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Microbial Community Structure and Bacterial Lineages Associated with Sulfonamides Resistance in Anthropogenic Impacted Larut River

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Abstract: Anthropogenic activities often contribute to antibiotic resistance in aquatic environments. Larut River Malaysia is polluted with both organic and inorganic pollutants from domestic and industrial wastewater that are probably treated inadequately. The river is characterized by high biochemical oxygen demand, chemical oxygen demand, total suspended solids, ammonia, and heavy metals. In our previous study, sulfonamides (SAs) and sulfonamide resistance genes (*sul*) were detected in the Larut River. Hence, in this study, we further examined the microbial community structure, diversity of sulfonamide-resistant bacteria (SARB), and their resistance genes. The study also aimed at identifying cultivable bacteria potential carriers of *sul* genes in the aquatic environment. Proteobacteria (22.4–66.0%), Firmicutes (0.8–41.6%), Bacteroidetes (2.0–29.4%), and Actinobacteria (5.5–27.9%) were the most dominant phyla in both the effluents and river waters. SARB isolated consisted only 4.7% of the total genera identified, with SAR Klebsiella as the most dominant (38.0–61.3%) followed by SAR Escherichia (0–22.2%) and Acinetobacter (3.2–16.0%). The majority of the SAR Klebsiella isolated from the effluents and middle downstream were positive for *sul* genes. *Sul* genes-negative SAR Escherichia and Acinetobacter were low (<20%). Canonical-correlation analysis (CCA) showed that SAs residues and inorganic nutrients exerted significant impacts on microbial community and total *sul* genes. Network analysis identified 11 SARB as potential *sul* genes bacterial carriers. These findings indicated that anthropogenic activities exerted impacts on the microbial community structure and SAs resistance in the Larut River.

Keywords: anthropogenic water pollution; Larut River; microbial community structure; sulfonamide resistance genes

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1. Introduction

The increasing community-acquired infections of antibiotic-resistant bacteria (ARB) and occurrence of environmental ARB and their resistance genes (ARGs) have raised concern about antibiotic resistance in natural environments [1–4]. Aquatic environments are suffering from different types and levels of anthropogenic antibiotic pollution, including discharge of untreated and crudely treated municipal, hospital, agricultural, livestock, and pharmaceutical industries wastewaters [5]. Subsequently, aquatic environments may represent the origin, amplifier, and/or reservoir of the environmental, human, and/or animals ARB and ARGs as well as act as a bioreactor facilitating the emergence and dissemination of ARGs transfer between the pathogenic and non-pathogenic bacteria [6–8].

There are three possible types of selection for antibiotic resistance in aquatic environments [9]: (i) natural selection for antibiotic resistance through the production of antibiotics by aquatic organisms (fungi, bacteria) [10]; (ii) anthropogenic selection of ARB and ARGs through anthropogenic activities [11]; and (iii) introduction of ARB and ARGs from anthropogenic sources [12]. Studies have shown that exposure of bacteria to a sub-inhibitory concentration of antibiotics may play a significant role in the selection of antibiotic resistance [13,14]. However, ARB with resistance genes on plasmids or genomes could remain in the environments with or without selective pressures [15,16]. These discrepancies could be due to more complicated mechanisms of development and preservation of ARB and ARGs in the environment. Thus, understanding the survival of ARB from anthropogenic sources as well as the persistence of ARGs in the natural environment is essential to more accurately assess the role of aquatic environments as reservoirs of resistance.

Most studies to date on ARGs in the aquatic environment are those that confer resistance to beta-lactam antibiotics, aminoglycosides, fluoroquinolones, tetracyclines, and macrolides [17]. In fact, studies related to sulfonamide resistance genes (*sul*) in surface waters were rarely reported, particularly for anthropogenic polluted rivers. There are numerous investigations on the composition of the bacterial assemblage and ARGs in rivers indicating that external pollution and environmental factors affect the bacterial community assembly [18,19]. However, most studies reported data either on the types and detection frequency of ARG or ARB. Despite metagenomic analysis that has been conducted in some studies to characterize the acquired resistome in aquatic environments, understanding the water quality, variation of bacterial communities, and anthropogenic factors that affect the fate of ARGs in the aquatic environment remain scarce.

Sulfonamides (SAs) have been applied in humans and veterinary as antibiotic prophylaxis for almost a century [20]. SAs possess a high excretion rate, high solubility, and persistence in the environment [21,22]. Bacterial SA resistance is mediated by the horizontal transfer of foreign *dhps* gene or a part of it or via plasmids that carry *sul* genes encoding alternative drug-resistant variants of the dihydropteroate synthase (DHPS) enzyme [23]. SA binds to DHPS to inhibit dihydrofolic acid formation and thus halts bacterial growth. Distribution profiles of *sul* genes vary in aquatic bacterial assemblages [24]. However, whether the ARGs carriers are potential harmful pathogens or harmless non-pathogens remains unresolved while the emergence and spread of ARGs have been a rising threat in recent decades [25,26].

In our recent monitoring work, we revealed that sulfamethoxazole (SMX), sulfadimethoxine (SDM), and sulfadiazine (SDZ) are the most prevalent SAs residues detected in the Larut River. Our findings have shown that SMX-resistant (SMX^r) heterotrophic bacteria and SMX^r-enteric bacteria, as well as the ratio of SMX^r bacteria to total bacteria, increased at sites receiving wastewater effluents [14]. As SAs residues are also prevalent here, we hypothesized that environmental bacterial assemblage, SARB, and *sul* genes are similarly influenced by the anthropogenic influence. To better understand the impact of environmental bacterial assemblage on the SAs resistance genes and the potential *sul* gene bacterial carriers in anthropogenically impacted Larut River, we used the culture-dependent method to identify and examine the diversity of culturable SAs-resistant (SAR) isolates and their *sul* genes. A comparison of culturable to total microbial community profile structure was carried out to identify the potential *sul* gene bacterial carriers. We also determined the factors affecting the bacterial community composition and the fate of *sul* genes in this river. The results from this study will be useful to strategize effective mitigating strategies for combating environmental antibiotic resistance dissemination and transmission of resistant genes through the water system.

2. Materials and Methods

2.1. Sampling Sites

The samples were collected from six sampling sites located upstream (S1a) and downstream (S1b and S1c) of Larut River, Taiping, Perak, including wastewater effluents from zoos, hospitals, and slaughterhouses (Figure 1). These wastewaters were collected from the major outlet flowing into the adjacent river, and they were mainly greywater. Larut River is the only river from the Larut hill flowing through the city, receiving the wastewater effluents that are eventually discharged into the Straits of Malacca. Thus, this river was selected as a model to study the anthropogenic impacts on SAs resistance in the aquatic environment [14]. Grab sampling was conducted for three consecutive months to collect the water samples with an acid-washed bucket. Although each grab sample represents a snapshot of the environment, the average of the three different months should adequately reflect the environmental condition. All samples were placed in sterilized sampling bottles and kept on ice until analysis in the laboratory. A concurrent study to measure the physicochemical (temperature, pH, salinity, dissolved oxygen) and inorganic nutrient parameters (ammonium (NH_4), nitrite (NO_2), nitrate (NO_3), phosphate (PO_4)) was conducted, and the results have been published [14].

