Hindawi
Aquaculture Research
Volume 2023, Article ID 6255586, 17 pages
https://doi.org/10.1155/2023/6255586

Research Article

Bacterial Diversity and Antibiotic Resistance Genes Associated with the Different Farming Systems of Black Tiger Shrimp (*Penaeus monodon*) in Bangladesh

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Received 25 February 2023; Revised 3 August 2023; Accepted 8 August 2023; Published 30 August 2023

Academic Editor: Mohamed Abdelsalam

Microbial community inhabiting the intestine of the shrimp (*Penaeus monodon*) and their surrounding environments (e.g., water and sediment) is considered as a key contributing factor for the sustainable farming of shrimp. Indiscriminate application of antibiotics in aquaculture is a growing concern due to the emergence of antibiotic resistant bacteria (ARB), more specifically the antibiotic resistance genes (ARGs). The present study investigates the microbiome composition and 19 ARGs from four different shrimp farming systems; (i) cluster, (ii) extensive, (iii) semi-intensive, and (iv) improved extensive in the southwest coastal region of Bangladesh. In doing so, the study applied advanced 16S rRNA-based metagenomic sequencing to study the bacterial composition. Moreover, gene specific polymerase chain reaction (PCR) was employed to detect the ARGs in shrimp, water, and sediments of different farming systems. In the current study, bacteria from the phylum Proteobacteria and Firmicutes were predominant among the samples ($n = 12$) collected from the different farming systems followed by Actinobacteria, Bacteroidetes, and Cyanobacteria. Firmicutes was the predominant phylum in the gut of shrimp cultured in the cluster (relative abundance 53.33%) and semi-intensive (relative abundance 59.2%) culture systems. Results indicated that the bacterial community structure was significantly ($p < 0.05$) distinct among gut, sediment, and water samples as well as the farming systems. The shared operational taxonomic unit (OTU) in the sediment sample (16,495) was nearly double than the gut (7,931) and water (8,513) bacterial communities. The improved extensive farming system showed 1,289 (11.05%) shared OTUs among gut, sediment, and water followed by semi-intensive (6.87%), cluster (6.27%), and extensive (5.46%) farming system. Among the tested ARGs, *sul1*, *cat*, *gyrA*(*C*), *tetA*, *tetC*, *tetX*, *ere*(*A*), *vanR*, and *dfrA1* were predominant in water and sediment samples. Semi-intensive farming system had the highest prevalence of ARGs (21.05%) while the lowest prevalence was found in extensive (5.26%) farming system. Overall, the study provides a comprehensive scenario of bacterial composition and growing emergence of ARGs in shrimp farming of Bangladesh. Therefore, the production strategy must focus on the alternatives of antibiotic for shaping the shrimp cultivation technique more sustainable.

1. Introduction

Shrimp is one of the most valuable export items for the growing economy of Bangladesh. The southwest region of Bangladesh is known as the shrimp culture zone which covers more than 80% of the total shrimp farming area of the country [1]. This region provides nearly 261,928 metric ton (MT) which is 87% of the total shrimp production (300,893 MT) of Bangladesh [1]. Because of domestic and international demand for shrimp, farmers of the region as

well as other coastal parts of the country are tending to be involved in shrimp farming. The rapid expansion of tiger shrimp culture practices in Bangladesh has been initiated, primarily using four types of farming systems including extensive, improved extensive, semi-intensive, and cluster farming systems [2].

However, one of the major obstacles for the sustainable development and productivity of this sector 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 dishearten them which in many cases limit the production [3]. 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 (ARGs) [4]. Previous study reported that, the use of multiple classes of antibiotics including fluoroquinolone, quinolones, sulfonamides, tetracyclines, nitrofurantoin, and chloramphenicol in shrimp farms [5], are primary reason for the growth of antimicrobial resistance (AMR). 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 [6]. ARGs is recognized as one of the topmost threats to food safety and global health by the World Health Organization [7]. Therefore, responsible use of antibiotics is central to reduce the emergence of AMR phenomenon in aquaculture. Besides, alternative strategies such as (i) use of probiotics or (ii) nutritional modulation of microbiome in shrimp and cultured environment need to be promoted to tackle the AMR.

Microbial community regulates various physiological functions including nutrient acquisition [8, 9] and immune response of aquatic organisms [10]. Moreover, microbial community in the gut is considered as a key indicator of the homeostasis and health status of shrimp [11, 12]. Furthermore, correlation between disease incidence and surrounding microbiota (water and sediment) has been reported from various studies [13–18]. On the top of that, gut, sediment, and water microbial community could be a potential source for screening of novel aquaculture probiotics [19]. Therefore, microbiome analysis is considered as an effective approach to characterize the microorganisms and take proper initiative to alleviate and prevent the incipient diseases [20, 21].

A comprehensive study for the identification of the microbial communities of shrimp aquaculture including shrimp's organs, water, and sediment has not been done yet; therefore, scientific basis of tackling disease-causing ARB still remained unknown. Therefore, the present study is aimed to identify and characterize the microbial community composition and ARGs associated with extensive, improved extensive, semi-intensive, and cluster farming of the shrimp in Bangladesh.

2. Materials and Methods

2.1. Sampling Sites and Sample Collection. The samples (sediment, water, and shrimp) were collected randomly from four different farming systems (extensive, improved extensive, semi-intensive, and cluster) of the Khulna and Satkhira district of Bangladesh from November 12 to November 17, 2021 (Figure 1).

According to DoF [1], Satkhira region covers nearly 25% of the total shrimp cultivation area and produces 26% (78,668 MT) of the total shrimp production. Khulna district encompasses around 22% of total shrimp cultivable areas where the production is 70,934 MT (23% of the total production) [1]. Moreover, all types of shrimp cultivation are commonly observed in these regions where lower stocking density and management are often described as extensive shrimp farming whereas artificial stocking with no or low supplementary feeding is considered as improved extensive culture system [22]. Periodic application of fertilizer and providing feed regularly in a semi-controlled environment is often categorized as a semi-intensive shrimp farming [23]. Apart from these, a newly developed farming strategy called cluster farming is gaining popularity in these regions where a group of farmers jointly contribute to asset and labor and get mutual benefits and participate in decision making process for farming a specific species in a specific region [22].

Shrimps (*Penaeus monodon*) were obtained through the netting process from every pond (Supplementary Table S1). Then the samples were labeled and stored in ice immediately after collection. Shrimp rearing water (1.0 L) was collected in a sterilized bottle from the surface, middle, and bottom of the pond and mixed as one sample and filtered through 0.2 μm filter paper. Sediment was collected from the pond in a sterile zipper bag (Supplementary Table S1). Samples from each farm were collected from earthen ponds. Only in the semi-intensive farming system, the ponds were PPE (polypropylene-based geomembrane) lined. In addition, water quality parameters including temperature, pH, salinity, turbidity, dissolved oxygen, conductivity, oxidation–reduction potential, pH per volume, and seawater specific gravity of all shrimp farming ponds were measured using a U-50 multiparameter water quality checker, HORIBA Advanced Techno Co., Ltd., Japan (Supplementary Table S1).

2.2. DNA Extraction. Total genomic DNA from sediment (0.5 *g*), water (500 mL), and gut (individual gut, ∼0.5 g) of black tiger shrimp was extracted by using a Pure Link™ Genomic DNA Purification Kit (Invitrogen, USA) with some modifications, e.g., including incubation at 65°C for 8 min followed by bead beating for 5 min and again incubation for 2 min and then final bead beating for 5 min. Besides all centrifugation steps were carried out at 14,000 *g* for 90 s. The purity and concentration of genomic DNA were determined by the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

2.3. Bacterial 16S rRNA Gene Amplification and Sequencing. A total of 36 DNA samples from gut, sediment, and water were sent to Novogene Biological Information Technology Co. (Tianjin. China) for amplicon metagenomics sequencing. The samples were labeled as SS for sediment, SG for shrimp gut, and SW for water (Supplementary Table S1). Sequencing libraries were constructed using Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) following manufacturer's protocol. Illumina NovaSeq 6000

