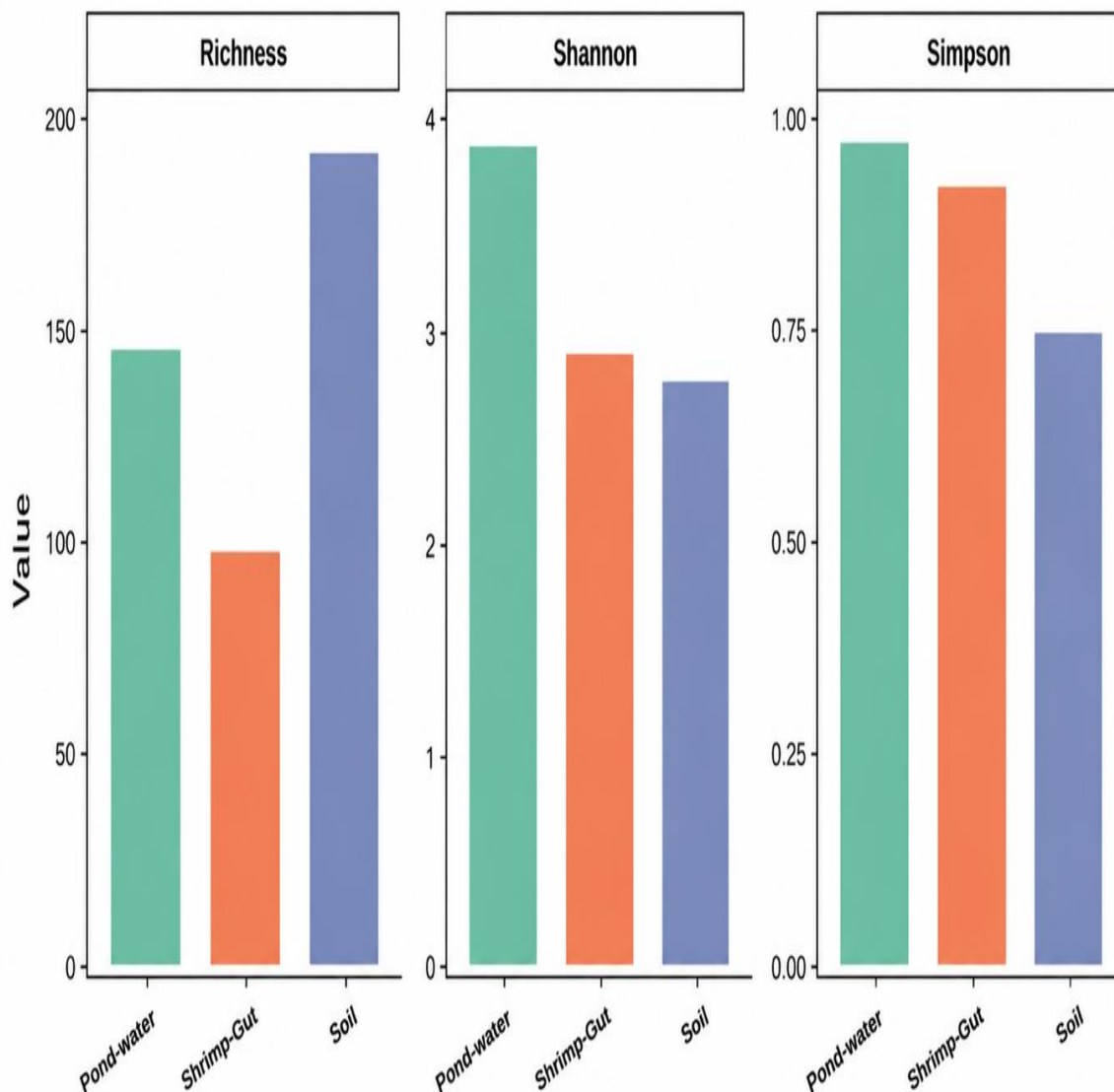


Fig 10 : Alpha diversity metrics of shrimp and water samples at the three shrimp farms