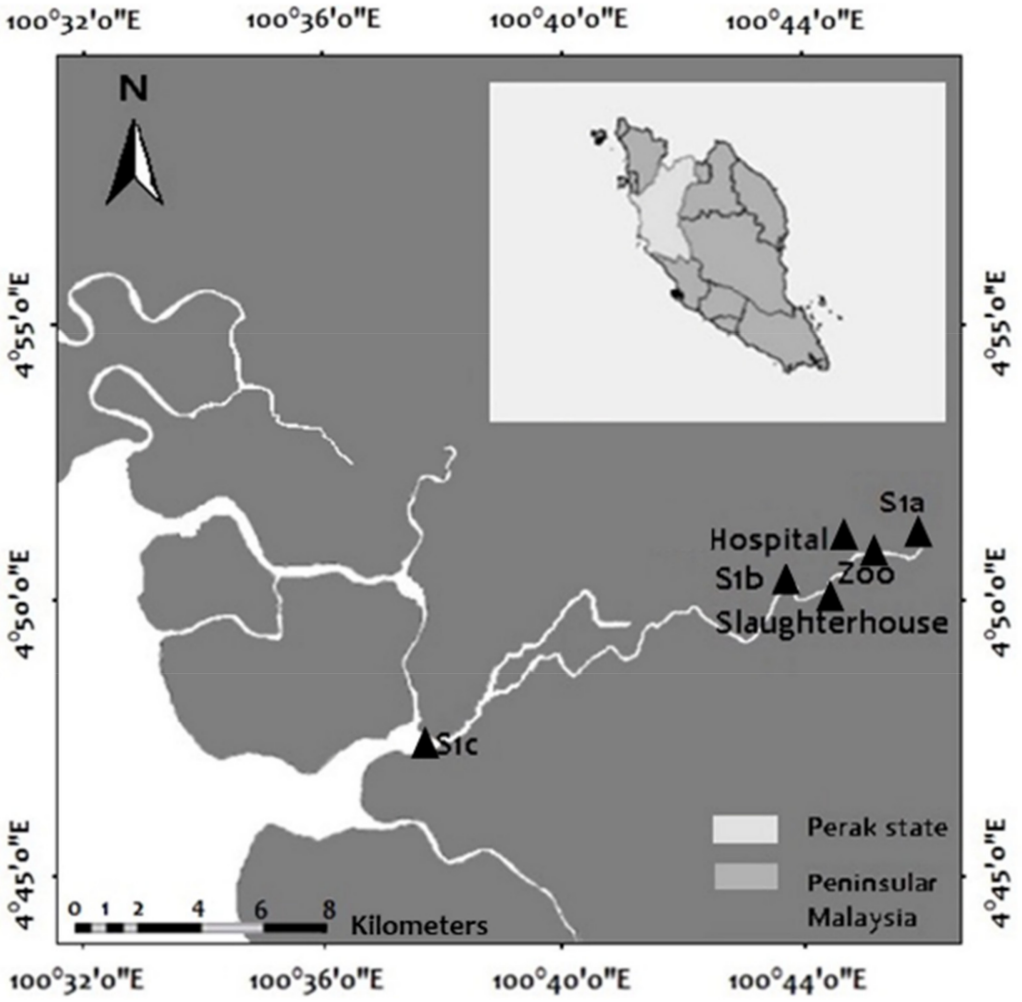


Figure 1. Sampling sites map.

2.2. Metagenomics DNA Extraction

A total of 500 mL of water samples were filtered through a 0.22 µM Millipore GTTP (Merck, Kenilworth, NJ, USA) membrane filter. Total genomic DNA was extracted using PowerWater® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA with concentration >30 ng μL^{-1} was sent for 16S metagenomics analysis.

2.3. Microbial Community Structure

The microbial community structure was analyzed using the Illumina MiSeq platform (Majorbio, China). The marker region of the bacteria was amplified using 338F and 806R primers with unique barcodes. PCR amplification was conducted at 95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. The libraries were constructed using the Illumina MiSeq PE250/300 Platform according to standard protocols. Sample libraries were pooled in equimolar and paired-end sequenced (2 × 250/300 bp) on an Illumina MiSeq platform. The raw "Fastq" files were demultiplexed and quality filtered using Quantitative Insights into Microbial Ecology (QIIME, version 1.9.1). Operational taxonomic units (OTUs) were clustered with 97% similarity cut-off using the UPARSE in the USEARCH software (version 7.1, http://drive5.com/uparse/, accessed on 24 January 2019) [11]. All quality sequence "Fasta" files were checked and removed for chimeric sequences by the UCHIME reference algorithm [7]. The taxonomic information of each 16S rRNA gene sequence was analyzed by Ribosomal Database Project Classifier (RDP Classifier 11.1, http://rdp.cme.msu.edu/, accessed on 31 December 2019) [16] against the SILVA rRNA databases 123 (SSU123) using a confidence threshold of 0.7 [27]. The diversity indices (Chao1 and Shannon indices) were calculated using Mothur (version v.1.30.1, https://mothur.org/wiki/, accessed on 31 December 2019) [28]. The microbial community heat map was drawn using Microsoft Excel. Bray-Curtis distance matrix was calculated using PAST software [29], and hierarchical cluster analysis was presented as a dendrogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) [30].

2.4. Identification of Culturable Sulfonamides-Resistant Bacteria

A total of 278 culturable SAR isolates were isolated from water samples collected from the six sampling sites. The culturable SAR bacterial isolation was carried out using tryptic soy agar (TSA), and CHROMagar™ Orientation (COR) supplemented with 60 µg mL^{-1} SMX in triplicates [31]. The culturable SAR isolates were cultured in nutrient broth supplemented with 60 µg mL^{-1} SMX at 37 °C for 18–24 h prior to DNA extraction using the boiling method [32]. The 16S rDNA genes were amplified using PCR (Applied Biosystem, CA, United States) with 27F and 1492R primers (Table 1) [4] and subjected to 16S rRNA Sanger sequencing. Basic Local Alignment Search Tool (BLAST) was used for bacterial identification [8].

Table 1. Primers sequences for SAR isolates identification and *sul* genes detection.

Target Genes	Primer	Sequence	Size (bp)	Reference
16S rDNA	27F	5′-AGAGTTTGATCMTGGCTCAG-3′	1500	[4]
	1492R	5′-GGTTACCTTGTTACGACTT-3′		
sul1	Sul1F	5′-CGGCGTGGGCTACCTGAACG-3′	433	[33]
	Sul1R	5′-GCCGATCGCGTGAAGTTCCG-3′		
sul2	Sul2F	5′-GCGCTCAAGGCAGATGGCATT-3′	293	[33]
	Sul2R	5′-GCGTTTGATACCGGCACCCGT-3′		
sul3	Sul3F	5′-CCCATACCCGGATCAAGAATAA-3′	143	[34]
	Sul3R	5′-CAGCGAATTGGTGCAGCTACTA-3′		

2.5. Detection of SAR Genes (sul1, sul2 and sul3) in the Sulfonamides-Resistant Bacteria

The presence of *sul* genes was determined using PCR (Applied Biosystem, California, United States) with gene-specific primers (Table 1). Amplification conditions for the *sul* genes were as follows: pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C (for *sul1* and *sul2*) [33] and 63 °C (*sul3*) [34] for 30 s and extension 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. Gel electrophoresis was performed on 1.5% agarose gel.

2.6. Statistical Analyses

Canonical-correspondence analysis (CCA) was used to explore the impacts' probable links among SAs residues, environmental physicochemical parameters, microbial community structure, and total *sul* genes in the Larut River [14]. The relationship between the categories was represented in a two-dimensional graph. Network visualization was performed based on the logarithm-transformed culturable SARB population and the abundance of their *sul* genes using Gephi 0.9.2 to measure the associations between these parameters.