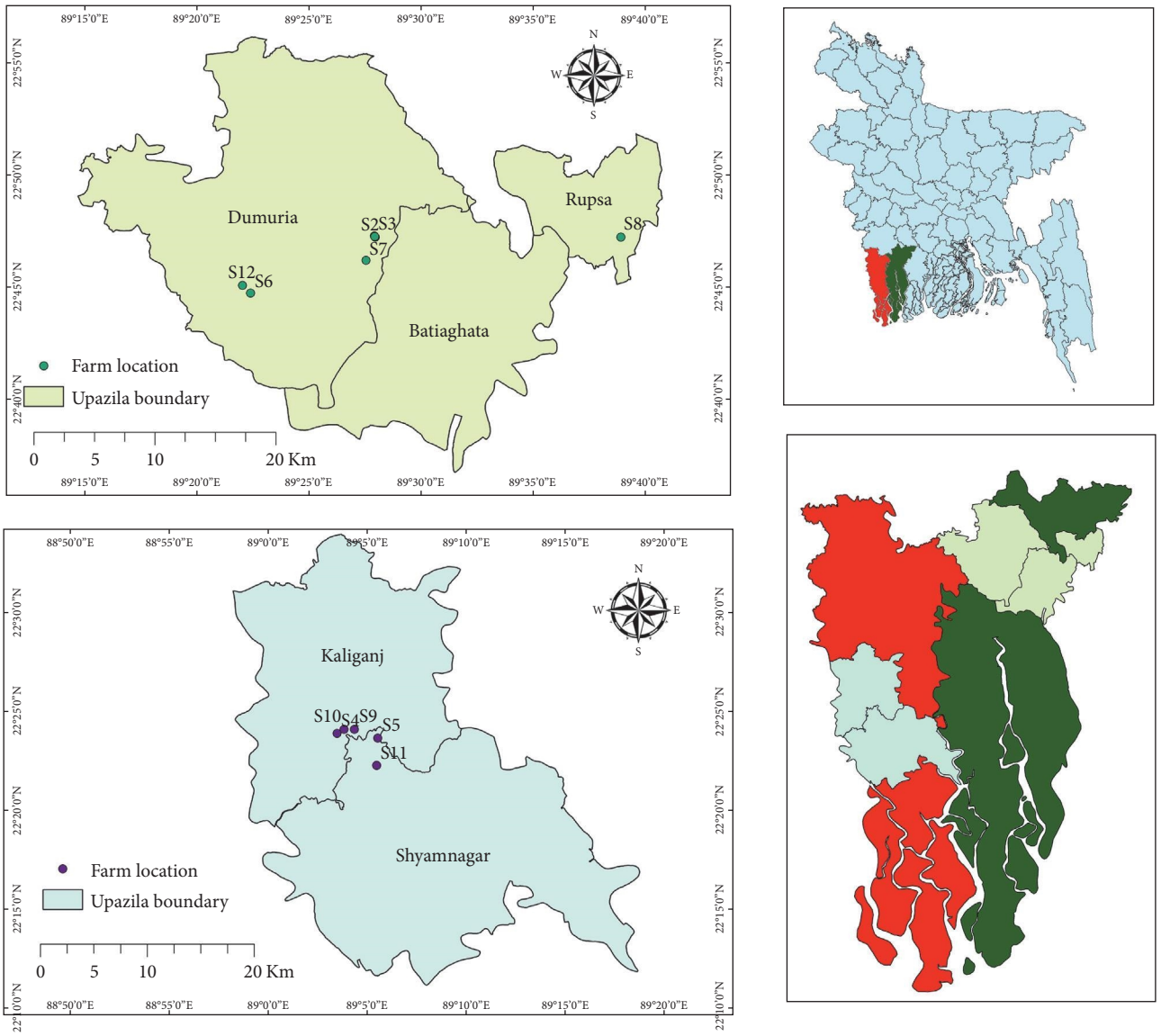


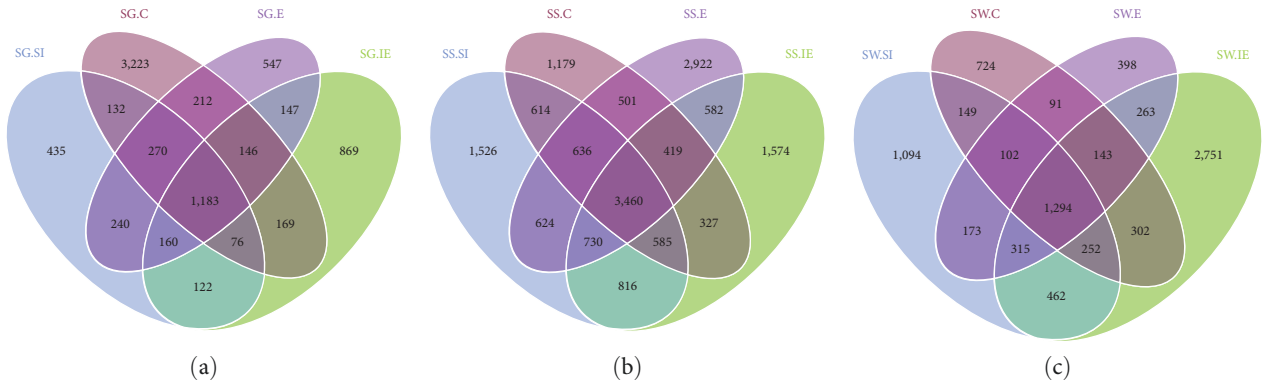
FIGURE 1: Map illustrating the sampling sites at Khulna (S1–S3, S6–S8, and S12) and Satkhira (S4–S5 and S9–S11). S1–S12 indicates the location of 12 farms from where the samples were collected.

platform was used to sequence the library. The sequencing was performed on the Illumina paired-end platform to generate 250 bp paired-end raw reads (Raw PE) and the V3–V4 region of the 16S rRNA gene was amplified by using the linker primer sequences CCTAYGGGRBGCASCAG and GGACTACNNGGGTATCTAAT. The sequences from three sample groups (i.e., sediment, water, and gut) have been deposited in the NCBI Sequence Read Archive (SRA) Database under accession number PRJNA976638 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA976638/).

2.4. Sequenced Data Processing. Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using fast length adjustment of short reads (FLASH V1.2.7) [24] (http://ccb.jhu.edu/software/FLASH/), a very fast and accurate analysis tool, which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment, and the splicing sequences were called raw tags. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags [25], according to the QIIME (V1.7.0) [26], (http://qiime.org/scripts/split_libraries_fastq.html) quality controlled process.

The tags were compared with the reference database (SILVA138 database, http://www.arb-silva.de/) using UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) [27] to detect chimera sequences (https://drive5.com/usearch/manual/chimeras.html). The chimera sequences were removed [28] and the effective tags were finally obtained.

2.5. Operational Taxonomic Unit (OTU) Cluster and Taxonomic Annotation. Sequence analysis was performed by Uparse software (Uparse v7.0.1090, http://drive5.com/uparse/) [29], using



(a) (b) (c)

FIGURE 2: Bacterial community profile among water, sediment, and gut at OTU level detected from four different farming systems. (a) Venn diagram of gut bacterial communities among four different farming systems; (b) Venn diagram of sediment bacterial communities among four different farming systems; (c) Venn diagram of water bacterial communities among four different farming systems; farming system (C: cluster; E: extensive; SI: semi-intensive; IE: improved extensive).

all the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. The sequences which could not be possible to assign to any taxonomic group were assigned to "Others."

For each representative sequence, QIIME (Version 1.7.0, http://qiime.org/scripts/assign_taxonomy.html) [30], in Mothur method was performed against the SSUrRNA database of SILVA138 Database (http://www.arb-silva.de/) [31], for species annotation at each taxonomic rank (Threshold: 0.8–1) [32], (kingdom, phylum, class, order, family, genus, and species).

To obtain the phylogenetic relationship of sequences of representative OTUs, the MUSCLE [33] (Version 3.8.31, http://www.drive5.com/muscle/) tool was used.

OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were performed based on the output normalized data of OTUs abundance. Alpha diversity was applied in analyzing the complexity of biodiversity using Chao1, Shannon, Simpson, and abundance–based coverage estimator (ACE) which were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Beta diversity analysis was used to evaluate the differences of samples in species complexity, beta diversity of both weighted and unweighted UniFrac were calculated by QIIME software (Version 1.7.0).

2.6. Identification of Antibiotic Resistance Genes in Shrimp, Sediment, and Water Samples. A background study was conducted through literature review and field survey for screening of commonly used antibiotics in shrimp aquaculture (Supplementary Table S2). Besides, common microbiota in shrimp aquaculture and their antibiotic resistance potential has also been taken into consideration during the literature review (Supplementary Table S3). Finally, 19 ARGs [34–37] related to 10 antibiotic groups (ciprofloxacin, chloramphenicol, sulfonamide, quinolones, aminoglycoside, tetracycline, beta-lactams, macrolide, bacitracin, vancomycin, and trimethoprim) were selected for the current study. Primer sequence (Supplementary Table S4) was collected from the literature and polymerase chain reaction (PCR) amplification was performed for the detection of ARGs. PCR amplification was carried out by preparing 50 μL of the PCR reaction by using 25 μL of Onetaq Quick-Load 2x Master Mix with standard buffer (New England Biolab Inc. USA), 2.5 μL of both forward and reverse primers (10 μM primer; final primer concentration 400 nM), 15 μL of nuclease-free water (Molecular Biology Grade, Cytiva, USA), and 5 μL of template DNA. The thermal cycles and time profile for PCR reaction were shown in (Supplementary Table S4) for each gene. Finally, the PCR products were run on a 1% agarose gel containing ethidium bromide (0.5 μg mL^{-1}) and visualized under UV illuminator (protein simple α imager, USA), and the amplified product was compared with a 1 kb and 100 bp DNA ladder (Promega, Madison, WI, USA) to detect the presence of the target genes.

2.7. Correlation Analysis. The correlation analysis between resistance genes and bacterial communities in different aquaculture systems was conducted using the GraphPad Prism version 8.

3. Results

3.1. Overview of 16S rRNA Gene Sequencing. A total of 5,407,111 raw tags were generated from the sequencing of 35 samples including gut, sediment, and water (one sample SG8 did not pass the quality control step). A total of 4,331,968 effective tags were identified in 35 samples. The alignment was performed at an average length of 417 bp.

The highest number of OTUs (16,495) was found in sediment samples followed by water (8,513) and gut (7,931). A total of 3,460 (20.98%), 1,294 (15.20%), and 1,183 (14.92%) OTUs common to the four farming systems were detected in sediment, water, and gut samples, respectively (Figure 2).

