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Isolation and molecular characterization of bacterial species from Sikog waterfall, Padawan, Sarawak

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ABSTRACT

Aims: Bacteria are microorganisms that are commonly distributed in any environment. They are also found abundantly in marine environments such as waterfalls and rivers. Some bacteria participate in various biological activities and possess no health risk; however, other species could be pathogenic and have been directly associated with various diseases in animals and humans. Therefore, it is crucial to analyze the antibiotic resistance profiles of bacteria in the research area based on regularly used antibiotics in clinical and agricultural contexts to establish a data baseline for health providers and public usage.
Methodology and results: Water samplings were done twice and collected from upstream, midstream, and downstream of the Sikog waterfall. A total of ninety isolates were isolated and analyzed using $(GTG)_5$ genetic fingerprinting to determine the genetic similarities. Based on the dendrogram generated using Gelj Version 2.0 software, 41 bacterial isolates were subjected to 16S rRNA gene sequencing for species identification. The Kirby-Bauer disk diffusion method was implemented to determine the level of susceptibility toward certain antibiotics. Sequence analysis was performed using BLAST, revealing that the isolates constitute 17 genera, including *Pseudomonas*, *Alcaligenes*, *Stenotrophomonas*, *Staphylococcus*, *Bacillus*, *Lysinibacillus*, *Rossellomorea*, *Citrobacter*, *Enterobacter*, *Kosakonia*, *Klebsiella*, *Escherichia*, *Serratia*, *Cronobacter*, *Aeromonas*, *Chromobacterium* and *Kocuria*. According to the overall antibiotic susceptibility analysis, streptomycin (10 µg) exhibited the highest rate of resistance among bacterial isolates, with 36.84%, followed by penicillin (10 units) (36.36%), rifampicin (5 µg) (27.27%) and ampicillin (10 µg) (26.32%).
Conclusion, significance and impact of study: The research findings revealed the predominant bacteria found in the recreational water of Sikog waterfall and their antibiotic susceptibility, which could be helpful in the treatment of bacterial infections for future clinical reference. Simultaneously, the public, particularly communities in the study area, should be informed about the potential health risk associated with diverse resistant enteric bacteria in the recreational water.

Keywords: 16S rRNA gene sequencing, antibiotic resistance, bacteria, $(GTG)_5$ PCR, Sikog waterfall

INTRODUCTION

The abundance of waterfalls in Sarawak is due to the topographical structure, climatic circumstances, and river conditions. Sikog waterfall, which is located in Padawan, Kuching, Sarawak, was chosen as a research site because it is a popular spot for outdoor activities such as camping and trekking. It is a place of indescribable beauty. Nevertheless, the nearby village still depends on the natural mountain river stream for their everyday needs and water supply. River water is also a vital resource for agriculture since various farms cultivate commodities such as pepper and other vegetation along the river.

Furthermore, this natural water body is also a reservoir for various microbes. Most bacteria found on the soil and surface water are non-pathogenic and play critical functions in marine food webs and they undertake essential ecosystem processes such as nitrogen, carbon, and sulphur transformation. For instance, some *Acinetobacter* species found on surface water and soil have been associated with useful functions in the natural environment, such as oil spill detoxification, soil fertility improvement and microflora in human and plant bodies (Adewoyin and Okoh, 2018). Despite their natural presence in aquatic habitats, some bacteria have been linked to human infections through ingestion or exposure to contaminated water from recreational activities (Nollet and De Gelder, 2000). Despite the persistent efforts to ensure water safety, waterborne outbreaks continue to be reported globally (Ramírez-Castillo *et al*., 2015).

Pathogenic bacteria and their antibiotic resistance associated with recreational water have been a significant

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public health concern. Baquero *et al.* (2008), as described by Delgado-Gardea *et al.* (2016), stated that enteric bacteria could be found in the human gastrointestinal tract as well as in the faeces of warm-blooded animals and these bacteria are usually washed off into the water environment due to runoff from farms and rural settlements with agricultural and industrial pollutants. Waterborne illness outbreaks are most common in economically disadvantaged communities, where water supply and sanitation are often insufficient (Cabral, 2010). Multiple resistant bacteria are prevalent in recreational water, posing a direct threat to anyone that comes into contact with it through a potential transfer of resistance to human and animal strains (Lesley *et al*., 2016).