3. Results and Discussion

3.1. Diversity of the Microbial Community

Metagenomics sequencing from six samples obtained 50997 to 68919 reads (Supplementary Table S1). These reads were clustered into 499 to 1227 OTUs (>97% sequence similarity). The rarefaction curves of OTUs were saturated for all of the sampling sites (Supplementary Figure S1). The microbial community composition varied among the sites. Two outcomes corresponding to anthropogenic inputs were observed: (i) wastewater effluents supported the microbial community diversity in which the bacterial diversity and species richness were pronouncedly increased in effluents from hospital (Chao index (Chao 1):1278; Shannon index (H): 4.91) and slaughterhouse (Chao 1:1254; H: 5.24); compared to zoos (Chao 1, 633; H, 3.87) as well as river water. (ii) Lower bacterial diversity and species richness by which Comamonas was found to be present predominantly in effluent from the zoo.

Cluster analysis indicated that the microbial community composition in zoo effluent and S1b, which received all wastewater effluents from the zoo, hospital, and slaughterhouse, were more similar compared to S1a (Figure 2). This could be due to the influence of effluent discharge that contained more animal waste as Lye et al. [14] detected SMX and SDM among the commonly used SAs in these wastewaters. In contrast, at S1c, which is further downstream and near the river mouth, salinity may be the important factor in shaping the bacterial community structure to be similar to the microbial community in hospital wastewater, which is characterized by high TDS [35]. Studies have shown shifts in aquatic bacterial diversity according to salinity gradient [36,37]. However, further research is needed to confirm this. The distinct bacterial community profile observed in slaughterhouses may be due to poultry waste, and the distinctly lower DO levels (<LOQ) [31] as DO is an important environmental driver that influences bacterial communities in various marine and freshwater ecosystems [38,39].

In this study, we identified 32 phyla, 40 classes, and 509 genera. Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria dominated in both effluents and river waters (Figure 3a–c). These four phyla were followed by six other major phyla, including Acidobacteria, Cyanobacteria, Parcubacteria, Verrucomicrobia (except for zoo effluent), Chloroflexi, and Saccharibacteria. The dominant phyla detected in this study are reportedly predominant in soil and various aquatic environments [40–44]. However, the dominant bacterial phyla in the aquatic ecosystem generally varied depending on the type of water samples and anthropogenic pollutants [41,45,46]. Within the Proteobacteria phylum, Alphaproteobacteria was enriched in S1a (37.3%) and S1c (24.9%), Betaproteobacteria in zoo effluent (33.3%) and S1b (60.0%) while Gammaproteobacteria in zoo (14.7%) and slaughterhouse (16.2%) effluents and S1c (13.4%). These findings

concurred with previous studies that reported the survival of the Proteobacteria in wastewater as members of this phylum are known to have versatile metabolic pathways and intense biodegradative activity [47–50]. For Firmicutes, Bacilli was enriched in S1a (28.2%) and slaughterhouse effluent (20.6%), while Clostridia was only in the slaughterhouse effluent (18.3%). The lower DO conditions in the slaughterhouse effluent might have provided more suitable conditions for the growth of these sporeformers [51]. In Bacteroidetes, Flavobacteriia predominated in the zoo effluent. A similar finding has been reported by Bondarczuk and Piotrowska-Seget [45] in a lake receiving wastewater, and the presence of Flavobacteriia is frequently associated with the high availability of organic matter [45,52]. Actinobacteria were enriched to >10% abundance in all of the effluents and river waters except in S1a. The members of this phylum are well known for tolerance to low levels of organic carbon, and thus, they can survive in a wide range of habitats [53–55].

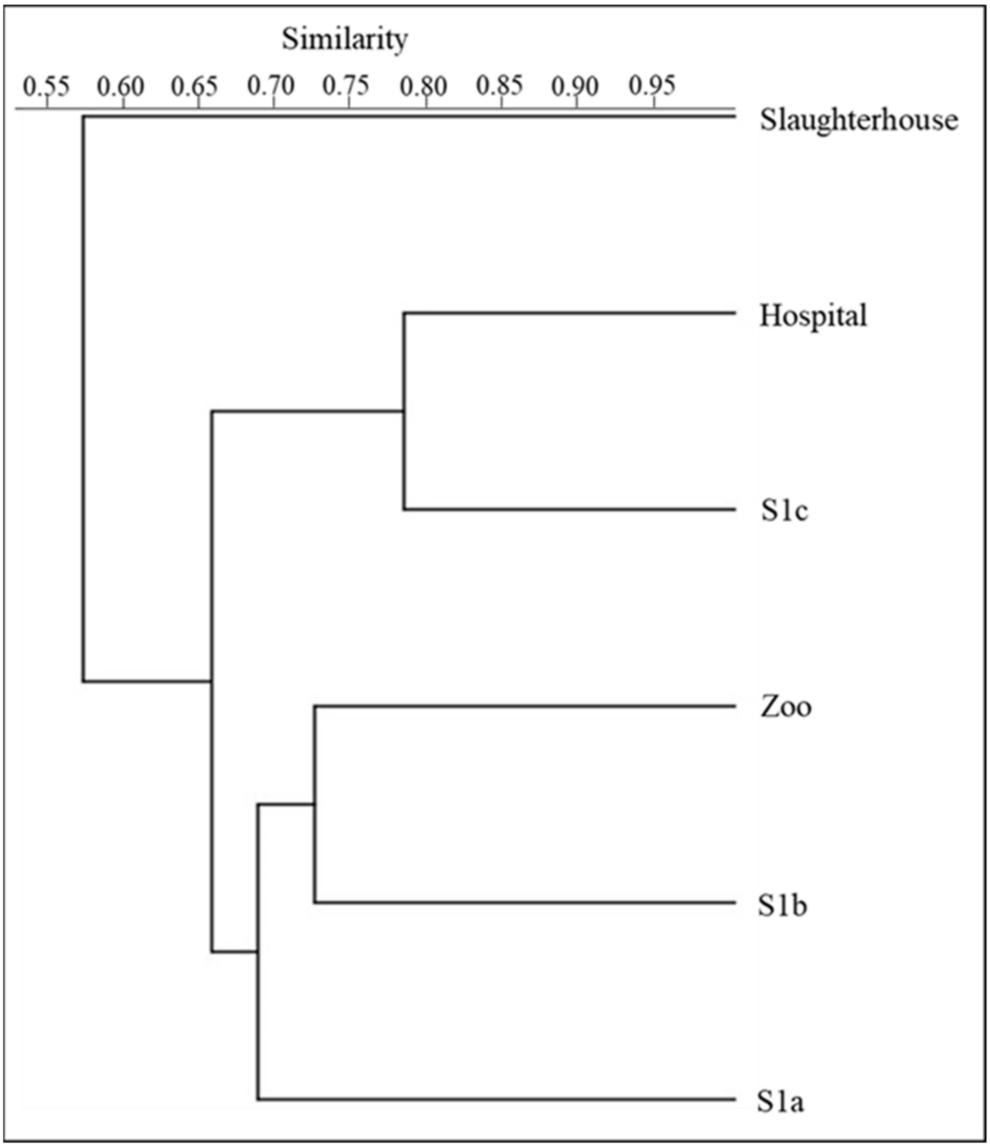


Figure 2. Bray-Curtis distance constructed by PAST software for clustering of sampling sites based on the phyla abundance.