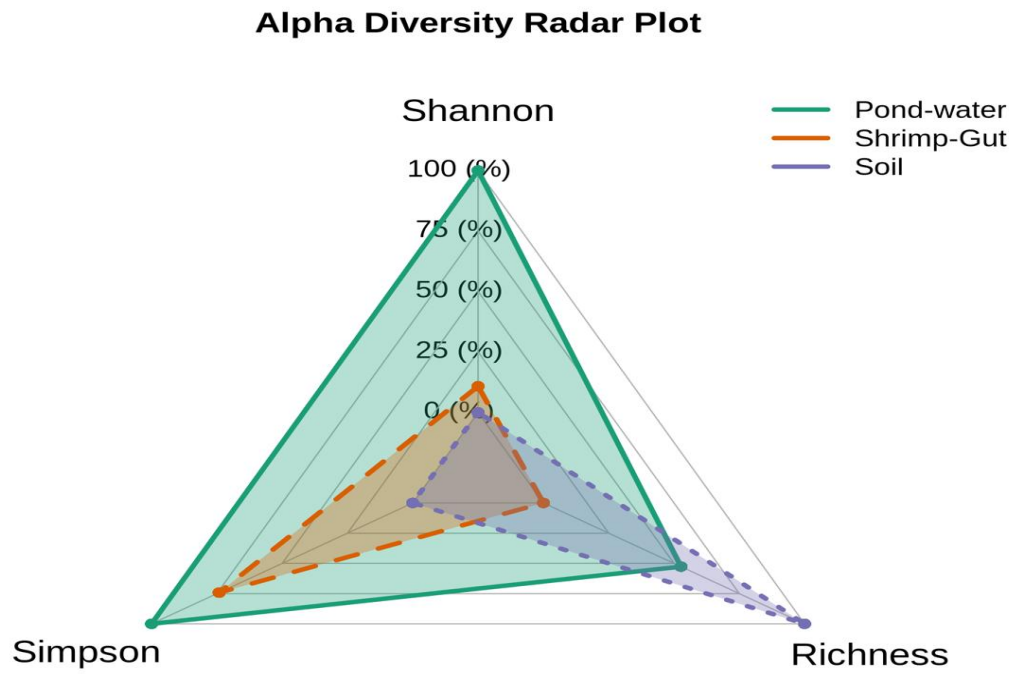


Fig 11:Radar plot

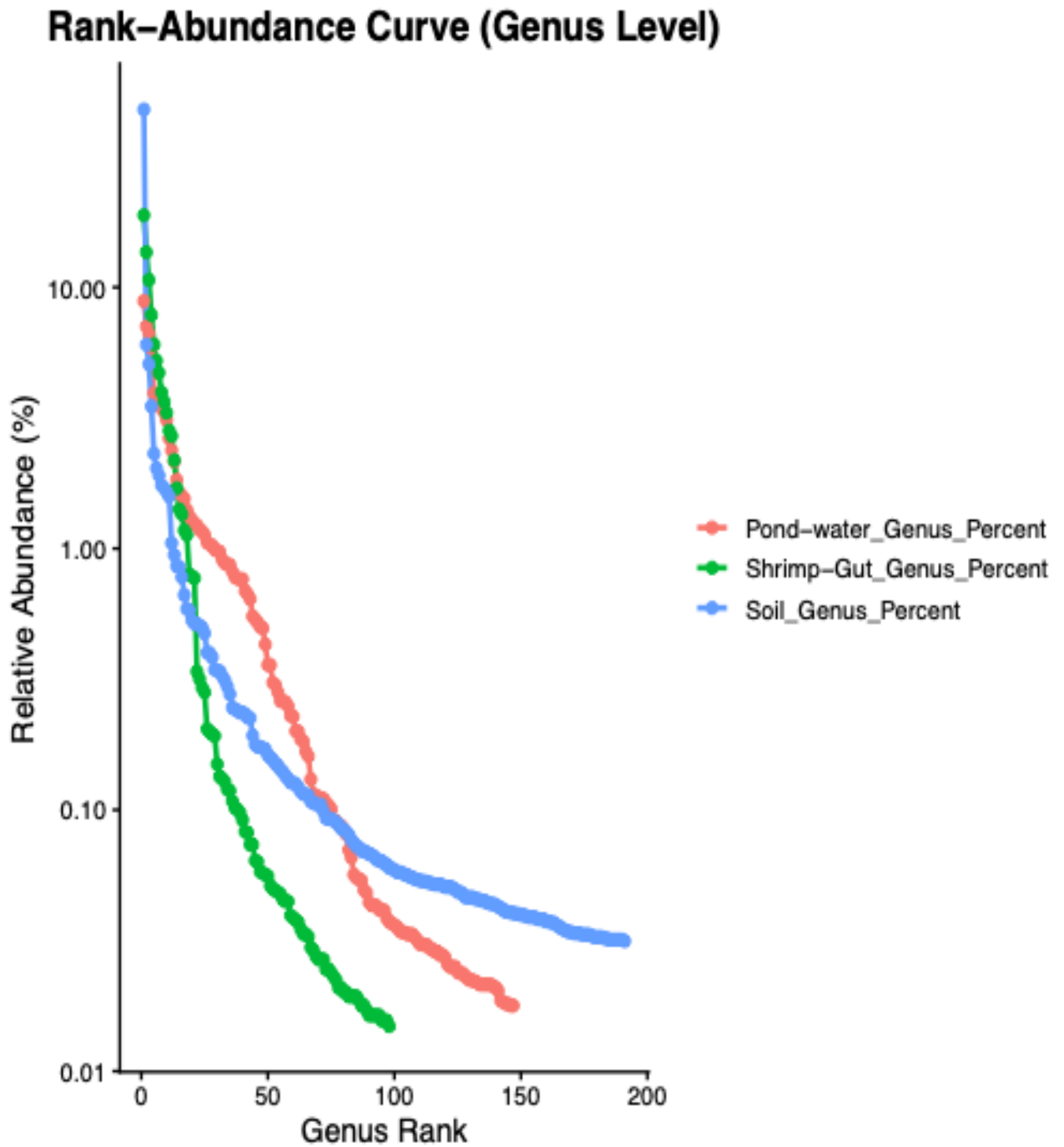


Fig 12: Abundance-based coverage estimator

The differential taxonomic distribution of shrimp gut, sediment and pond water from the respective sites were explored by heatmap analysis . To assess the variation in bacterial community composition between samples, a heatmap of the top genera based on log-transformed and Z-score standardized relative abundance data were generated. This transformation was performed across rows, within genera allowing visualization of deviations relative to each genus's average across samples. Hierarchical clustering was applied to both taxa and samples to reveal compositional relationships. Genera were annotated by phylum to provide taxonomic context fig 14.