The cluster farming (Figure 3(a)) showed 768 common OTUs (out of 12,254) and 4,724, 570 and 3,793 unique OTUs in sediment, water, and gut samples, respectively. A total 680 OTUs were shared between gut and sediment samples, while 170 OTUs were common between gut and water samples in the cluster farming. The highest number of OTUs (1,549) shared between water and sediment samples in the cluster farming.

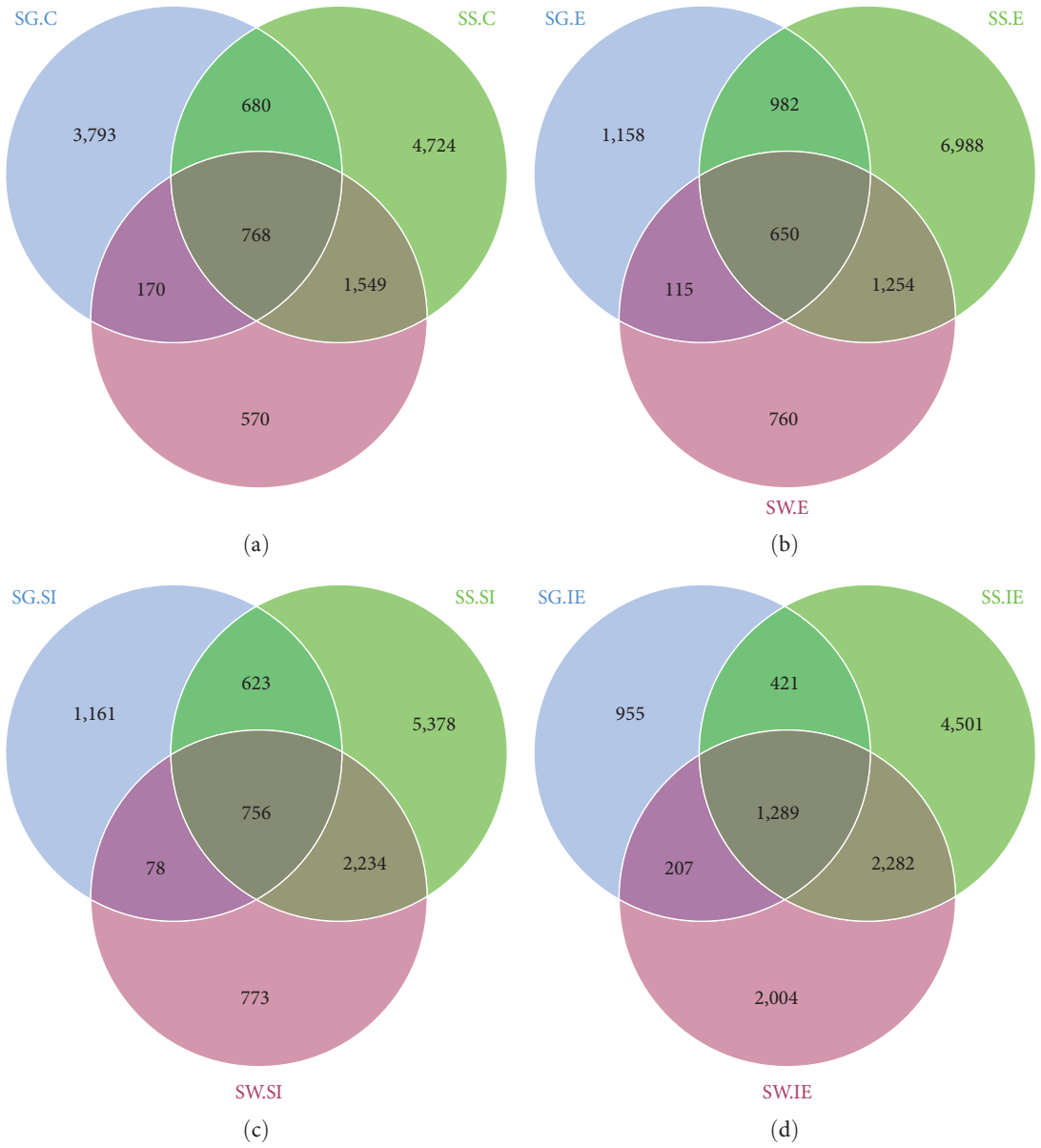


Figure 3: Bacterial community profile among water, sediment and gut at OTU level of each farming system; (a) Venn diagram of bacterial communities among gut, sediments, and water (SG, SS, and SW) of cluster farming system; (b) Venn diagram of bacterial communities among gut, sediments, and water (SG, SS, and SW) of extensive farming system; (c) Venn diagram of bacterial communities among gut, sediments, and water (SG, SS, and SW) of semi-intensive farming system; (d) Venn diagram of bacterial communities among gut, sediments, and water (SG, SS, and SW) of improved extensive farming system; farming system (C: cluster; E: extensive; SI: semi-intensive; IE: improved extensive).

A total 11,907 OTUs were found in the extensive farming systems where 5.46% (650) were common among sediment, water, and gut samples (Figure 3(b)). Among 11,003 OTUs in the semi-intensive farming system, 6.87% (756) were common, while 48.88% (5,378), 7.03% (773), and 10.55% (1,161) unique OTUs had been found across the sediment, water, and gut samples, respectively (Figure 3(c)). A total of 11,659 OTUs were detected in the improved extensive aquaculture system, in which 11.05% (1,289) shared by the three sample types (Figure 3(d)).

3.2. Microbial Community Abundance in Shrimp and Shrimp Farms at Phylum Level. At the phylum level, a total of 42 phyla were observed and the abundance of 7 phyla were identified at a level of >1%. The dominant phyla (relative abundance > 1%) in the gut of *P. monodon* was Proteobacteria (39.29%), Firmicutes (33.68%), Actinobacteria (8.87%), Cyanobacteria (5.29%), Bacteroidetes (3.65%), Verrucomicrobia (2.61%), and Chloroflexi (1.07%) (Figure 4). These dominant phyla accounted for 94.49% of the bacterial tags of the gut samples. Others belonged to the phyla which have a relative abundance of less than 1%. By comparison, Proteobacteria (37.62%) was the predominant phyla among shrimp, gut, sediment, and water samples.

In the context of overall farming systems, the dominant phyla (relative abundance >5%) in the gut of shrimp were Firmicutes (53.33%), Proteobacteria (17.25%), and Actinobacteria

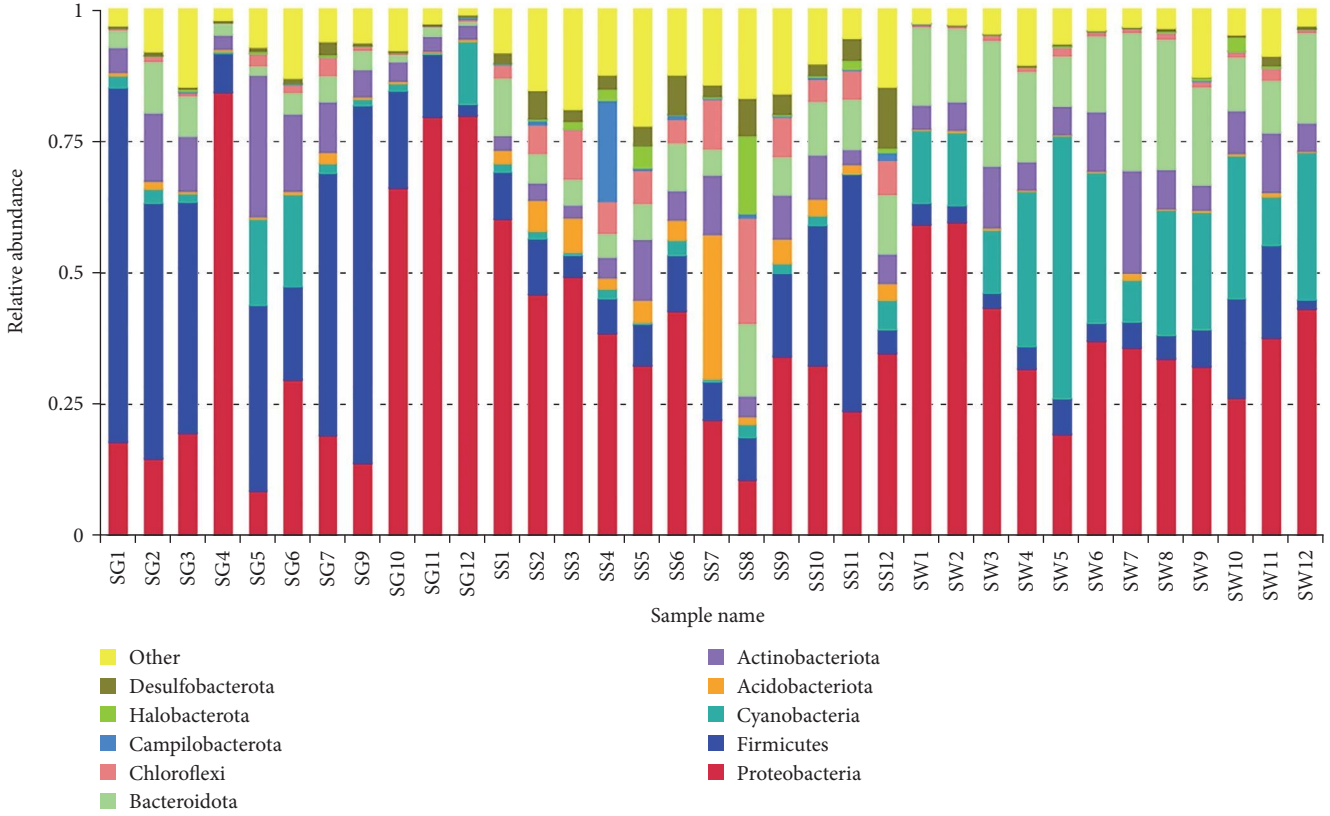


FIGURE 4: The relative abundance of top 10 phyla among all samples (gut, sediment, and water). Here, sample name is in the *X* axis and *Y* axis denotes the relative abudance and the remaing phyla rather than top 10 phyla were grouped as "Others." As shown in the figure, gut, sediment, and water (SG, SS, and SW).