Phylum	S1a	Zoo	Hospital	Slaughterhouse	S1b	S1c
Acidobacteria	1.8	0.0	3.3	0.1	0.5	4.5
Actinobacteria	5.5	10.0	19.8	18.7	14.7	27.9
Aminicenantes	0.0	0.0	0.0	0.1	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0
Bacteroidetes	2.0	29.4	4.5	4.0	8.6	5.1
Caldiserica	0.0	0.0	0.1	0.0	0.0	0.0
Chlamydiae	0.0	0.0	0.1	0.0	0.0	0.0
Chlorobi	0.0	0.0	0.1	0.0	0.0	0.2
Chloroflexi	1.4	0.1	2.3	3.0	0.5	7.2
Cyanobacteria	0.2	0.0	3.4	0.2	0.6	2.1
Deferribacteres	0.0	0.0	0.0	0.0	0.0	0.3
Deinococcus-Thermus	0.2	0.1	0.1	1.0	0.0	0.1
Elusimicrobia	0.0	0.0	0.1	0.0	0.0	0.0
Firmicutes	29.0	8.1	18.1	41.6	0.8	3.4
Fusobacteria	0.1	0.1	0.1	0.0	0.0	0.0
Gemmatimonadetes	0.3	0.0	0.5	0.0	0.3	0.8
Gracilibacteria	0.0	0.0	0.0	0.0	0.2	0.0
Hydrogenedentes	0.0	0.0	0.0	0.0	0.0	0.0
Latescibacteria	0.0	0.0	0.1	0.0	0.0	0.2
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.1
Marinimicrobia	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.1	0.1	0.1	0.1
Nitrospirae	0.1	0.0	0.2	0.0	0.1	0.1
Parcubacteria	0.2	0.0	0.6	0.7	2.0	0.3
Planctomycetes	0.1	0.0	0.3	0.0	0.0	0.2
Proteobacteria	56.3	51.7	38.4	22.4	66.0	44.5
Saccharibacteria	0.5	0.3	3.0	3.6	3.3	0.2
Spirochaetae	0.0	0.0	0.0	0.1	0.0	0.2
Synergistetes	0.0	0.0	0.0	0.1	0.0	0.0
Tenericutes	0.0	0.0	0.3	0.1	0.2	0.0
Thermotogae	0.0	0.0	0.0	0.0	0.0	0.0
Verrucomicrobia	0.1	0.0	1.1	0.1	0.1	1.9
Unassigned	2.0	0.0	3.3	4.0	1.9	0.5
Total	100	100	100	100	100	100

Scale: 0.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0

(**a**)

Figure 3. *Cont*.

Class	S1a	Zoo	Hospital	Slaughterhouse	S1b	S1c
Acidobacteria	1.8	0.0	3.3	0.1	0.5	4.5
Actinobacteria	5.5	10.0	19.8	18.7	14.7	27.9
Alphaproteobacteria	37.3	3.7	5.4	3.3	2.1	24.9
Anaerolineae	0.7	0.0	1.3	1.2	0.3	5.1
Ardenticatenia	0.0	0.0	0.0	0.0	0.0	0.1
Bacilli	28.2	1.6	6.1	20.6	0.3	3.1
Bacteroidia	0.0	0.3	1.2	3.1	0.6	0.4
Betaproteobacteria	13.6	33.3	25.0	2.5	60.0	0.9
Caldilineae	0.0	0.0	0.0	0.3	0.0	0.4
Caldisericia	0.0	0.0	0.1	0.0	0.0	0.0
Chlorobia	0.0	0.0	0.1	0.0	0.0	0.0
Chloroflexia	0.1	0.0	0.2	0.3	0.0	0.0
Chthonomonadetes	0.0	0.0	0.0	0.0	0.0	0.0
Clostridia	0.6	2.5	5.9	18.3	0.4	0.3
Cytophagia	0.7	0.0	0.2	0.0	1.0	1.6
Deferribacteres	0.0	0.0	0.0	0.0	0.0	0.3
Dehalococcoidia	0.0	0.0	0.0	0.0	0.0	0.0
Deinococci	0.2	0.1	0.1	1.0	0.0	0.1
Deltaproteobacteria	0.4	0.0	0.8	0.4	1.6	4.2
Elusimicrobia	0.0	0.0	0.1	0.0	0.0	0.0
Epsilonproteobacteria	0.0	0.0	0.2	0.0	0.3	0.5
Erysipelotrichia	0.2	3.8	2.5	1.6	0.1	0.0
Flavobacteriia	0.9	28.2	2.6	0.6	4.6	1.6
Fusobacteriia	0.1	0.1	0.1	0.0	0.0	0.0
Gammaproteobacteria	4.9	14.7	6.9	16.2	2.0	13.4
Gemmatimonadetes	0.3	0.0	0.5	0.0	0.3	0.8
Ignavibacteria	0.0	0.0	0.0	0.0	0.0	0.1
Ktedonobacteria	0.0	0.0	0.0	0.0	0.0	0.0
Mollicutes	0.0	0.0	0.3	0.1	0.2	0.0
Negativicutes	0.1	0.2	3.6	1.0	0.0	0.0
Nitrospira	0.1	0.0	0.2	0.0	0.1	0.1
Opitutae	0.0	0.0	0.0	0.0	0.0	0.0
Phycisphaerae	0.0	0.0	0.1	0.0	0.0	0.1
Planctomycetacia	0.0	0.0	0.1	0.0	0.0	0.0
Spartobacteria	0.0	0.0	0.0	0.0	0.0	1.1
Sphingobacteriia	0.4	0.9	0.4	0.2	2.5	1.0
Synergistia	0.0	0.0	0.0	0.1	0.0	0.0
Thermomicrobia	0.1	0.1	0.2	0.1	0.0	0.0
Thermotogae	0.0	0.0	0.0	0.0	0.0	0.0
Unassigned	3.6	0.4	11.7	10.0	8.4	6.8
Verrucomicrobiae	0.0	0.0	0.9	0.0	0.0	0.7
Total	100	100	100	100	100	100

(**b**)

Figure 3. *Cont*.