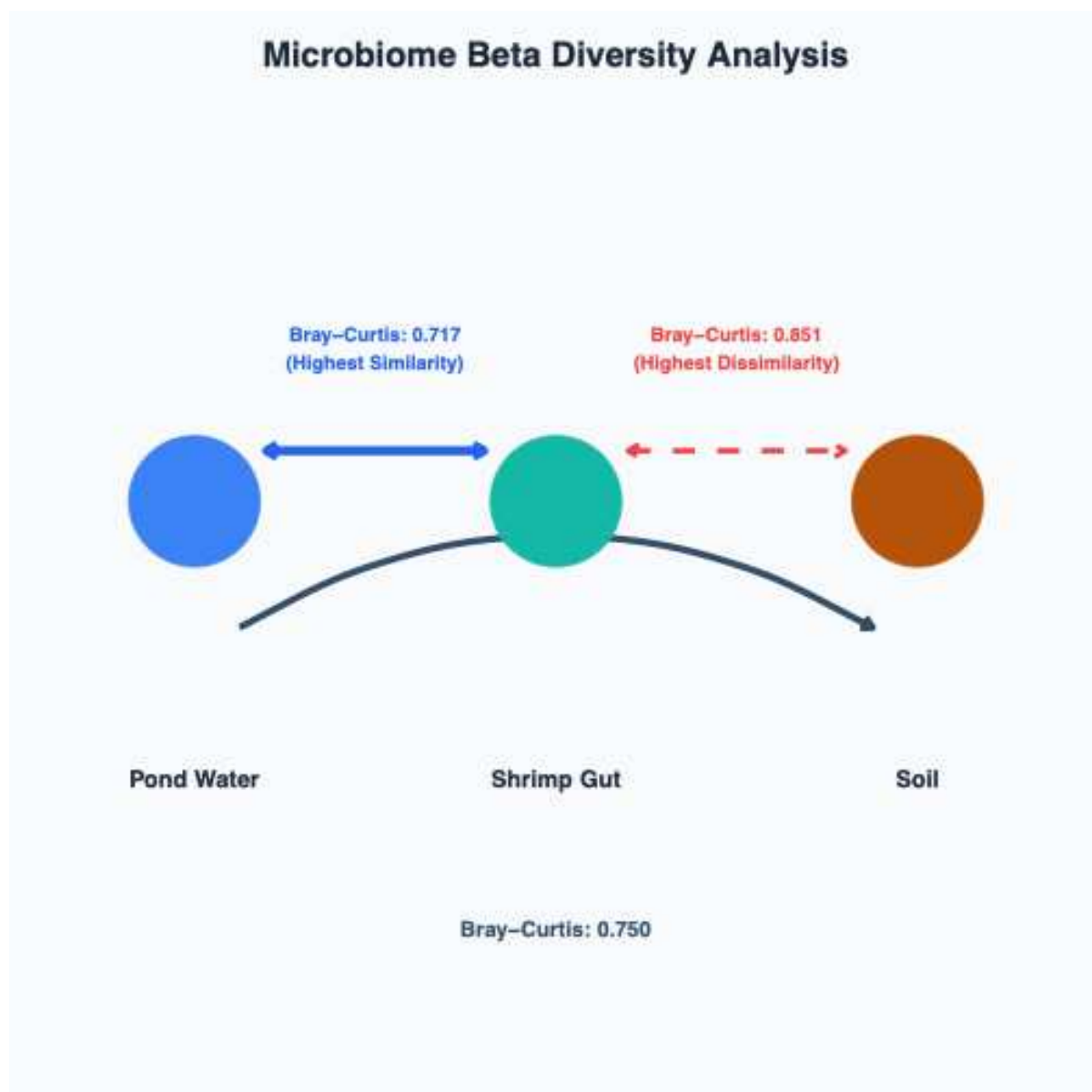


Fig 13: Beta diversity

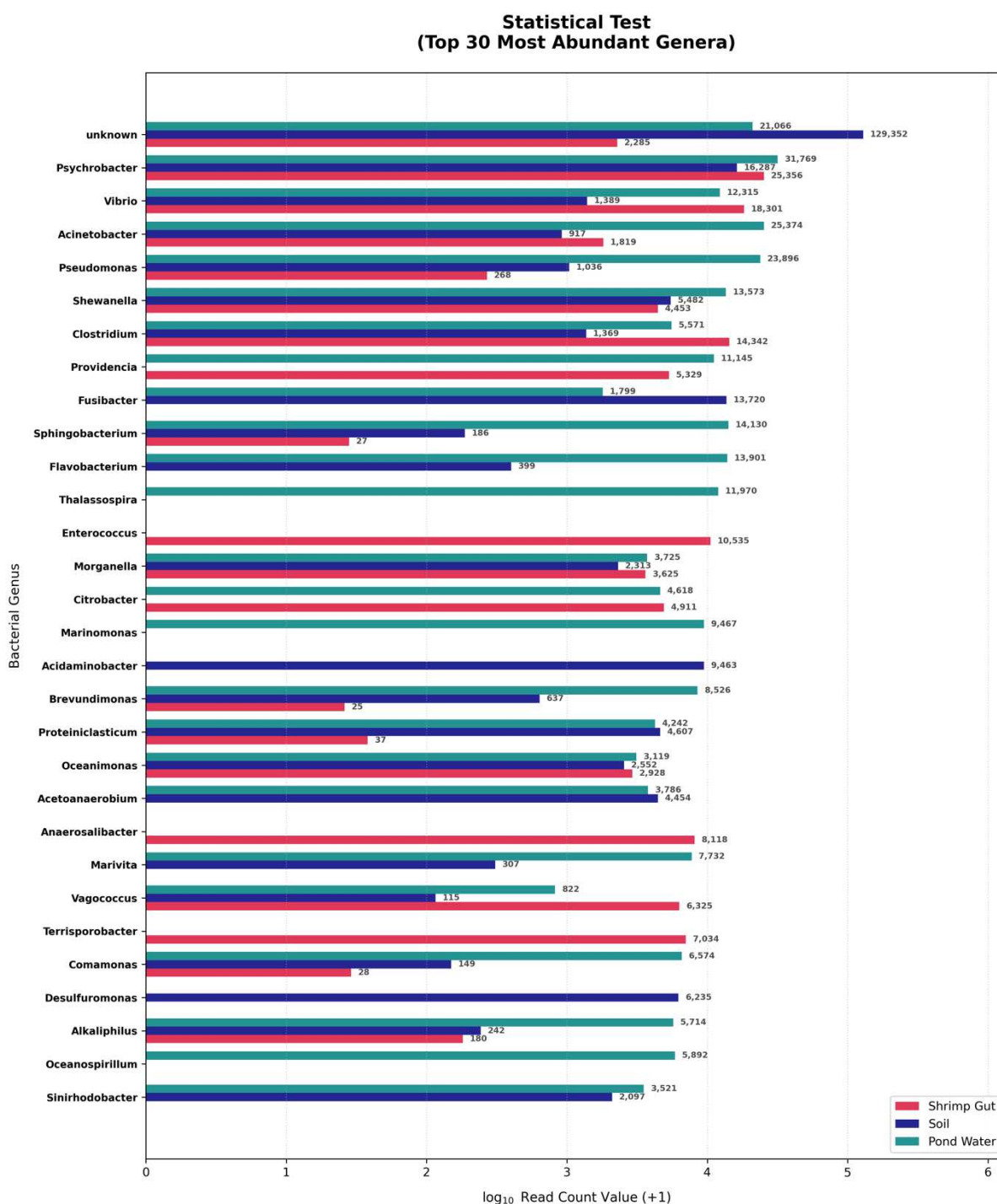


Fig 14 : Hierarchical clustering is shown as heatmap generated by MicrobiomeAnalyst using Euclidean distance measure and Ward clustering algorithm. Color intensity indicates abundance, ranging from red (high abundance) to blue (low abundance).