(9.50%) in the cluster farming system; Proteobacteria (40.75%), Firmicutes (20.13%), and Actinobacteria (14.87%) in the extensive farming system; Firmicutes (59.02%), Proteobacteria (16.33%), and Actinobacteria (7.46%) in the semi-intensive farming system; Proteobacteria (75.19%) and Firmicutes (10.69%) in the improved extensive farming system (Figure 5).

Firmicutes were the predominant phylum in the shrimp gut of cluster (53.33%) and semi-intensive farming systems (59.02%). On the other hand, the extensive and improved extensive farming systems were dominated by Proteobacteria which accounted for 40.75% and 75.19%, respectively. Therefore, the relative abundance of dominant gut bacterial phylum was different among the farming systems.

In sediment sample, the dominant phyla (relative abundance >5%) were Proteobacteria (57.71%), Firmicutes (7.80%), and Bacteroidetes (7.14%) in the cluster farming system; Proteobacteria (37.79%), Firmicutes (8.28%), and Actinobacteria (7.14%) the in extensive system; Proteobacteria (22.14%), Chloroflexi (12.44%), Acidobacteria (11.269%), Firmicutes (10.45%), and Bacteroidetes (8.53%) in the semi-intensive system; Proteobacteria (30.17%), Firmicutes (25.36%), and Bacteroidetes (10.29%) in the improved extensive farming system (Figure 5).

The microbial flora dominated (relative abundance >5%) in water sample were Proteobacteria (53.98%), Bacteroidetes (17.52%), and Cyanobacteria (13.37%) in the cluster system; Cyanobacteria (36.13%), Proteobacteria (29.28%), and Bacteroidetes (13.59%) in the extensive system; Proteobacteria (33.83%), Bacteroidetes (23.21%), Cyanobacteria (18.06%), and Actinobacteria (10.66%) in the semi-intensive system; Proteobacteria (35.44%), Cyanobacteria (21.63%), Firmicutes (12.79%), and Bacteroidetes (12.45%) in the improved extensive farming system (Figure 5). The heatmap also showed the relative abundance of bacterial phylum and genus throughout the samples and farming systems (Figure 6). The heatmap showed the presence of *Lactococcus* in the gut samples of cluster and semi-intensive farming systems.

3.3. Microbial Community Abundance throughout Samples and Farming Systems at Genus Level. A total of 312 genera were identified and among them the top 10 genera were selected to construct the relative abundance graph (Figure 7). The dominant genera (relative abundance >1%) in the shrimp gut (Figure 7) were *Rickettsiella*, *Shewanella*, *Candidatus_Bacilloplasma*, *Clostridium*_sensu_stricto_1, *Candidatus_Hepatoplasma*, *Romboutsia*, *Vibrio*, *Cyanobium*, *Photobacterium*, *Lactococcus*, *Muribaculaceae Chloroplast*, *Akkermansia*, *Mycobacterium*, *Lactobacillus*, *Candidatus_Hepatincola*. Rheinheimera, *Paenisporosarcina*, *Aeromonas*, *Shewanella*, *Paraclostridium*, and *Pseudarcobacter* were identified as the dominant genera in sediment samples (Figure 7). The predominant genera (relative abundance >1%) in water group (Figure 7) were *Rheinheimera*, *Cyanobium*, *Chloroplast*, *Flavobacterium*, *Pseudomonas*, *Oceanobacter*, and *Candidatus_Aquirestis*.

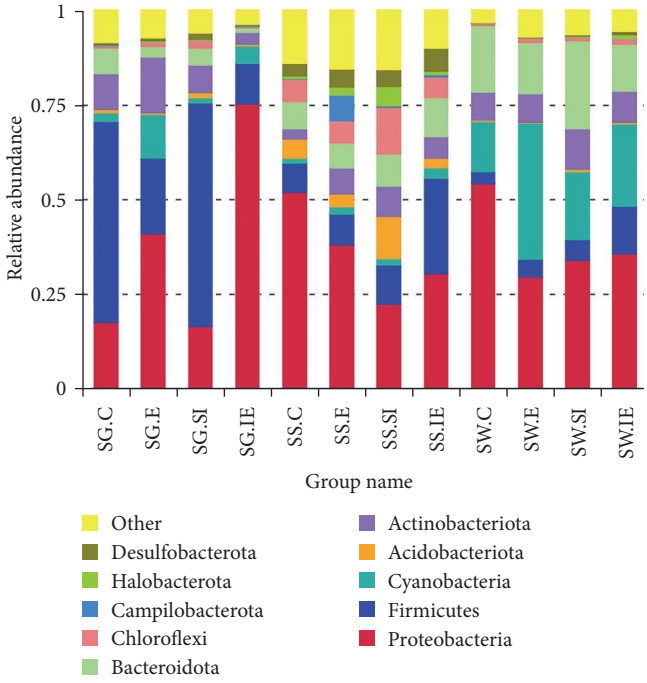


FIGURE 5: Relative abundance of top 10 phylum for 4 different farming systems. Here, group name (farming system) is in the *X* axis and *Y* axis denotes the relative abudance and the remaing phyla rather than top 10 phyla were grouped as "Others." As shown in figure, gut, sediment, and water (SG, SS, and SW); farming system (C: cluster, E: extensive, SI: semi-intensive, and IE: improved extensive).

The dominant genera in the shrimp gut were*Candidatus_Hepatoplasma*, *Clostridium*_sensu_stricto_1, *Candidatus_Bacilloplasma*, *Lactococcus*, *Muribaculaceae*, *Candidatus_Hepatincola*, *Lactobacillus*, and *Rickettshiella* in the cluster farming system; *Rickettshiella*, *Cyanobium*, Chloroplast, *Romboutsia*, *Mycobacterium*, and Muribaculaceae, in the extensive system; *Candidatus_Bacilloplasma*, *Clostridium*_sensu_stricto_1, *Romboutsia*, *Lactococcus*, *Rickettshiella*, *Muribaculaceae*, *Cetobacterium*, *Akkermansia*, *Clostridia*, and *Candidatus_Hepatoplasma* in the semi-intensive system; *Rickettsiella*, *Shewanella*, *Vibrio*, *Photobacterium*, and *Cyanobium* in the improved extensive farming system (Figure 8).

The microbial communities dominated in the sediment were *Rheinheimera*, *Pseudomonas*, *Aeromonas*, *Paraclostridium*, and *Bacillus* in the cluster farming system; *Pseudomonas*, *Pseudarcobacter*, *Shewanella*, *Rheinheimera*, and *Aeromonas* in the extensive system; *Methanosercina*, *Pseudomonas*, *Rheinheimera*, and *Methanogenium* in the semi-intensive system; *Paenisporosarcina*, *Pseudomonas*, *Pontibacter*, *Rheinheimera*, and Chloroplast in the improved extensive farming system (Figure 8).

In water sample, the predominant genera were *Rheinheimera*, *Flavobacterium*, *Cyanobium*, *Pseudomonas*, and *Nodosilinea* in the cluster farming system; *Cyanobium*, Chloroplast, *Rheinheimera*, and *Flavobacterium* in the extensive system; *Flavobacterium*, *Cyanobium*, Chloroplast, *Rheinheimera*, *Oceanobacter*, and *Pseudomonas*, in the semi-intensive system; Chloroplast, *Cyanobium*, *Rheinheimera*, *Pseudomonas*, *Flavobacterium*, *Clostridium*_sensu_stricto_1, and *Vibrio* in the improved extensive farming system (Figure 8). Evolutionary tree for different farming systems and samples presents in the supplementary materials (Supplementary Figures S1 and S2).

3.4. Alpha Diversity of Microbial Community throughout Samples and Farming Systems. Boxplots were formed to analyze the difference of alpha diversity indices between the groups. *t*-Test, Wilcox and Tukey tests (*t*-test and Wilcox test are for two groups while Wilcox and Tukey tests are for more than two groups) were performed for the analysis of significance of the difference between groups.

Among shrimp gut, sediment, and water samples, the bacterial diversity in sediment was higher than those of gut and water. The diversity indices and species richness of bacterial communities among all samples were in the following order: SS > SW > SG.

In case of farming systems, in the gut of shrimp, the diversity index (Shannon index and Simpson index) (Figures 9 (a) and 9(b)) and species richness (Chao1 and ACE) (Figures 9 (c) and 9(d)) were ordered as follows farming systems: cluster > semi-intensive > extensive > improved extensive farming system.