Genus	S1a	Zoo	Hospital	Slaughterhouse	S1b	S1c
Acidovorax	0.3	4.2	0.9	0.0	0.3	0.0
Acinetobacter	1.6	10.2	0.6	0.4	0.3	0.4
Actinomyces	0.0	0.0	0.1	1.3	0.0	0.0
Allochromatium	0.0	0.0	0.0	9.9	0.0	0.0
Alpinimonas	0.1	0.0	0.0	0.2	1.2	0.0
Arthrobacter	0.4	0.8	0.3	1.3	0.0	0.0
Bacillus	5.3	0.0	0.0	0.4	0.0	0.0
Bifidobacterium	0.0	0.0	10.6	2.4	0.0	0.0
Brevibacillus	11.1	0.0	0.0	0.0	0.0	0.0
Brevundimonas	12.3	1.5	0.0	0.1	0.0	0.0
Candidatus	0.1	0.0	0.6	0.0	0.5	15.5
Chryseobacterium	0.3	7.2	0.4	0.0	0.0	0.0
Chryseomicrobium	0.0	0.1	0.0	0.2	0.1	2.0
Cloacibacterium	0.2	3.8	1.7	0.2	1.0	0.0
Collinsella	0.0	5.3	5.0	0.1	0.0	0.0
Comamonas	0.4	22.9	12.7	0.4	0.1	0.1
Corynebacterium	0.1	0.3	0.0	2.2	0.0	0.0
Empedobacter	0.0	3.5	0.0	0.0	0.0	0.0
Enterobacter	0.1	0.3	2.8	0.4	0.0	0.0
Enterococcus	0.1	0.0	0.7	1.4	0.0	0.0
Escherichia-Shigella	0.0	0.3	0.4	1.3	0.0	0.0
Fictibacillus	10.7	0.0	0.0	0.0	0.0	0.0
Flavobacterium	0.2	9.9	0.1	0.0	1.2	0.0
Holdemanella	0.0	0.0	1.4	0.0	0.0	0.0
Illumatobacter	0.0	0.0	0.0	0.0	0.0	1.1
Lactobacillus	0.0	0.0	0.6	1.5	0.1	0.0
Leeia	0.1	0.0	6.7	0.0	0.0	0.0
Leucobacter	0.2	0.9	0.0	3.1	0.0	0.0
Limnohabitans	0.8	0.0	0.0	0.0	3.5	0.0
Lysinibacillus	0.0	0.1	0.0	2.0	0.0	0.0
Marinicella	0.0	0.0	0.0	0.0	0.0	1.1
Massilia	5.7	0.1	0.0	0.0	0.0	0.0
Minor	13.1	12.9	17.6	20.8	11.8	12.0
Nosocomiicoccus	0.0	0.0	0.0	1.0	0.0	0.0
Peptoniphilus	0.0	0.0	0.0	1.3	0.0	0.0
Polynucleobacter	0.2	0.0	0.0	0.0	11.9	0.0
Proteiniclasticum	0.0	0.0	0.1	1.7	0.0	0.0
Pseudomonas	1.6	1.3	0.5	0.0	0.3	0.9
Romboutsia	0.0	0.0	1.2	0.0	0.0	0.0
Sediminibacterium	0.1	0.0	0.0	0.0	1.7	0.0
Solobacterium	0.0	3.5	0.2	0.1	0.0	0.0
Soonwooa	0.2	2.4	0.0	0.1	0.0	0.0
Sphingobium	19.4	0.0	0.0	0.0	0.0	0.0
Streptococcus	0.0	0.0	4.4	9.7	0.0	0.0
Thiobaca	0.0	0.0	0.0	3.1	0.0	0.0
Thiothrix	0.0	0.0	1.8	0.0	0.0	0.0
Unassigned	15.1	8.6	28.4	33.0	65.6	66.5
Total	100	100	100	100	100	100

(**c**)

Figure 3. Microbial community structure by metagenomics approach at (**a**) phylum, (**b**) class, and (**c**) genus level. Only classified bacterial genera with >1% were displayed. The genera with <1% were grouped as minor, while those that could not be classified were grouped as unclassified.

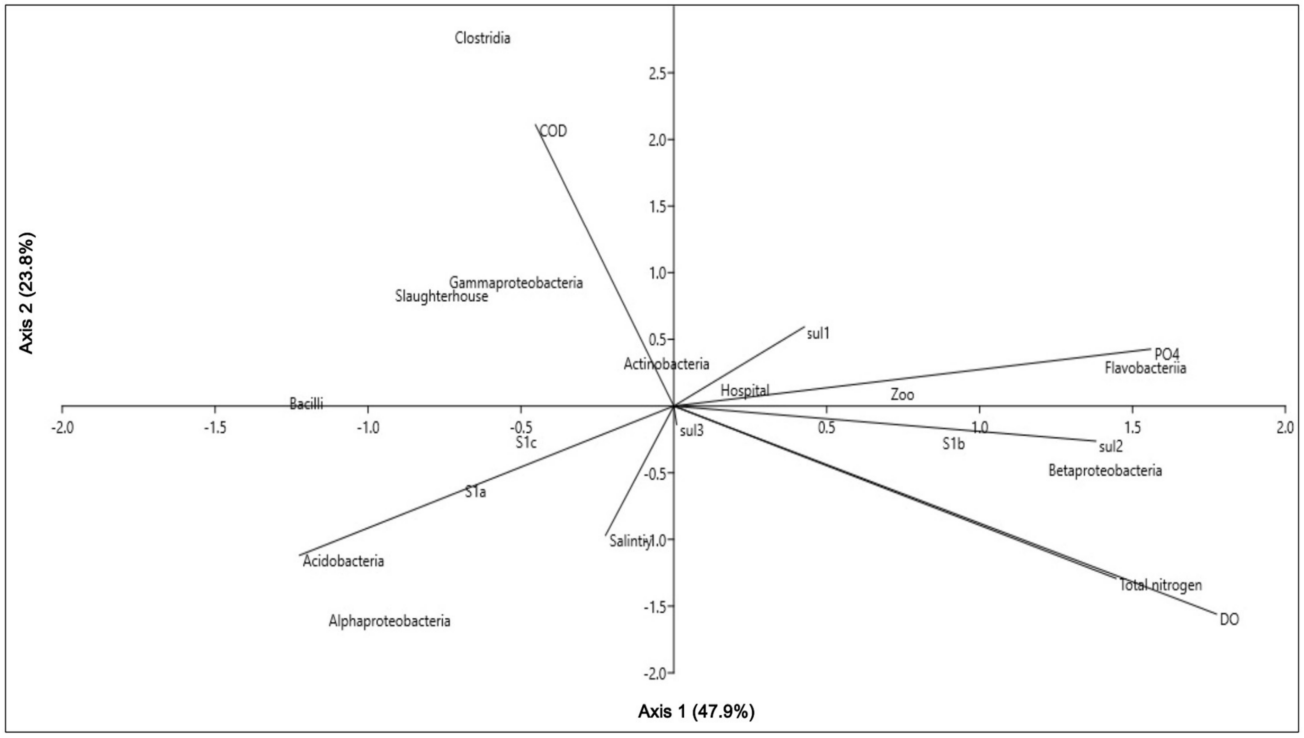
3.2. Environmental Physicochemical Parameters, Sulfonamides Residues and Their Relations to Microbial Community

CCA results (Figure 4a) showed that the microbial community was significantly shaped by salinity, DO, chemical oxygen demand (COD), phosphate (PO_4), and total nitrogen (TN). The results are in agreement with the findings of previous studies that the changes in the abundance and composition of the microbial bacterial community can be attributed to the in situ physicochemical parameters [56–58]. Salinity and DO are important drivers of bacterial diversity and composition [59–61]. In the riverine waters, Betaproteobacteria and total *sul2* genes showed a positive association with DO, while Acidobacteria was associated with salinity. On the contrary, a negative correlation was observed between DO and Clostridia and Bacilli. Moreover, COD also had a significant influence on Actinobacteria, Proteobacteria (Gammaproteobacteria), and Firmicutes (Bacilli and Clostridia) from the slaughterhouse effluent. Similar observations have been reported by Liu et al. [62]. The relationship between bacteria and PO_4 is rarely reported [63,64]. In the present study, we found that Flavobacteriia and total *sul1* genes in the zoo and hospital effluents were associated positively with PO_4. This correlation could be attributed to phosphorus sources. Zheng et al. [64] have revealed the role of phosphorus in shaping the microbial composition and function in activated sludges. TN has been associated with Betaproteobactria concurring with Guo et al. [65], who reported that a higher nitrogen source could enrich Betaproteobacteria.