4. Discussion

In the present study the observed bacterial diversity and the prevalence of specific taxa, in particular locations of the southern coastal areas of Kerala like Munroe Island, Anjuthengu and Neyyatinkara emphasized the convoluted relationship between the antibiotic resistance and the microbial communities. These findings have significant inference for understanding the prevalence of antibiotic resistant genes within the studied aquaculture system. The existence of Proteobacteria across all examined locations aligns with the existing literature, which often cites Proteobacteria as a dominant phylum in aquatic environments [28-29]. This widespread nature can be attributed to the diverse metabolic capabilities of Proteobacteria, allowing them to thrive in various environmental conditions. In specific site like Munroe Island and Anjuthengu the marked prevalence of the species *Vibrio parahaemolyticus* is noteworthy. It is a major pathogen in aquaculture farms causing high mortality rates in shrimps which leads to significant economic losses. Furthermore, the distinct presence of *Sinirhodobacter* species with strain HNIBRA609 in sediment samples from Neyyatinkara, the southern coastal area of Kerala enables the notable presence of these species. As a photosynthetic bacterium, it can be used for environmental bioremediation in shrimp ponds, helping to manage waste and improve water quality. It functions as a potent probiotic and functional feed additive to improve growth, immunity and survival rates, especially against diseases like Acute Hepatopancreatic Necrosis Disease (AHPND) [30]. Our investigation into the distribution of antimicrobial resistance (AMR) genes across multiple geographical locations revealed a complex and diverse landscape of resistance mechanisms. This study encompassed a wide array of antibiotic resistance types including Norfloxacin, Ciprofloxacin, Sulfadiazin, Sulfamethoxazole, Trimethoprim and Sulfadimidin across three Southern coastal locations in Kerala, i.e., Munroe Island, Anjuthengu

and Neyyatinkara. The findings demonstrated significant variability in the presence and prevalence of specific AMR genes among the studied locations. The genes conferring resistance to Norfloxacin like qnr S in shrimp gut, qnr S8 in sediment and qnr B in pond water and Sulfamethoxazole resistant genes like sul 1 in shrimp gut, sul 1 and 2 in sediment samples and sul 2 in pond water were identified in Munroe Island.

The observed ARG distribution may reflect differences in antimicrobial exposure, environmental conditions, microbial community composition or other local selective pressures, however antimicrobial usage and residues were not measured in this study. The region specific antimicrobial resistance has been described in earlier studies on *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Klebsiella pneumoniae*. Trimethoprim resistant genes, like dfr A12, dfr A1 and dfr A and which are unique to Anjuthengu underscore the heterogeneity in the distribution of resistance genes. This gene is one of the key genes related to antibiotic resistance and has been well studied as an indicator of antibiotic resistance in various water sources [31-32]. Sulfadiazine conferring resistance to sul1 and sul 2 genes both in shrimp gut and sediment, pond water shows the sulfadiazine resistant gene sul 1 is found in the coastal area of Anjuthengu. The study revealed the distribution of these resistance genes is not uniform across the regions, indicating a complex interaction of factors such as local antibiotic usage patterns, environmental conditions, and genetic exchange mechanisms that might contribute to this varied distribution. The observed ARG distribution may reflect differences in antimicrobial exposure, environmental conditions, microbial community composition or other local selective pressures; however, antimicrobial usage and residues were not measured in this study. Antimicrobial resistance is a major global public health problem, which develops when pathogens acquire ARGs, primarily through genetic recombination between commensal and pathogenic microbes. The resistome is a

collection of all ARGs. The findings highlight the necessity for monitoring and oversight to track and manage the spread of AMR in these specific areas. Understanding the patterns of resistance gene prevalence can aid in developing leverage to control the expanding issue of antibiotic resistance in diverse geographical locations. The distinct distribution of genes in processes like aging, replication, repair, and cell motility across studied locations emphasizes the unique ecological characteristics of each site. This diversity can be attributed to a multitude of factors, including environmental conditions, the presence of specific microbial communities, and local selective pressures. The variability observed in the data reflects the adaptive responses of organisms to their respective habitats. The genes related to antimicrobial resistance, particularly in regions like Munroe Island and Neyyatinkara, raises significant public health concerns. This observation suggests that these areas might be reservoirs of drug resistant organisms, potentially due to the overuse of antibiotics. The data calls for a more targeted approach towards monitoring and managing antimicrobial resistance in these regions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis depicted a comparative identification across the three distinct locations of the southern coastal areas of Kerala. The analysis reveals the frequency of processes ranging from aging and replication to metabolism and drug resistance. A significant portion of the genes fell into unclassified categories, pointing to the existence of unknown or poorly understood biological processes in these regions. This observations reveals wide approach for future research aimed at uncovering novel biological functions and mechanisms, which could have far reaching implications on understanding ecosystem dynamics and organismal adaptations. It opens comprehensive method for future research to explore these uncharacterized functions, which could lead to ground breaking discoveries in microbial ecology and biology. The distinct distribution of various biological processes in three different southern coastal locations of Kerala were uniformly identified. We also found unclassified pathways, possibly linked with