Cabral (2010) reported that *Aeromonas hydrophila* had been identified as an opportunistic pathogen that can cause gastroenteritis, septicaemia, meningitis, and wound infections. It can cause intestinal disorders in children under five, the elderly and immunocompromised people. In addition, opportunistic pathogens such as *Escherichia coli*, *Leptospira* species, *Campylobacter* species, *Pseudomonas* species and *Vibrio cholerae* may be present in naturally occurring recreational water (Cabral, 2010). The public, particularly in the research area, should be informed of the possible risk of genetically diverse intermediate and resistant enteric bacteria present in recreational water. Hence, monitoring the presence of indicator bacteria for pathogenic bacteria and their level of susceptibility to antibiotics routinely used in clinical settings is crucial to ensure the continued effectiveness of treatment for waterborne diseases.

Antibiotic-resistant bacteria have emerged as a significant public health concern worldwide. Increased antibiotic resistance is a certainty, as antibiotic-resistant genes are ubiquitous in various environmental bacterial populations and not limited to clinical settings. Although resistance determinants in soil and environmental bacteria cause no harm to human health, their mobilization to a new host and expression in various circumstances, such as their transfer to plasmids and integrons in pathogenic bacteria, could constitute a significant concern (Peterson and Kaur, 2018).

The aim of this study was to determine the distribution of environmental bacteria in recreational water. The phylogeny of the isolates was determined using the $(GTG)_5$ DNA fingerprinting and the isolates were further identified using 16S rDNA sequencing. $(GTG)_5$ fingerprinting has various applications in a variety of fields and one of them is to analyze the genetic similarity between bacterial isolates to learn more about their epidemiology (Heras *et al*., 2016). It is also beneficial for screening a variety of bacterial strains because it has the best discriminatory power among different rep-PCR variants and can type a broad range of Gram-negative bacteria (Kathleen *et al*., 2014). This study also further details the antibiotic susceptibility of bacteria isolates against different antibiotics.

MATERIALS AND METHODS

Sample collection

Water samples were collected from the surface water of Sikog waterfall, Padawan, Sarawak. The GPS coordinates for the sampling sites are shown in Table 1, while the locations of samplings are illustrated in Figure 1. Water sampling was done twice, and nine samples were collected for each sampling. A total of eighteen water samples were collected at different spots at a depth of 12 cm below the water surface. Water samples were kept at 4 °C upon arrival in the laboratory for further analysis.

Bacterial isolation

Bacterial isolation was accomplished utilizing a membrane filtration technique described by Lihan *et al*. (2020), in which 18 water samples were serially diluted with phosphate buffer saline before being filtered through a 47 mm pore-size membrane. Membrane filters were then transferred to a Hi-Chrome agar and incubated overnight at 28 °C. About five pure colonies were randomly picked from each plate and were subcultured, purified, and stocked in glycerol.

DNA isolation

Bacterial DNA (deoxyribonucleic acid) was extracted using the boiling centrifugation method or heat shock, as described by Lesley *et al*. (2016), with minor modifications. A small colony of bacteria was first transferred to a PCR (polymerase chain reaction) tube containing 40 µL of nuclease-free water and centrifuged at 13,500 rpm for 1 min. The bacterial suspension was then boiled for 10 min using a heater block at 100 °C and recentrifuged for 1 min at 13500 rpm. About 2 µL of DNA template was pipetted into a new sterile PCR tube containing the $(GTG)_5$ PCR master mix.

Rep-PCR genomic fingerprinting

Modifications were made to the $(GTG)_5$ PCR protocol published by Lihan *et al*. (2017). A total of 40 µL of PCR master mix consisting of 4 µL of 5× *Taq* Green buffer, 4 µL of 25 mM $MgCl_2$, 0.3 µL of 25 mM dNTPs, 2 µL of 25 µM $(GTG)_5$ primer (5′-GTGGTGGTGGTGGTG-3′), 27.4 µL of nuclease-free water, 0.3 µL of Go*Taq* DNA polymerase and 2 µL of DNA template. $(GTG)_5$ PCR amplification was then carried out with an initial denaturation at 95 °C for 2 min, 30 amplification cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 5 min and a final elongation step at 72 °C for 5 min. 5 µL of the amplified PCR product were then electrophoresed on 1% (w/v) agarose gel in 1× Tris-Borate-EDTA (TBE) buffer at 80 V, 400 mA for 40 min and 1 kb DNA ladder was used as a DNA marker. The bands were visualized with an ultraviolet (UV) transilluminator, and scoring was done to construct a dendrogram using GelJ Version 2.0 software.