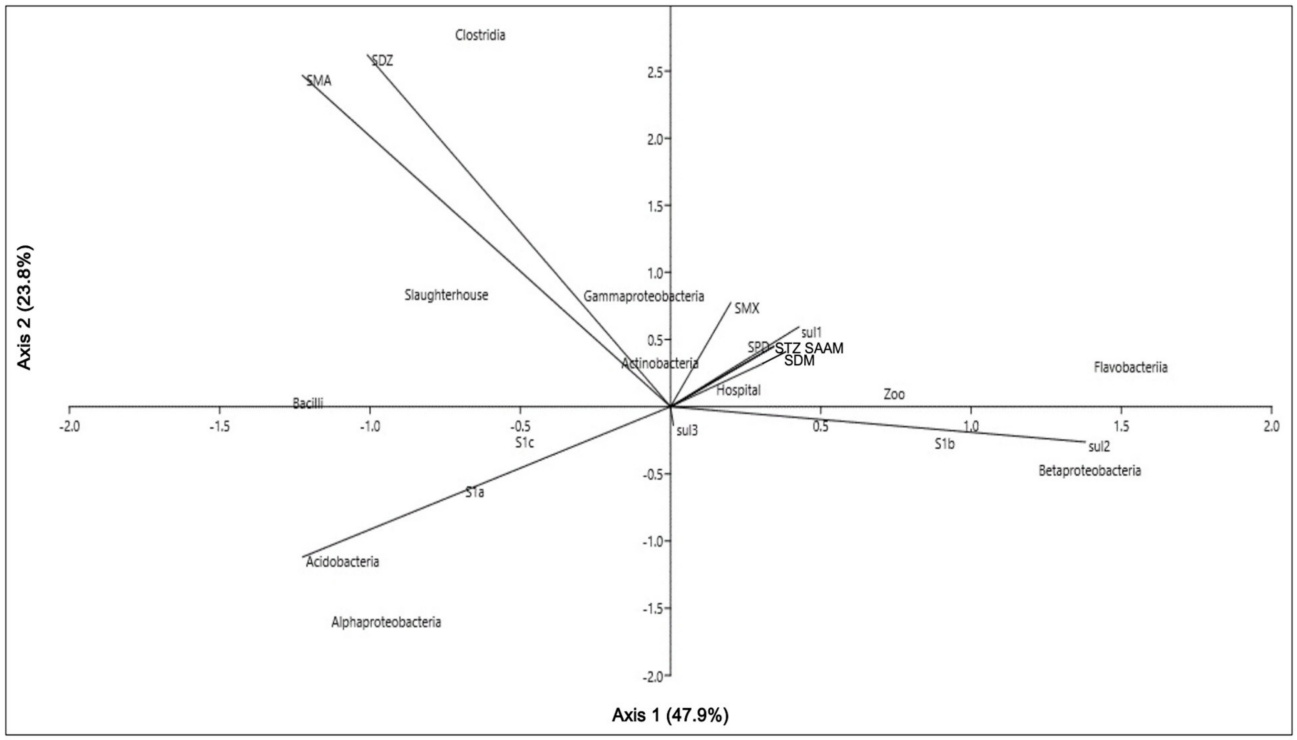
Our results also showed that microbial community was significantly shaped by SMA, SDZ, SMX, SPD, SDM, STZ, and SAAM, in which 47.9% of the variations could be explained by CCA1 and 23.7% by CCA2 (Figure 4b). The key antibiotics detected in the hospital (SPD, SMX, SDM, STZ, and SAAM) exhibited significant correlations with the microbial community and total *sul1* genes. In the slaughterhouse effluent, Actinobacteria and Gammaproteobacteria showed positive associations with SDZ and SMA. These observed correlations were similar to the works of Xiong et al. [43], Guan et al. [66], Visca et al. [67], and Xu et al. [44]. Differences in the response by the observed microbial community to SA were probably due to the development of antibiotic resistance mechanisms in indigenous bacteria under antibiotic selective pressure [68].

3.3. Identification of Culturable Sulfonamide-Resistant Bacteria

We had isolated a total of 278 SARB that belonged to 24 genera, which consisted of only 4.7% of the total number of genera identified with the metagenomics approach (Figure 5). SAR Klebsiella (38.0%–61.3%) were predominant in both the effluents and the Larut River. The majority of the SAR Klebsiella isolated was mainly K. pneumoniae (Supplementary Table S2). Our results concurred with previous studies that reported K. pneumoniae is able to survive a broad ecological range, which could be attributed to their highly diverse genome [25,69–72]. The SAR Escherichia (0–22.2%) and Acinetobacter (3.2–16.0%) were more frequently isolated from the effluents than the Larut River. Among these SARB, Acinetobacter sp., Bacillus sp., and Pseudomonas sp. are common bacterial genera intrinsically resistant to SAs, whereas Serratia sp., Alcaligens sp., Aquitalea sp., and Delftia sp. are poorly characterized in terms of SAR resistance. In this study, approximately half of the SARB found in the effluents from the zoo, hospital, and slaughterhouse were not able to persist in the river water. This was evident in the reduction in SARB isolated from wastewater effluents and S1b to S1c [14]. The reduction in SAR could be attributed to environmental factors and reduced antibiotic selection pressure [10,31,73,74] that may have limited their distribution and persistence along the Larut River.



(**a**)



(**b**)

Figure 4. Canonical correspondence of relationships between the dominant class and total *sul* genes with (**a**) environmental physicochemical parameters and (**b**) SAs residue.

Genus	S1a	Zoo	Hospital	Slaughterhouse	S1b	S1c
Acinetobacter	3.2	16.0	6.3	11.7	6.7	6.9
Aeromonas	0.0	0.0	0.0	3.3	2.2	6.9
Alcaligenes	0.0	0.0	1.6	0.0	0.0	0.0
Aquitalea	0.0	0.0	0.0	0.0	2.2	0.0
Bacillus	9.7	4.0	0.0	5.0	4.4	10.3
Chryseobacterium	3.2	0.0	0.0	0.0	0.0	0.0
Citrobacter	0.0	2.0	1.6	0.0	4.4	0.0
Comamonas	0.0	4.0	3.2	0.0	6.7	0.0
Delftia	0.0	0.0	0.0	0.0	0.0	6.9
Elizabethkingia	0.0	0.0	1.6	0.0	0.0	0.0
Enterobacter	0.0	0.0	7.9	20.0	4.4	3.4
Escherichia	0.0	16.0	22.2	13.3	11.1	6.9
Exiguobacterium	6.5	0.0	0.0	0.0	0.0	0.0
Klebsiella	61.3	38.0	41.3	38.3	48.9	44.8
Kocuria	0.0	0.0	1.6	0.0	0.0	0.0
Macrococcus	0.0	2.0	1.6	0.0	0.0	0.0
Micrococcus	0.0	2.0	0.0	0.0	0.0	0.0
Pseudomonas	9.7	2.0	1.6	0.0	0.0	6.9
Serratia	3.2	0.0	0.0	0.0	0.0	0.0
Shewanella	0.0	0.0	0.0	0.0	0.0	3.4
Shigella	0.0	0.0	0.0	1.7	0.0	0.0
Soonwooa	0.0	2.0	0.0	0.0	0.0	0.0
Stenotrophomonas	0.0	0.0	0.0	3.3	0.0	0.0
Wautersiella	0.0	2.0	0.0	0.0	0.0	0.0
Unassigned	3.2	10.0	9.5	3.3	8.9	3.4
Total	100	100	100	100	100	100

Figure 5. Culturable sulfonamides-resistant bacteria population by identification of 16S Sanger sequencing. The culturable that could not be identified were grouped as unclassified.