genetic information processing and the metabolism process. The variation in amino acid metabolism across different locations highlights the metabolic flexibility and adaptability of the residing organisms allowing them to thrive in diverse environmental conditions. The Cluster of Orthologous Groups (COG) analysis, encompassing three distinct geographical locations, unveiled a rich tapestry of functional biodiversity. This diversity, evident in the distribution of various COG categories, reflects the intricate interplay between microbial communities and their respective environments. It highlights the universality of certain metabolic functions, which serve as the foundation for microbial survival and proliferation, irrespective of geographical variances.

From KEGG and COG analysis observations revealed the genes involved in cellular processes, particularly cell cycle control, cell division, and chromosomal dynamics. The marked presence of these categories indicates active cellular mechanisms, potentially as a response to local environmental pressures or genomic instabilities. The variation observed in defense mechanisms and signal transduction pathways among the different locations indicates the adaptive strategies employed by microbial communities [33,34,35]. The efflux pump related COG categories and KEGG pathways associated with drug resistance were clearly elevated in three studied locations with specific ARGs. This variability could stem from the need to respond to specific local environmental conditions, such as nutrient availability, the presence of antimicrobial agents, or other ecological pressures [36]. The differential expression of these categories highlights the role of local environmental factors in shaping the functional capabilities of microbial communities. The statistical analysis revealed the exact abundance of specific samples in respective farm sites. While addressing the lack of biological replicates in our study, the issue is driven by the high cost of sampling, logistical challenges in remote areas like the supply of high quality inputs like broodstock, post larvae (PL) seeds, and feed like wheat bran, probiotics

,fish meal [37].These supply inputs are vulnerable to climate events, political instability and pandemic related restrictions. Shrimp are highly perishable, they require immediate icing and rapid transport. Also the prioritization of rapid pathogen screening, leads to data gaps regarding the true prevalence, persistence and horizontal transfer of resistance gene.

5.Conclusion

In conclusion, this study offers critical insights into the bacterial diversity across three southern coastal areas of Kerala, India through a metagenomics approach. In addition to this, the spatial distribution of AMR, illustrating a complex antibiotic resistance has been depicted. This study features the intricate relationship between organisms and their environments, as evidenced by the diversity of biological processes observed in different locations. This emphasizes the need for local environmental considerations in both ecological and biological research and for region-specific strategies to address public health challenges, such as AMR .While the study provides valuable insights, it is limited by its focus on specific resistance genes and geographical locations. Future research should broaden to include a wider array of resistance determinants and environmental samples to fully comprehend AMR dynamics. This expansion is crucial for the global fight against the growing threat of antimicrobial resistance and for developing effective infection control and management strategies. Understanding the diverse functional profiles of microbial communities and their adaptability will be crucial to approaching the ecological dynamics and assuring the environmental viability. The presence of disease related categories like cancer and endocrine disorders in the studied data from specific locations suggests potential environmental or genetic factors influencing disease prevalence in these locations. These findings could be instrumental in guiding further epidemiological studies to explore the environmental contributions to disease etiology.

6.Data availability

WGS data of the Shrimp gut, sediment and pond water samples from specified sites were submitted to the National Centre for Biotechnology Information(NCBI;<https://submit.ncbi.nlm.nih.gov/subs/bioproject/>)under Bio Project accession number PRJNA1435634.

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