In sediment sample (Figure 9), the diversity index (Shannon index and Simpson index) (Figures 9(a) and 9(b)) of bacterial diversity was ordered as follows farming systems: extensive > semi-intensive whereas the Shannon index was higher in the improved extensive system than cluster system. On the contrary, Simpson index was slightly higher in the cluster system than improved extensive farming system. The species richness (Chao1 and ACE) (Figures 9(c) and 9 (d)) of bacterial diversity was in the following order: semi-intensive > cluster > extensive > improved extensive farming system.

The diversity index (Shannon index and Simpson index) (Figures 9(a) and 9(b)) of bacterial community in water group was ordered as follows: improved extensive > semi-intensive > extensive > cluster farming system. The species richness (Chao1 and ACE) (Figures 9(c) and 9(d)) was ordered as follows; improved extensive > semi-intensive > cluster > extensive farming system. The Good's coverage estimation indicated that in each sample, 98%–99% of bacterial species were obtained.

3.5. Relationship between Bacterial Communities of Different Farming Systems. The relationship of bacterial communities among shrimp gut, sediment, and water along with their changes in farming system were detected by Nonmetric Multidimensional Scaling (NMDS) (Figure 10).

The ordination biplot based on NMDS showed the clustering between bacterial communities based on OTU detection of each sample (gut, sediment, and water). Compared to sediment (SS), the bacterial communities in the gut (SG), and water (SW) were more closely clustered (Figure 10).

3.6. Antibiotic Resistance Genes Associated with Different Farming Systems. A total of 19 ARGs were selected for the detection of the antibacterial resistance in shrimp aquaculture (Supplementary Table S5). Of the 19 ARGs, nine genes

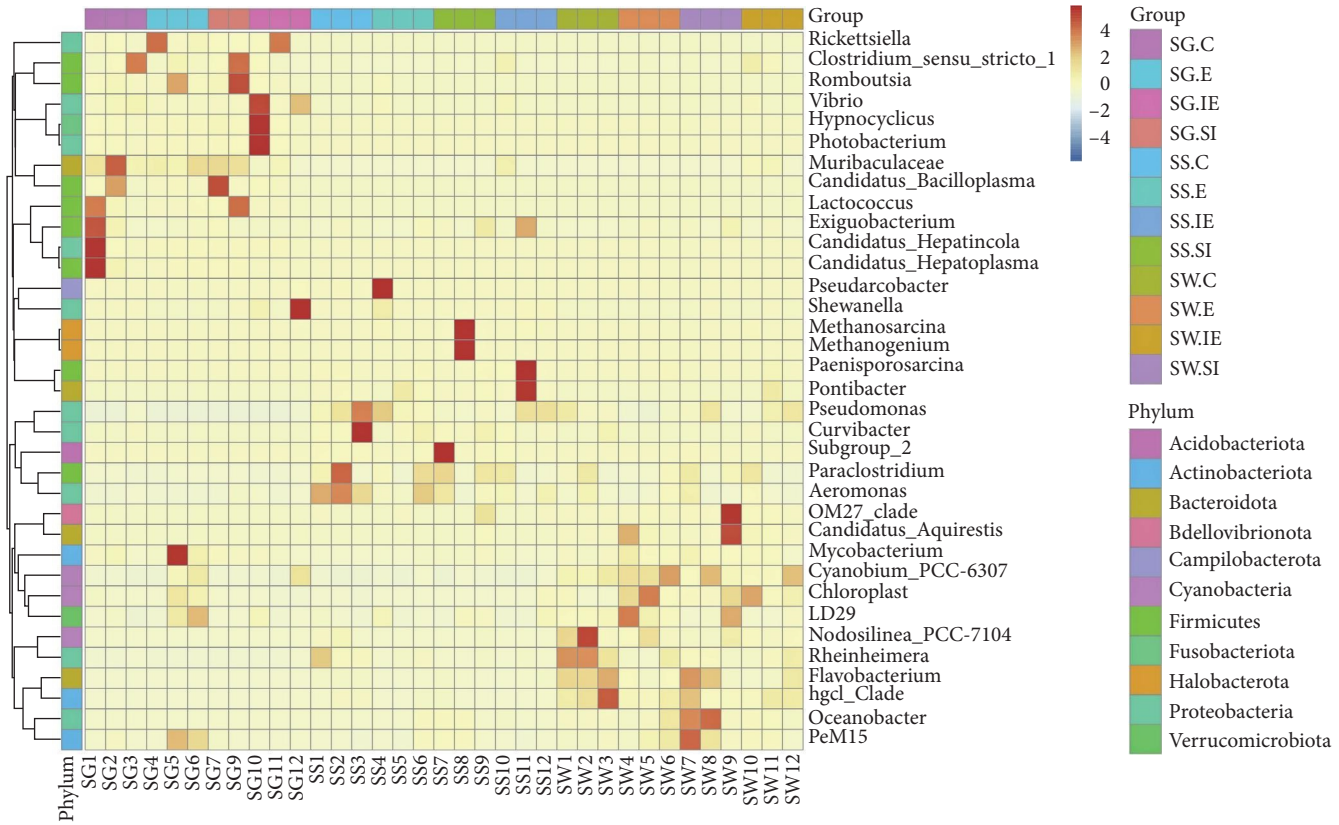


Figure 6: The OTU taxa heatmap shows the relative percentage of bacterial abundance of gut, sediment, and water from four different farming systems, while the horizontal and vertical direction was the sample information and annotation of the bacterial phylum. The top bar group represents the combined abundance of gut, sediment, and water from four different farming systems. As shown in the figure, gut, sediment, and water (SG, SS, and SW); farming system (C: cluster; E: extensive; SI: semi-intensive; IE: improved extensive).

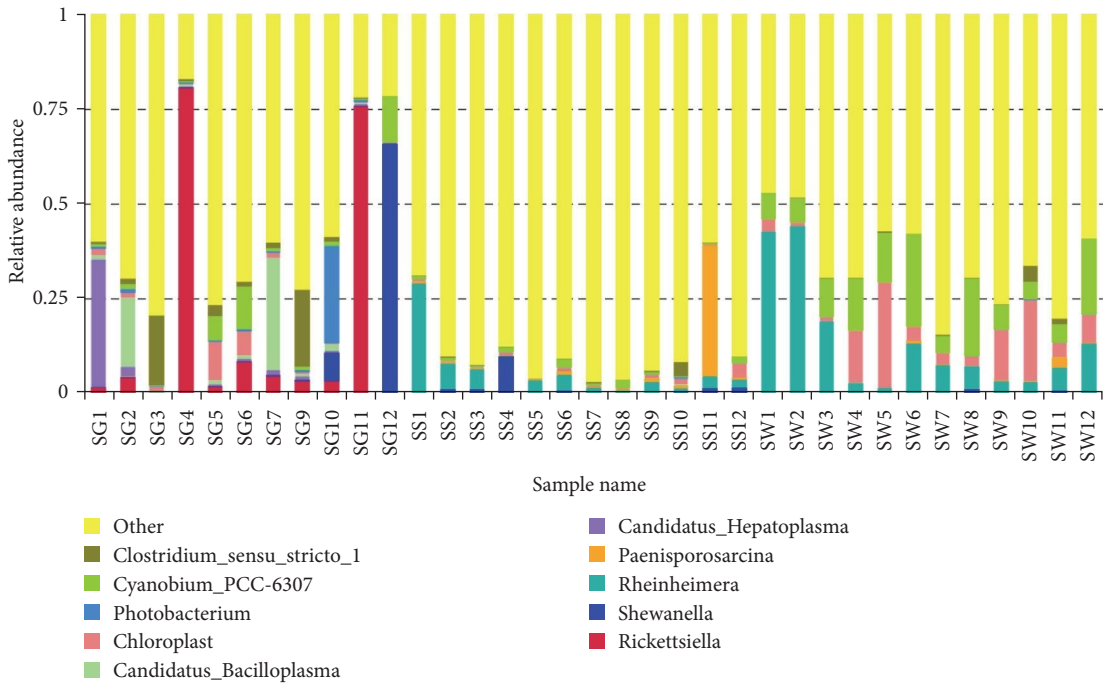


Figure 7: Relative abundance of top 10 genus among all samples (gut, sediment, and water). Here, sample name is in the X axis and Y axis denotes the relative abundance and the remaining genus rather than top 10 genus were grouped as "Others." As shown in the figure, gut, sediment, and water (SG, SS, SW); farming system (C: cluster; E: extensive; SI: semi-intensive; IE: improved extensive).