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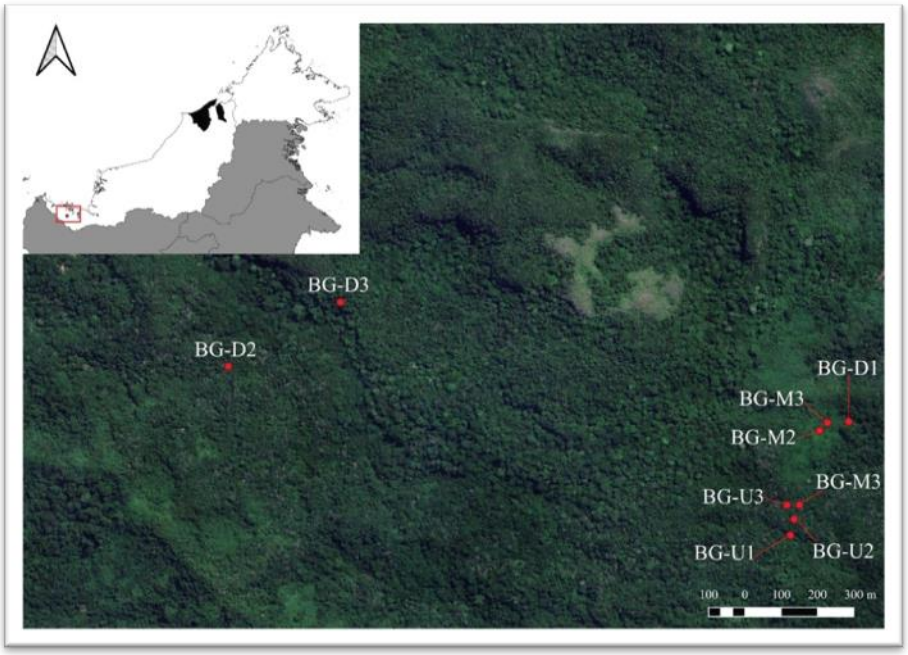

Figure 1: Location of sampling points in Sikog waterfall, Padawan, Kuching, Sarawak.

Table 1: GPS coordinates for water sampling in Sikog waterfall, Padawan, Sarawak.

Sampling site	Station	Coordinates
Upstream	1	01°20'08.9" N 110°19'53.4" E
	2	01°20'10.3" N 110°19'53.7" E
	3	01°20'11.6" N 110°19'53.1" E
Midstream	1	01°20'18.4" N 110°19'54.2" E
	2	01°20'18.2" N 110°19'56.0" E
	3	01°20'18.9" N 110°19'56.7" E
Downstream	1	01°20'19.0" N 110°19'58.6" E
	2	01°20'23.9" N 110°20'03.3" E
	3	01°20'29.6" N 110°20'13.3" E

DNA purification

DNA products were purified using the QIAquick® Gel Extraction Kit. The DNA band was first cut using a sterile scalpel, placed in a 2.0 mL PCR tube and weight. Three gel volumes of QG buffer were then added to the PCR tube. The gel was subsequently melted by placing the PCR tube in a 50 °C water bath for 15 min. One gel volume of isopropanol was added to each PCR tube and then flicked to mix. The mixtures were then put into Econospin™ spin columns and centrifuged for an additional 1 min at 10 000 rpm. After discarding the supernatant, 500 µL of QG buffer was added to the sample and recentrifuged at 10 000 rpm for another 1 min. The supernatant was discarded before each tube was added with 750 µL of PE buffer. The Econospin™ spin columns were recentrifuged to remove the residual supernatant and then transferred into new sterile 1.5 mL centrifuge tubes. Approximately 50 µL of EB buffer was pipetted into the membrane centre and left for 1 min to elute the DNA, followed by 1 min centrifugation at 10 000 rpm. Following the disposal of the spin column, DNA was subsequently purified.