3.4. Detection of sul1, sul2, and sul3 Genes in the Culturable Sulfonamide-Resistant Isolates

The *sul* genes were detected in all sites with a distribution pattern of *sul2* (24.5%) > *sul1* (21.2%) > *sul3* (4.0%) (Figure 6a). The majority of the SARB harboring *sul* genes were found in the zoo (80.0%), hospital (90.5%), and slaughterhouse (75.0%) effluents. SARB isolated from the zoo and slaughterhouse effluents harbored *sul2* gene, whereas the hospital wastewater effluent was dominated by the SARB with *sul1* gene. These findings indicated that SAR is common in the aquatic environment of Larut River and is in agreement with Suzuki et al. [75], who showed that *sul1* and *sul2* are ubiquitous in aquatic bacteria. Notably, the SARB in these wastewater effluents was found to harbor multiple *sul* genes. Contrarily, SARB that harbored no *sul* gene in their genome was more prevalent in the upstream and further downstream river, suggesting that the SARB carrying *sul* genes was associated with anthropogenic activities. Furthermore, a decreasing trend was observed in SARB harboring multiple *sul* genes from the immediate downstream (S1b) to further downstream (S1c). The decreased abundance of SARB that possessed *sul* genes and the *sul* genotypes at S1c might be due to the reduction in SA selection pressure where Björkman and Andersson [76] reported that resistance is associated with metabolic cost suggesting a decline of ARB with lower antibiotic use. Schulz zur Wiesch et al. [77] reported that gene-based resistance in bacteria strains is often costly, and the fitness loss may be reflected in a reduced growth rate in vivo [78] or in vitro [79,80] and transmission rate [81] in the absence of antibiotics. Studies also revealed that the cost of resistance is among the most important factor determining the rate and extent of resistance emergence [9,79,82–84].

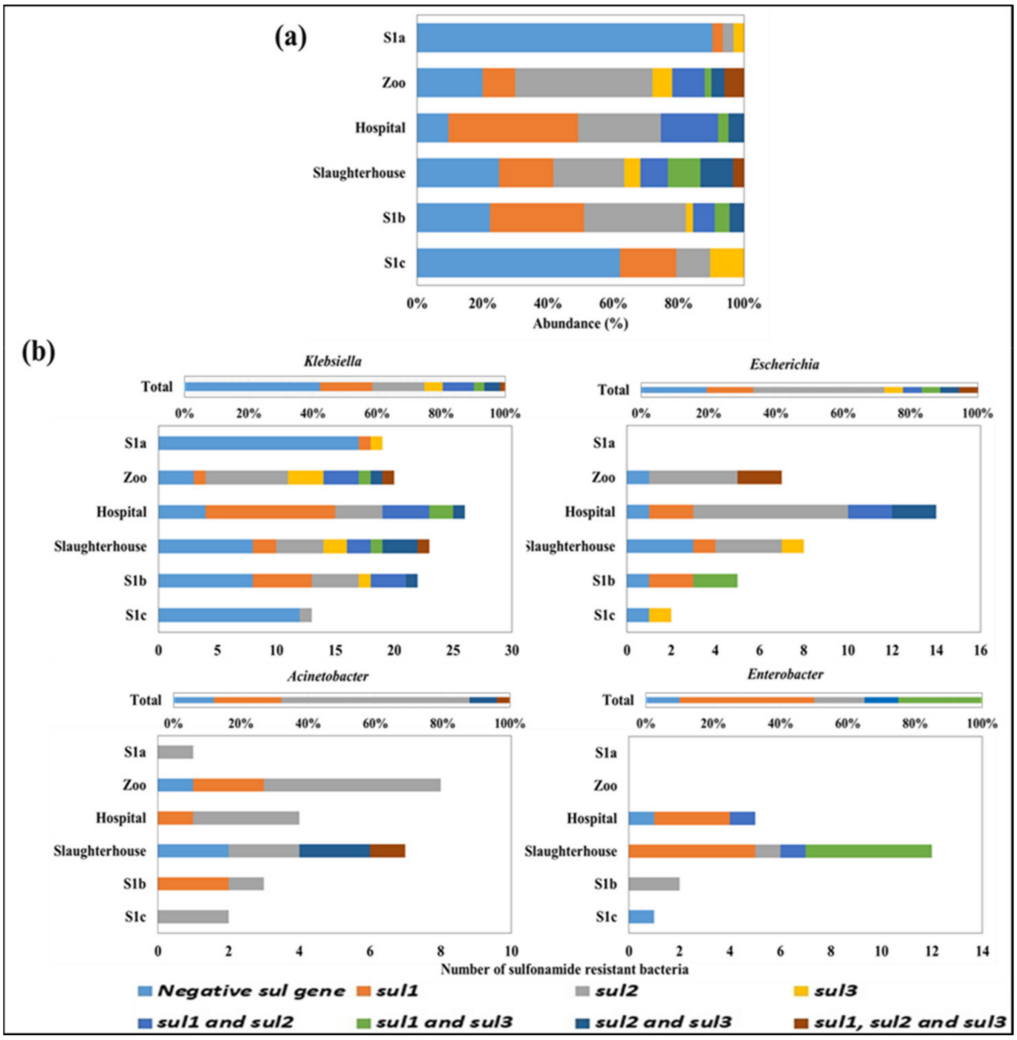


Figure 6. (**a**) Sul genotypes across sampling sites and (**b**) in the most abundant sulfonamides-resistant bacteria.

Interestingly, all the combinations of *sul* genes in the culturable SARB were found in the wastewater effluents. In this study, ~40% of the SAR Klebsiella did not carry *sul* genes and were mainly detected in S1a and S1c while the rest were harboring *sul* genes, i.e., mainly *sul1* and *sul2* (~32%) (Figure 6b). These results indicated that SAR in Klebsiella in wastewater environment did not rely on *sul* genes but other SAR mechanisms. With the rising cases of community-acquired infections of resistant bacteria, it is interesting to look into the SAR mechanisms, including folate synthesis and efflux systems in Klebsiella in the aquatic environments, to predict and counteract the emergence and future evolution of antibiotic resistance in these bacteria.

We observed different *sul* genotypes and *sul* genes distribution patterns in SAR Acinetobacter, Escherichia, and Enterobacter. However, the majority (>80%) of these SARB harbored *sul* genes suggesting that SAR of these bacteria were mostly dependent on resistance genes. The SAR Acinetobacter that dominated in the zoo (16.0%) and slaughterhouse (11.7%) effluents were consistent with previous studies that reported Acinetobacter as a potential environmental *sul* genes reservoir from animal sources (pig slurry and manured agricultural soils). Our findings also suggested a potential link for dissemination of sul genes between the natural environments, clinical, and agricultural settings. However, direct evidence of ARGs dissemination between the environmental and clinical resistome is rare as these ARGs may undergo several rounds of evolutions between the natural and clinical environments [85,86].

3.5. Identification of Potential sul Genes Bacterial Carrier

A total of 19 nodes (8 *sul* genotypes and 11 SARB) and 43 edges were obtained through the network analysis. Acinetobacter, Aeromonas, Bacillus, Comamonas, Citrobacter, Enterobacter, Escherichia, Klebsiella, Macrococcus, Pseudomonas, and Stenotrophomonas were identified as potential *sul* genes carriers and found associated with more than one *sul* genotype except for SAR Macrococcus and Pseudomonas that were associated with only one *sul* genotype (Figure 7).