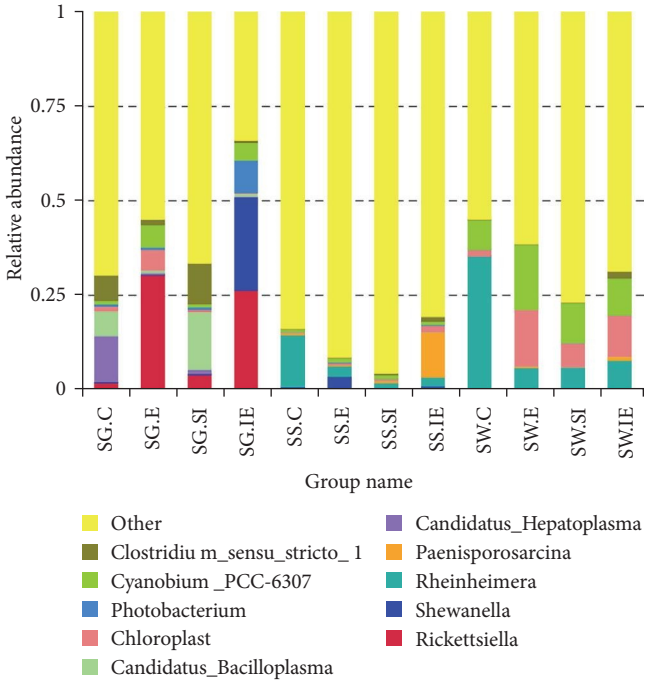


FIGURE 8: Relative abundance of top 10 genus for 4 different farming system. Here, group name (farming system) is in the *X* axis and *Y* axis denotes the relative abundance and the remaining genus rather than top 10 genus were grouped as "Others." As shown in the figure, gut, sediment, and water (SG, SS, SW); farming system (C: cluster; E: extensive; SI: semi-intensive; IE: improved extensive).

were detected in the sediment, gut, and water samples of different farming systems. Among the samples, the highest number (8) of ARGs (*sul1*, *cat*, *gyrA(C)*, *tetA*, *tetC*, *tetX*, *ere(A)*, and *vanR*) was detected in the water sample of semi-intensive shrimp farm. In the case of sediment samples, four ARGs were detected in both semi-intensive (*cat*, *tetA*, *tetC*, and *tetX*) and cluster (*sul1*, *cat*, *tetC*, and *vanR*) shrimp farms (Table 1). In the gut of shrimp, only sulfonamide resistance gene (*sul1*) was detected in the semi-intensive and cluster farms. The most prevalent ARGs were *sul1* which was detected in all the shrimp farming systems. Regardless of samples in total of two, four, five, and eight ARGs were detected in the extensive, improved extensive, cluster, and semi-intensive shrimp farming systems, respectively (Table 1).

The correlation analysis between the number of ARGs detected and OTU abundance was conducted, and no significant correlation ($p > 0.05$) was found (Figure 11).

4. Discussion

4.1. Comparative Analysis of Microbial Communities among Gut, Sediments, and Water. The positive contribution of microbiome to the immunity [38], and metabolism of host has a great potential to reduce the excess and random use of antibiotics in fish and shellfish culture. Considering the role played by microorganisms in the aquatic environment, the better understanding of microbial dynamics and their function in the aquatic bodies is necessary to develop microbe-based strategies for the sustainable shrimp farming [19, 39]. Therefore, the present study underpins the importance of studying microbiome associated with shrimp rearing water and sediments.

Earlier studies reported that the dominant phyla associated with *P. monodon* gut were identified Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria [40–43]. Similarly, in the present study, the predominant phyla in shrimp gut were Proteobacteria (39.29%), Firmicutes (33.68%), Actinobacteria (8.87%), and Bacteriodetes (3.65%). However, it was reported that in healthy shrimp, gut communities are generally dominated by Proteobacteria along with lower abundance of Firmicutes [41]. The phylum Firmicutes was also reported in shrimp gut followed by Proteobacteria in the previous studies [44, 45], but the relative abundance was lower. On the contrary, in our study, the phylum Firmicutes was the second highest dominant phyla identified in shrimp gut although the shrimp were healthy. Since the feed ingredients (Supplementary Table S1) used in Bangladesh are entirely different from those of other countries in the world, it might be the possible reason for these types of patterns of bacterial colonization in gut. Besides, the probiotics supplemented in feeds (Supplementary Table S1) prepared by the different companies in Bangladesh are also different in composition.

In sediment, the dominant bacterial phyla that were reported in previous studies were Proteobacteria, Bacteroidetes, Acidobacteria, Verrucomicrobia, Planctomycetes, Firmicutes, Cyanobacteria, and Actinobacteria [46]. In this investigation, the top 10 phyla were identified as Proteobacteria (35.45%), Firmicutes (12.97%), Bacteriodetes (8.12%), Chloroflexi (7.38%), Actinobacteria (5.90%), Acidobacteria (5.56%), Desulfobacteria (4.60%), Halobacteria (2.20%), and Camipilobacteria (2.05%); therefore, consistent with earlier studies (Figures 4 and 5). Here, some rare phyla like Chloroflexi, Desulfobacteria, Halobacteria, and Camipilobacteria were detected in sediment samples. The variation of geographical locations and administering of different types of feed, soil properties etc. might be the possible reason for the presence of such phyla in the sediment. During shrimp farming, the sediment bacterial community is known to play a very essential role in recycling nutrients with the constant accretion of organic matter in the aquatic ecosystem [47]. In this study, Desulphobacterales and Rhodobacterales were present in the sediment of shrimp culture pond, which are regarded as heterotrophs and have ability to degrade organic compounds in the aquatic ecosystem [47, 48]. In addition, the members of Rhodobacteraceae have successfully been applied in shrimp culture pond [49].

In general, Proteobacteria, Bacteroidetes, Acidobacteria, Cyanobacteria, Planctomycetes, Actinobacteria, and Tenericutes were reported as the predominant bacterial phyla found in water samples of shrimp culture pond [50, 51]. The present study depicted that the dominant phyla in shrimp rearing water were Proteobacteria (38.13%), Cyanbacteria (22.30%), Bacteriodetes (16.69%), Actinobacteria (8.46%), Firmicutes (6.55%), and Verrucomicrobia (2.34%) which was consistent with the previous investigations. Compared to sediment [52], or gut [53], the Actinobacteria was found to be the dominant phylum in the water of the shrimp culture ponds. It was

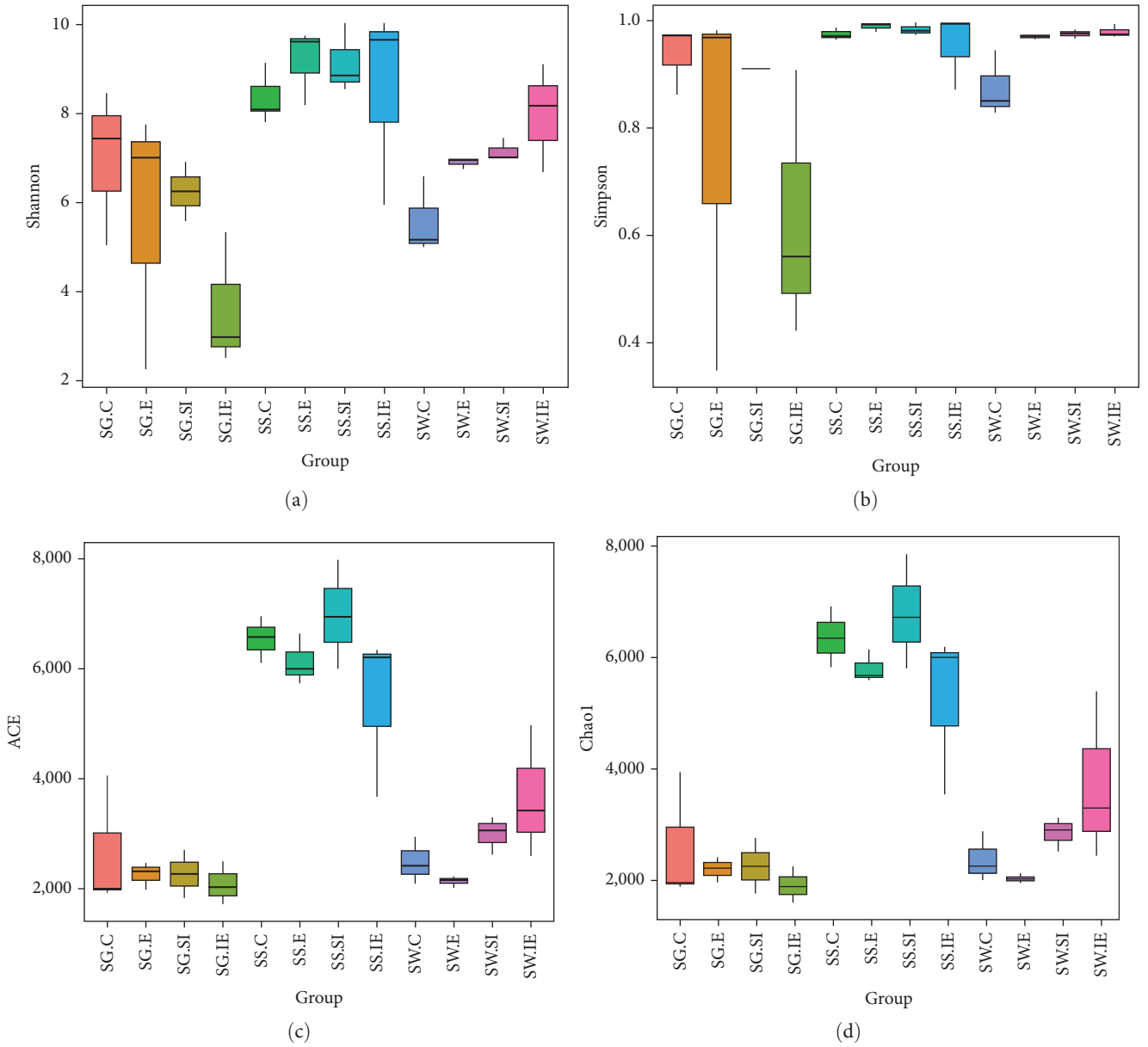


FIGURE 9: Boxplot shows different alpha diversity indices. These indices represents the bacterial diversity of gut, water, and sediment and their significant difference among four different farming systems: (a) Shannon diversity index, (b) Simpson index, (c) ACE index, and (d) Chao1 index. As shown in the figure, gut, sediment, and water (SG, SS, SW); farming system (C: cluster; E: extensive; SI: semi-intensive; IE: improved extensive).

reported that the substances that promotes positive growth and antibacterial effects are generally produced by the members of the phylum Actinobacteria [54].