16S rDNA sequencing

The 16S rRNA gene amplification using PCR and result analysis was performed using the method described by Kathleen *et al*. (2014), with minor modifications. A fragment of 16S rRNA was amplified by PCR using 27F (5'-CAGGCCTAACACATGCAAGTC-3') and 519R (5'-GWATTACCGCGGKGCTG-3') primer. A total of 41 DNA products were sent for gene sequencing, and nucleotide sequences were compared with the available sequence data in the Genbank using the Basic Local Alignment Search Tool (BLAST).

Antibiotic susceptibility testing

The disk diffusion method is the gold standard in determining the susceptibility of bacteria isolates toward various antibiotics (Lihan, 2020). Firstly, a pure bacteria

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culture was grown on Mueller Hinton Broth (MHB) and incubated overnight. The turbidity of bacteria suspension was determined using spectrophotometry and McFarland standards were used as a reference to adjust the turbidity to maintain the number of bacteria grown within a specified range. A sterile cotton bud was dipped into the overnight culture and then swabbed on the Mueller Hinton Agar (MHA). The plate was incubated overnight at 37 °C and the size of the inhibition zone formed around the antibiotic disk was measured. The antimicrobial susceptibility testing standards M02-A11, provided by CLSI (2021), were used to assess the diameter of the zone of inhibition. Supplementary Table S1 summarizes the antibiotics used in the testing. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as standard control strains in the AST. The diameter of the zone of inhibition was determined and interpreted as susceptible (S), intermediate (I) or resistance (R) (Salam *et al*., 2023).

RESULTS

Rep-PCR fingerprinting and 16S rRNA analysis

The banding profiles of 90 bacterial isolates isolated from Sikog waterfall are visualized in Supplementary Figure S1 and Figure S2. The dendrogram was constructed based on the Dice similarity method with a tolerance value of 5.0 using UPGMA linkage of $(GTG)_5$ PCR genetic fingerprinting. Based on Figure S1, forty-five isolates were grouped into 21 clusters with a similarity index of 73%. As indicated in Figure S1, there are a total of 21 clusters, with Cluster 19 forming the most significant group consisting of 8 isolates. Cluster 21, with a total of 5 isolates, was the second largest cluster, followed by Cluster 16, which consisted of 4 isolates.

On the other hand, 20 clusters are generated in Figure S2, with Cluster 20 forming the most significant group with a total of 9 isolates, followed by Cluster 12 (4 isolates). The agarose gel electrophoresis results for 16S rRNA PCR are illustrated in Figure S3a, Figure S3b, Figure S3c and Figure S3d. Table 2 details the identities of the selected isolates and the percentage of similarities compared using the NCBI Genbank Database, with 41 isolates having a similarity level of at least 97%, as Srinivasan *et al.* (2015) suggested that 97% is the acceptable cut-off value for species-level identification.

Antibiotic susceptibility testing

Antibiotic selection is contingent upon bacteria genera, as different bacteria groups may be intrinsically resistant to certain antibiotic classes, as defined by the Clinical and Laboratory Standards Institute (2021). The assessment of the bacterial susceptibility tested against twenty-seven antibiotics are demonstrated in Supplementary Table S2, Table S3, Table S4, Table S5, Table S6 and Table S7.

Based on the antibiotic susceptibility analysis (Table 4, 14 out of 41 (34%) bacterial isolates were resistant to at least one antibiotic. The highest rate of resistance was against streptomycin (36.84%), followed by penicillin (36.36%), rifampicin (27.27%) and ampicillin (26.32%). While cefepime, ciprofloxacin, levofloxacin, norfloxacin, nalidixic acid, trimethoprim-sulphamethoxazole, chloramphenicol, ertapenem, amikacin, cephalothin, ceftriaxone, cefotaxime and ofloxacin have the highest susceptibility rates (Table 3).