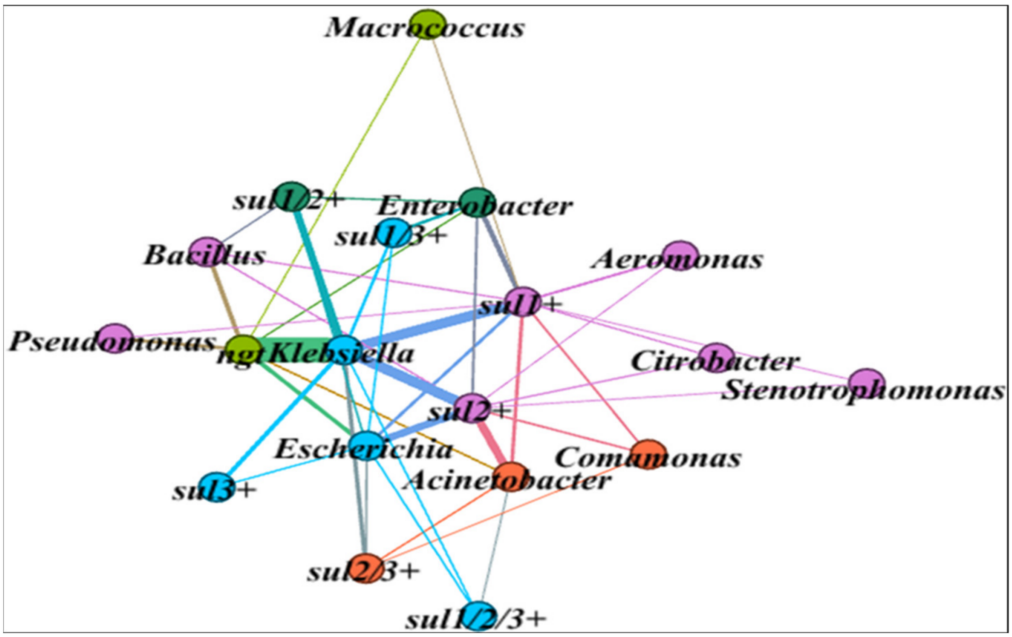


Figure 7. Identification of potential sul genes bacterial carriers by network analysis.

In this study, a comparison of culturable SARB population to total microbial community profile structure was carried out to identify the potential *sul* gene bacterial carriers in this river. The culturable method employed has recovered most of the same dominant bacterial phyla in the total microbial community (Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes). However, the results showed that the most prevalent SAR bacterial genera isolated from the culturable SARB were different from the total community. Klebsiella and Citrobacter were not detected in the total microbial community, whereas Macrococcus and Stenotrophomonas were detected at <1% across the sampling sites (Supplementary Figure S2). These results are in agreement with Suzuki et al. [75] that reported culturable bacteria are not a major component of the total bacteria community and are selected by the culture method causing biases in bacterial diversity. Furthermore, conventional culture methods for monitoring ARB and ARGs only revealed $\leq$0.1% of the true bacterial community [87,88]. Thus, the relationships between ARGs and bacteria species cultivated found are limited to a fraction of the overall river bacterial community.

Notably, nine out of eleven (81.8%) identified potential *sul* genes bacterial carriers belonged to Proteobacteria while the other two carriers belonged to Firmicutes. Those carrying *sul* genes were dominated by culturable SAR Klebsiella, Escherichia, Acinetobacter, and Enterobacter and might serve as reservoirs of *sul* genes and disseminate these genes among the microbial community. It is noted that the abundance and distribution of *sul* genotypes in the culturable SARB could be largely affected by the *sul* genotypes in SAR Klebsiella, which were present as the most dominant culturable SARB. The potential environmental *sul* genes carriers in Proteobacteria, as well as reservoirs, includes Acinetobacter, Aeromonas, Enterobacter, Escherichia, Klebsiella, and Pseudomonas, and their positive correlation with *sul1* has been reported [66,89–92]. For Comamonadaceae and Aeromonadaceae, strong correlations with *sul1* and *sul2* have also been reported [19].

A correlation was also reported between Firmicutes, particularly Bacillus and Clostridia, and *sul1* and *sul2* [50,66,92]. Interestingly, this present study had identified SAR Macrococcus that belonged to Firmicutes as one of the *sul* gene bacterial carriers associated with *sul1*. Generally, animal origin Macrococcus has been reported to have resistance to methicillin (*mecB* gene) and TMP (*cfr* gene) [93,94]. Therefore, this is the first study to show Macrococcus resistance to SA. Further studies are needed to confirm the emergence and dissemination of the *sul* genes in this genus in the aquatic environment.

Aeromonas, Comamonas, Pseudomonas, and Stenotrophomonas were also detected in the Larut River by both culturable and metagenomics approaches suggesting that these bacteria are the common *sul* genes carriers residing in Larut River. Furthermore, studies have also revealed the ability of Acinetobacter, Bacillus, Brevundimonas, Comamonas, Escherichia, Klebsiella, Pseudomonas, and Stenotrophomonas for the degradation of SA in various environments and acclimated membrane reactors [26,95,96]. The catabolism of SAs in these bacteria is important not only for antibiotic degradation to remove pollutants in the environment but also for antibiotic resistance as the enzymes involved in degradation are a potential resistance mechanism [97]. Kim et al. [98] revealed that SA metabolism may have evolved in SARB, which has already acquired the class 1 integron under SA selection pressures.

Masco et al. [99] reported that Bifidobacterium is intrinsically resistant to SMX. There have been reports of resistance of SA in Sphingobium, Brevibacillus, Candidatus, and Polynucleobacter [100,101]. Correlations between Candidatus and *sul1* and *sul2* and Polynucleobacter with *sul2* have also been reported [102,103]. Thus, even though these bacteria were not isolated in the present study, they could be the important *sul* genes bacterial carriers in the Larut River. Therefore, further studies using more variety of culture media and targeting more diverse bacteria are needed to study the antibiotic-resistant profile.

Martínez [3] reported that natural environments represent reservoirs of ARGs, and changes in these ecosystems might be relevant for the emergence of previously unknown resistance determinants in bacterial pathogens. However, the effect of environmental changes on the dynamics of the bacteria population and their ARGs have received less attention [104]. Past research has focused on antibiotic resistance in pathogenic bacteria, where the information about the extent to which commensal and non-pathogenic bacteria can act as antibiotic resistance reservoirs is lacking [105]. Therefore, more studies on whether the anthropogenic activities might enrich the ARB population in the natural environments and facilitate the transfer of ARGs will be important to address in the future. Moreover, Narciso-da-Rocha and Manaia [106] reported that there is still a significant difference between the ARB profile through culture-dependent and metagenomics analysis. The ARGs and their affiliated taxa are still very unclear [107]. Therefore, the combination of correlation analysis, culture-dependent, and metagenomics should be performed for further confirmation of the relationships between the ARGs and their bacterial hosts to the taxa level [92].

4. Conclusions

The zoo, hospital, and slaughterhouse wastewater effluents had exerted impacts on the bacterial microbial community and culturable SAR bacteria population in the Larut River. Our findings showed that the zoo, hospital, and slaughterhouse were potential sources of high diversity and number of potentially pathogenic and clinically important bacteria into the receiving river. The SARB isolated from the wastewater effluents harbored higher *sul* genes and displayed more diverse *sul* genotypes. The absence of *sul* genes was detected in ~40% of the culturable SAR *Klebsiella*, and more study is needed to understand their resistance mechanisms in the emergence and evolution of SAs resistance in aquatic environments. We have identified 11 *sul* genes carriers (Acinetobacter, Aeromonas, Bacillus, Comamonas, Citrobacter, Enterobacter, Escherichia, Klebsiella, Macrococcus, Pseudomonas, and Stenotrophomonas) that could act as reservoirs of antibiotic resistance. CCA revealed that SAs residues and inorganic nutrients exerted significant impacts on microbial

community and total *sul* genes. However, further studies are required to understand the development of resistance mechanisms and the relationships between opportunistic pathogens and ARGs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w14071018/s1, Table S1: Summary of diversity and richness of microbial community. Table S2: Sulfonamides resistant bacteria identified to species level. Figure S1: Rarefaction curves estimated at 97% of microbial community. Figure S2: Bacterial genera detected by 16S rDNA metagenomics.

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