In fact, the phylum Proteobacteria was the most dominant and efficient colonizers in gut [40, 42, 55], sediment [46, 56], and water [11, 42, 56], of the shrimp culture pond. It indicates that the phylum Proteobacteria could colonize in the aquaculture environment along with the gut of shrimp in different farming systems. Moreover, this phylum can be a successful target for probiotic screening in future.

4.2. The Relationship among Bacteria in Gut, Sediment, and Water at Genus Level. In the broader context e.g., at the phyla level, microbial communities inhabiting gut, sediment, and water may be found similar; however, at genus level this difference could be more significant. In the gut of shrimp, the dominated genera reported in the previous studies were *Actinotalea*, *Roseovarius* [41], *Labrenzia*, *Silicibacter*, *Vibrio*, *Listonella*, *Pseudoalteromonas* [43], *Sphingobium*, *Bradyrhizobium*, *Sphingomonas*, *Candidatus Bacilloplasma*, *Sphingopyxis*, *Serratia*, *Hyphomicrobium and Rhodococcus*, *Photobacterium*, *Roseivivax*, *Bacillus*, *Vibrio* [42], *Vibrio*, *Photobacterium damselae*, *Aeromonas* sp., *Actinomyces*, *Halospirulina*, *Propionigenium*, *Shewanella* [44], *Pseudoalteromoa* sp., *Vibrio* sp., *V. vulnificus*, *V. alginolyticus* [55], *Shewanella*, *Vibrio* sp. [37], *Shewanella*, *Vibrio*, *Bacteroides fragilis Bacteroides eggerthii*, *Bifidobacterium*, *Lactobacillus*, *Photobacterium damselae*, *Acinetobacter junii*, *Aeromonas*, *Marinobacter* sp., *Mesoflavibacter zeaxanthinifaciens*, *Shigella*

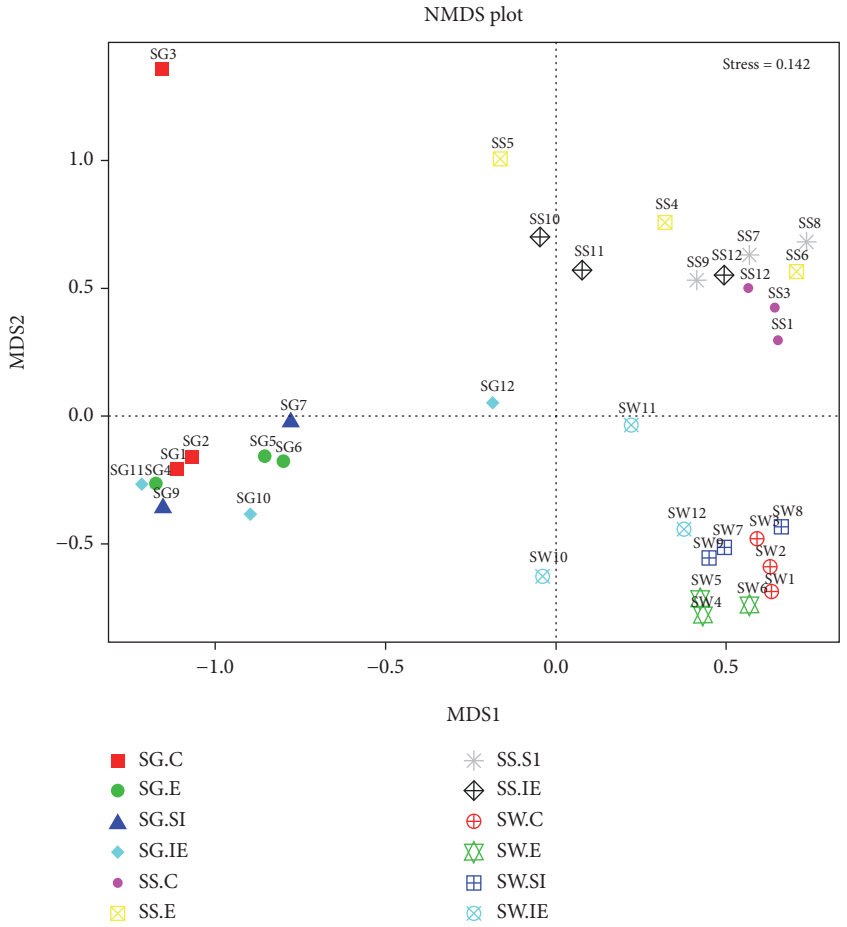


Figure 10: Nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity for bacterial community profiles. Each data point in the graph stands for a sample. The distance between data points reflects the extent of variation. Samples belongs to the same group are in the same color. When the value of stress factor is less than 0.2, it's considered that NMDS is reliable to some extent. Here, stress value was 0.142, so NMDS was reliable. As shown in the figure, gut, sediment, and water (SG, SS, and SW); farming system (C: cluster; E: extensive; SI: semi-intensive; IE: improved extensive).

flexneri, *Photobacterium* sp., *Propionibacterium* sp., *Actinomyces dentalis*, and *Fusibacter* sp.

The dominant genera, found in the current exploration, in shrimp gut were *Rickettsiella*, *Shewanella*, *Candidatus_Bacilloplasma*, *Clostridium_sensu_stricto_1*, *Candidatus_Hepatoplasma*, *Romboutsia*, *Vibrio*, *Cyanobium*, *Photobacterium*, *Lactococcus*, *Muribaculaceae Chloroplast*, *Akkermansia*, *Mycobacterium*, *Lactobacillus*, and *Candidatus_Hepatincola*. Therefore, the bacterial communities in the gut were more diverse and consistent with some extent to the previous results.

In sediment the predominant genera were *Sulfurovum*, *Robiginitalea*, *Desulfobulbus* [56], *Acinetobacter lwoffii*, *Vibrio* sp. [46], *Bacillus* [57], *Nitrospirae*, *Chlorobi*, *Pseudomonas*, and *Geobacter* [58]. The present study indicates the bacterial flora in sediment of shrimp culture pond were *Rheinheimera*, *Paenisporosarcina*, *Aeromonas*, *Shewanella*, *Paraclostridium*, and *Pseudarcobacter* which is totally distinct from the previous findings.

The predominant genera in water sample of shrimp culture pond reported in previous studies were *Shewanella*, *Pseudomonas*, *Flavobacterium*, *Nitrospirae*, *Chlorobi*, *Pseudomonas* [58], *Skeletonema*, *Thalassiosira*, *Chaetoceros* [57], *Limnothrix*, *Photobacterium*, *Roseivivax*, and *Bacillus* [42]. The current study indicated that the predominant genera were *Rheinheimera*, *Cyanobium*, *Chloroplast*, *Flavobacterium*, *Pseudomonas*, *Oceanobacter*, and *Candidatus_Aquirestis*. In addition, the genera in water group were also distinct from the previous studies.

4.3. Association between Microbial Communities and Shrimp Health in Different Farming Systems. In semi-intensive shrimp farming systems, the phylum Proteobacteria was reported to be the most abundant in the sediment sample of penaeid shrimp culture pond [46]. In the case of European sea bass semi-intensive farming, Proteobacteria was also the most abundant phylum [59]. However, in the current study, the relative abundance of Proteobacteria in the sediment sample of semi-intensive farming system was 22.14%, whereas in the cluster farming system the abundance was much higher (57.71%). The use of different feeds might be the possible reason for the opposite bacterial abundance pattern in these two systems.

Compared to the extensive, semi-intensive, and improved extensive farming system, Proteobacteria (53.98%) was also found to be the most abundant phyla in water samples of

Table 1: Antibiotic resistance gene profile in different shrimp farming systems.

Culture system	Sample ID	Name of the antibiotic groups and associated resistance genes																		
		Sulphonamide		Chloramphenicol		Quinolones		Aminoglycoside	Ciprofloxacin	Tetracycline				Beta-lactams	Macrolide			Bacitracin	Vancomycin	Trimethoprim
		sul1	*sul3*	*cat*	*cfr*	*qnrA*	*gyrA*	*aacA1*	*gyrA*(*c*)	*tetA*	*tetC*	*tet32*	*tetX*	*blaTem-1*	*mecA*	*ermB*	*ere* (*A*)	*bacA*	*vanR*	*dfrA1*
Cluster	**SS**	**P**	ND	**P**	ND	ND	ND	ND	ND	ND	**P**	ND	ND	ND	ND	ND	ND	ND	**P**	ND
	SG	**P**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	SW	**P**	ND	**P**	ND	ND	ND	ND	ND	**P**	**P**	ND	ND	ND	ND	ND	ND	ND	**P**	ND
Semi-intensive	**SS**	ND	ND	**P**	ND	ND	ND	ND	ND	**P**	**P**	ND	**P**	ND	ND	ND	ND	ND	ND	ND
	SG	**P**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	SW	ND	ND	**P**	ND	ND	ND	ND	**P**	**P**	**P**	ND	**P**	ND	ND	ND	**P**	ND	**P**	ND
Improved extensive	**SS**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	**P**	ND
	SG	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	SW	**P**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	**P**	ND	ND	**P**
Extensive	**SS**	**P**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	SG	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	SW	**P**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	**P**	ND	ND	ND

SG = shrimp gut, SW = shrimp water (water of the shrimp culture pond), SS = shrimp sediment (sediment of the shrimp culture pond), P = positive, and ND = not detected.