MAR index assessment

The Krumperman (1983) formula was used to determine the multiple antibiotic resistance (MAR) indexes. The number of antibiotics the bacteria were resistant to was divided by the total number of antibiotics tested on the isolates. Results were interpreted as intermediate (I), susceptible (S) or resistant (R) based on the CLSI breakpoints table (Kathleen *et al*., 2016). As shown in Table 4, MAR ranges from 0 to 0.50, with *Lysinibacillus telephonicus* having the highest MAR index, as it exhibits resistance toward 5 out of 10 antibiotics tested, followed by *Alcaligenes aquatilis* (0.36) and *A. faecalis* (0.36).

DISCUSSION

The susceptibility of bacteria isolates was tested against twenty-seven antibiotics and the findings revealed that 14 of the 41 examined were resistant to at least one antibiotic, resulting in a 34% overall resistance rate. According to the findings, 46% (19/41) of the bacteria isolated from Sikog waterfall belong to the Enterobacteriaceae family. The broad family of Gram-negative bacteria known as Enterobacteriaceae predominately lives in the gastrointestinal tracts of humans and animals. As a result of their remarkable environmental adaptability, the bacteria in this group have been found to thrive freely in waters with limited energy sources, making them excellent indicators of faecal pollution of river water (Lihan *et al*., 2017). Antibiotic susceptibility testing of this bacterium group tested against 17 antibiotics found that 36.84% of isolates were resistant to streptomycin, followed by ampicillin (26.32%), imipenem (10.53%) and ticarcillin-clavulanate (5.26%), while the rest are susceptible. The World Health Organization (WHO) recently placed Enterobacteriaceae, along with *Acinetobacter* and *Pseudomonas aeruginosa*, on the list of pathogens with essential needs for developing new antibiotics to treat infections (Tacconelli *et al*., 2017). According to the MAR analysis (Table 4), all 19 isolates from this family have a MAR index of less than 0.20, which suggests that all of the isolates originated from lower antibiotic-contaminated sources (Kathleen *et al*., 2016). The different antibiotic-resistant patterns tested displayed by different bacteria strains in this study demonstrate how complex it is to comprehend antibiotic resistance in this study (Kathleen *et al*., 2016).

Cronobacter sakazakii formerly known as *Enterobacter sakazakii* in 1980 by Japanese microbiologist Richii Sakazakii is one of the bacteria species isolated from the waterfall (Iversen *et al.*, 2007). It is an opportunistic pathogen that can infect full-term and

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Table 2: Sequence similarities result of bacterial isolates compared using NCBI Genbank database.

Sampling	Cluster	Total related isolates (≥73%)	Isolates chosen for sequencing	Species	Accession number	Percentage similarity (%)
1	1	1	SR1-D2-39	*Rossellomorea marisflavi*	MT102628	100
	2	1	SR1-U1-3	*Bacillus thurigiensis*	MT605582	100
	3	1	SR1-U3-12	*Enterobacter mori*	MN910304	100
	4	1	SR1-U3-13	*Enterobacter chengduensis*	CP043318	99
	5	3	SR1-U2-6	*Staphylococcus saprophyticus*	JQ043188	99
	6	1	SR1-D3-42	*Pseudomonas aeruginosa*	OMO21866	100
	7	1	SR1-M1-16	*Enterobacter cloacae*	MH266244	98
	8	1	SR1-U1-4	*Rossellomorea vietnamensis*	CP047394	98
	9	2	SR1-U3-15	*Citrobacter farmeri*	MH972183	99
	10	2	SR1-D2-40	*Kosakonia sacchari*	CP040677	100
	11	2	SR1-D3-43	*Bacillus pumilis*	MN704554	99
	12	1	SR1-U1-2	*Bacillus cereus*	MT605291	100
	13	2	SR1-D2-38	*Chromobacterium* sp.	MG685878	99
	14	2	SR1-U2-10	*Lysinibacillus telephonicus*	NR_157637	99
	15	3	SR1-D2-36	*Serratia marcescens*	MT185439	97
	16	4	SR1-D1-30	*Alcaligenes faecalis*	LC376950	100
	17	1	SR1-D3-45	*Rossellomorea aquimaris*	MG705812	100
	18	2	SR1-D3-44	*Stenotrophomonas acidaminiphila*	MN889037	99
	19	8	SR1-M3-27	*Alcaligenes aquatilis*	MT572474	100
	20	1	SR1-D1-31	*Aeromonas aquatica*	MG428980	99
	21	5	SR1-M3-29	*Kosakonia oryzae*	CP065358	98
2	1	1	SR2-D1-80	*Serratia marcescens*	MT645673	100
	2	1	SR2-U2-55	*Kosakonia oryzendophytica*	MN999999	100
	3	1	SR2-M1-64	*Cronobacter sakazakii*	MT903210	99
	4	2	SR2-D2-84	*Kocuria palustris*	JX077104	100
	5	1	SR2-M1-62	*Enterobacter cloacae*	MT613881	100
	6	1	SR2-M3-72	*Escherichia coli*	KY681423	100
	7	3	SR2-D2-83	*Stenotrophomonas terrae*	KT380555	100
	8	1	SR2-M3-71	*Enterobacter mori*	MW242734	100
	9	1	SR2-U3-57	*Kosakonia oryzendophytica*	MN999999	100
	10	3	SR2-U2-51	*Stenotrophomonas maltophilia*	LC379125	100
	11	2	SR2-U3-59	*Rossellomorea aquimaris*	AB376670	99
	12	4	SR2-U1-50	*Aeromonas encheleia*	MG428718	100
	13	1	SR2-M1-63	*Enterobacter asburiae*	MN691854	100
	14	3	SR2-D3-89	*Klebsiella pneumoniae*	KY417867	99
	15	1	SR2-D3-86	*Staphylococcus haemolyticus*	MT254764	100
	16	3	SR2-U1-48	*Escherichia coli*	CP054449	99
	17	3	SR2-U3-56	*Enterobacter soli*	MW947078	100
	18	3	SR2-M3-75	*Rossellomorea aquimaris*	KC335217	99
	19	1	SR2-D3-90	*Pseudomonas aeruginosa*	KX519541	100
	20	9	SR2-U1-49	*Escherichia coli*	CP054232	100

premature infants, and cause diseases like sepsis, necrotizing enterocolitis, and meningitis (Hunter *et al*., 2008). Although its natural habitat is unknown, a recent study on the occurrence of this organism reported that it had been isolated, with varying frequency, from nearly all environments including in the production of food, factories, and households, thus, confirming its widespread distributions (Zhou *et al*., 2012). Although *E. sakazakii* infection is low, the prognosis is poor, and infection is associated with significant morbidity and mortality (Drudy, 2006). Stock and Wiedemann (2002) states that *E. sakazakii* is susceptible to some antibiotics, including tetracycline, aminoglycosides, numerous β-lactams, chloramphenicol, antifolates and quinolone. Based on the AST result, *C. sakazakii* is indeed susceptible to tetracycline, β-lactams such as ticarcillin-clavulanate, aminoglycosides such as gentamicin, amikacin, chloramphenicol, and quinolones such as levofloxacin, nalidixic acid and norfloxacin. Pitout (1997), as reported by Drudy (2006), clarified that ampicillin resistance developed as a result of the acquisition of transposable elements and the production of β-lactamases. This report was confirmed as the isolates showed resistance when tested with ampicillin.

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Table 3: Percentage of bacterial resistance toward different antibiotics.

Antibiotics	Antibiotics Abbreviation	Total of resistant isolates (Total isolate tested)	Percentage of resistance (%)
Streptomycin	S	7(19)	36.84
Penicillin	P	4(11)	36.36
Rifampicin	RD	3(11)	27.27
Ampicillin	AMP	5(19)	26.32
Imipenem	IPM	4(27)	14.81
Erythromycin	E	1(11)	9.09
Ceftazidime	CAZ	2(27)	7.41
Aztreonam	ATM	2(27)	7.41
Meropenem	MEM	2(27)	7.41
Gentamicin	CN	2(36)	5.56
Ticarcillin-clavulanate	TIM	1(23)	4.35
Piperacillin	PRL	1(25)	4
Doxycycline	DO	1(32)	3.13
Tetracycline	TE	1(34)	2.94
Cefepime	FEP	0(4)	0
Cefotaxime	CTX	0(2)	0
Ceftriaxone	CRO	0(2)	0
Ertapenem	ETP	0(19)	0
Amikacin	AK	0(21)	0
Ciprofloxacin	CIP	0(16)	0
Levofloxacin	LEV	0(39)	0
Norfloxacin	NOR	0(34)	0
Nalidixic Acid	NA	0(19)	0
Ofloxacin	OFX	0(2)	0
Trimethoprim-sulphamethoxazole	SXT	0(14)	0
Chloramphenicol	C	0(32)	0
Cephalothin	KF	0(2)	0

Table 4: Multiple antibiotic resistant index (MAR) of bacterial isolates.