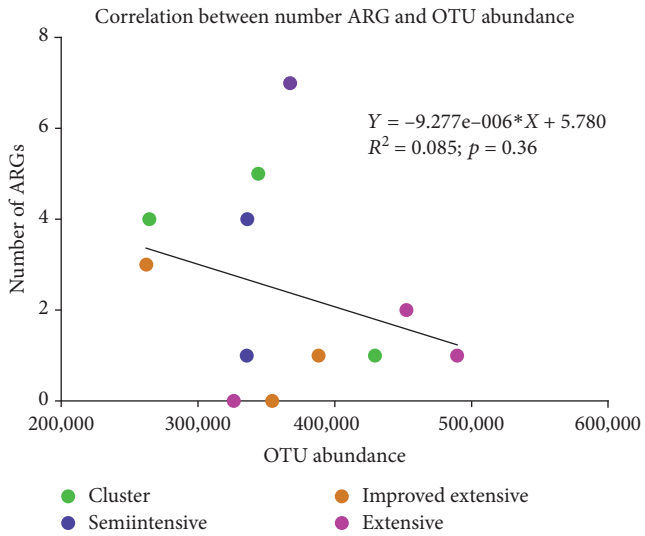


FIGURE 11: Correlation between the number of antibiotic resistance genes and OTU abundance.

cluster farming system ponds. The mineralization of organic compounds and the cycling of nutrients is occurred in the environment by the phylum Proteobacteria [60].

The relative abundance of Actinobacteria in semi-intensive pond water was higher compared to the all other farming systems. In the extensive farming system, the abundance of Actinobacteria was higher in the gut of shrimp, which is an indication of no application of antibiotics and chemicals. Moreover, the ratio of Firmicutes, Proteobacteria, and Actinobacteria in the gut of cluster system shrimp and semi-intensive shrimp was very similar. Although there is an abundance of Proteobacteria, bacterial disease may be prevented by the members of Firmicutes and Actinobacteria in the gut of shrimp. Besides Proteobacteria, the members of the phylum Actinobacteria can also be a successful target for probiotic screening or nutritionally bioaugmentation in water compared to the gut or sediment.

The phylum Bacteroidetes are a group of gut microbiomes which were found to be abundant in sediment and water samples, which corroborate with our results. Several species of the phylum Bacteroidetes have recently been reported with resistance against antibiotics [61], which has also the capacity to produce vitamin B12 in the gut of shrimp and finfishes [62]. In our findings, the sediment and water of cluster and semi-intensive farming system, the relative abundance of Bacteriodetes phylum was higher. Generally, shrimp disease [63], is caused by two opportunistic pathogens, *Vibrio* and *Photobacterium*, as they can carry the virulence gene responsible for causing disease. Although a single taxon can not be the sole reason for causing disease, as several environmental factors are also important for infections and outbreak. In our findings, the relative abundance of these two diseases causing taxa in the cluster and semi-intensive farming systems were less than 1%. In the surrounding environment and the gut of the host, the information regarding the function of the microorganism is crucial for direct screening of potential probiotics in the aquaculture.

4.4. ARGs and Their Prevalence throughout Shrimp Farming Systems. With the rapid expansion of the shrimp aquaculture sector over time, the arbitrary and excess use of antibiotics in the ponds for the prevention of infectious diseases has also been increased. The situation is leading to the emergence of ARB in fish and shellfish aquaculture. The presence of ARGs in the shellfish is a serious threat not only for the shrimp culture sector but also for interconnected One-Health aspect [64]. The resistant gene *cat* belongs to the antibiotic group chloramphenicol, which was previously reported as one of widely and heavily used antibiotics in shrimp aquaculture in Bangladesh [65]. The commonly used antibiotics in shrimp culture system in Bangladesh are sulfonamide, chloramphenicol, oxytetracycline, ciprofloxacin, and erythromycin [65]. Besides that, the use of other groups of antibiotics such as neomycin sulfate, chlortetracycline and doxycycline [66], sulfamethoxazole (SMX), erythromycin, trimethoprim, and tylosin [67] were also reported. In our study, the ARGs responsible for conferring resistance against antibiotics such as chloramphenicol, sulfonamide, ciprofloxacin, tetracycline, macrolide, vancomycin, and trimethoprim were detected shrimp's gut as well as rearing water and sediment which is consistent with the previous reports [65, 67]. The number of ARGs detected in four different farming systems were eight, five, three, and two ARGs in semi-intensive, cluster, improved extensive, and extensive system, respectively. Among the 8 semi-intensive and 42 extensive system surveyed by Aftabuddin et al. [65], found that most of the antibiotics were applied in semi-intensive shrimp farming systems. The only antibiotic, oxytetracycline was found applied in the extensive farming system. This means, antibiotics are heavily used in the semi-intensive systems in Bangladesh followed by the cluster systems. The heavy usages of antibiotics could alter the bacterial physiology as well as community dynamics and might enhance the bacterial resistance capacity against certain antibiotics [68]. In such scenario, the event of horizontal transfer of ARGs among bacterial communities could also be a major contributing factor for the emergence of AMR. However, extensive research is needed to identify the potential sources of ARGs in the aquaculture systems.

The resistant gene *cat* belonging to the chloramphenicol group was previously reported in the intestine of shrimp [69]. In our study, this gene was detected in the sediment and water samples of two farming systems. On the contrary, the sulfonamide group resistant genes were reported from the shellfish isolates and sediment of shrimp culture system [70, 71], which is consistent with our findings. In addition, the presence of resistant genes that belong to the tetracycline group were found in the sediment as reported by previous studies [72], which is also agreed with our results. The indiscriminate use of antibiotics in shrimp aquaculture is a serious threat in terms of consumer health and export.

5. Conclusion

The current study reveals the bacterial community structure in gut, sediment, and water associated with four different farming systems (semi-intensive, cluster, improved extensive,

and extensive aquaculture) of black tiger shrimp (*P. monodon*) for the first time in the southwest region of Bangladesh. The phyla Proteobacteria and Firmicutes were predominant where bacterial community structure was significantly distinct among gut, sediment, and water samples of the different farming systems. Of the tested 19 ARGs, *sul1*, *cat*, *gyrA*(*C*), *tetA*, *tetC*, *tetX*, *ere*(*A*), *vanR*, and *dfrA1* were predominantly detected in the water and sediment samples. Semi-intensive farming system had the highest prevalence of ARGs (21.05%) followed by the cluster (15.79%), improved extensive (7.02%), and extensive (5.26%) farming system. Our study indicates that the bacterial diversity is not only altered with the sample type (gut, sediment, and water) but also with the farming techniques. The variation of geographical location of the farm, types of feed, and management practices might be the crucial factors for regulation of the microbial community structure. In addition, the emergence of ARGs might be a growing concern for the sustainability of shrimp cultivation in Bangladesh.

Data Availability

The data supporting the findings of this study are accessible from the corresponding author upon request.

Ethical Approval

The research protocol was approved by the ethics committee of the Faculty of Biological Sciences, University of Dhaka, Dhaka-1000, Bangladesh (Reference no- 186/Biol.Scs.)

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Md. Zakaria contributed in the sample and data collection, methodology, formal analysis, bench work, writing–original draft, and visualization. Santonu K. Sanyal, Anwar Hossain, and Md. Inja-Mamun Haque contributed in the conceptualization, data analysis, investigation, and supervision. Md. Inja-Mamun Haque and Anwar Hossain contributed in the funding acquisition. Shankar Chandra Mandal, Kozo Watanabe, Santonu K. Sanyal, Anwar Hossain, and Md. Inja-Mamun Haque contributed in the writing–review and editing. Shankar Chandra Mandal contributed in the data analysis. Md. Inja-Mamun Haque and Anwar Hossain contributed equally to this work.

Acknowledgments

We would like to acknowledge Microbial Genetics and Bioinformatics Laboratory of Department of Microbiology, University of Dhaka, for high-tech instrumental support to conduct this study. Special appreciation to Professor Dr. Mahmud Hasan, Aquaculture Genomics Laboratory, Department of Fisheries, University of Dhaka, for providing the opportunity to use machinaries to carry out the research project. The authors wish to express a deep appreciation to the shrimp farmers and owners for providing samples and information to conduct the study. This research project was supported by the Ministry of Science and Technology, Government of the People's Republic of Bangladesh [Grant Nos.: 39.00.0000.009.14.011.20-ES-328/1674/2020-2021 and 39.00.0000.009.14.019.21-ES-359/1106/2021-2022].

Supplementary Materials

Table S1: Environmental parameter (water) and sample related information of four different farming systems. Table S2: Antibiotics usage in shrimp aquaculture of Bangladesh. Table S3: Antibiotics resistant bacteria in shrimp aquaculture of Bangladesh. Table S4: PCR thermal cycle and time profile for antibiotic resistance genes used in this study. Table S5: PCR product visualization under agarose gel electrophoresis of each detected genes. Figure S1: Evolutionary tree for all samples (gut, sediment, and water) at genus level illustrating 100 genus. Figure S2: Evolutionary tree for four different farming systems at genus level illustrating 100 genus. (*Supplementary Materials*)

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