Bacteria strains	Resistant pattern	MAR index *a/b
SR1-U2-10	P-E-TE-DO-RD	0.50
SR1-M3-27, SR1-D1-30	ATM-CAZ-MEM-CN	0.36
SR1-U1-2, SR2-D3-86	P-RD	0.20
SR1-U3-12,	IPM-S	
SR1-M3-29,	AMP-S	0.12
SR2-D3-89,	TIM-NA	
SR1-U2-3	P	
SR1-M1-16, SR2-U1-48, SR2-M3-72, SR2-U1-49, SR2-U3-56	S	
SR1-D2-36, SR1-D2-40, SR2-M1-64	AMP	0.10
SR1-D2-38	PRL	
SR2-M3-71, SR2-U1-50, SR2-D2-84	IPM	
SR1-U1-4, SR1-U2-6, SR1-U3-13, SR1-U3-15, SR1-D2-39, SR1-D1-31, SR1-D3-42, SR1-D3-43, SR1-D3-44, SR1-D3-45, SR2-U2-51, SR2-U2-55, SR2-U3-57, SR2-U3-59, SR2-M1-62, SR2-M1-63, SR2-M3-75, SR2-D2-83, SR2-D3-90	-	0.00

On the other hand, three subspecies of *Stenotrophomonas* were identified from the waterfall: *Stenotrophomonas acidaminiphila*, *Stenotrophomonas maltophilia* and *Stenotrophomonas terrae*. *Stenotrophomonas maltophilia*, formerly classified as *Pseudomonas maltophilia*, is an emerging opportunistic pathogen that has evolved into one of the leading multidrug-resistant pathogens responsible for various nosocomial infections (Patil *et al*., 2018). After *Pseudomonas* and *Acinetobacter*, *S. maltophilia* is the third most frequently encountered non-fermenting organism in clinical laboratories. It is often associated with substantial morbidity and a 43% mortality rate, particularly in immunocompromised patients, those in the intensive care unit and pulmonary sources of the isolate (Chavan *et al*., 2020). Treatment of *S. maltophilia* infections is difficult

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due to the organism's low antibiotic susceptibility. Additionally, it can develop resistance to currently used antimicrobials (Gil-Gil *et al*., 2020). On the other hand, Duan *et al*. (2020) reported that the organism exhibited low resistance to trimethoprim-sulfamethoxazole and levofloxacin, indicating that *S. maltophilia* is susceptible to trimethoprim-sulphamethoxazole and levofloxacin, as demonstrated in the study. Based on the MAR analysis, *S. acididamiphila*, *S. maltophilia* and *S. terrae* have a MAR index of less than 0.2, signifying that all isolates originated from lower antibiotic-contaminated sources (Krumperman, 1983).

Furthermore, *Pseudomonas aeruginosa*, an opportunistic pathogen, was isolated from Sikog waterfall. It is widely distributed in environments, posing a risk of nosocomial infections in cystic fibrosis patients. *Pseudomonas aeruginosa* is challenging to treat and eradicate due to its remarkable antibiotic resistance (Lim *et al*., 2019). Carbapenems-resistant *P. aeruginosa* is also one of the three bacterial species that require the development of new antibiotics (Tacconelli *et al*., 2017). It has been discovered that *P. aeruginosa* has a high intrinsic antibiotic resistance due to restricted outer membrane permeability, efflux systems that remove antibiotics from the cell and the production of antibiotic-inactivating enzymes such as β-lactamases (Breidenstein *et al*., 2011). In addition to its high intrinsic resistance to antibiotics, *P. aeruginosa* can acquire two types of inheritable traits that increase its resistance; horizontal transfer and mutational resistance (Breidenstein *et al*., 2011). *P. aeruginosa* is resistant to antibiotic groups such as aminoglycosides, quinolones, and β-lactams (Hancock and Speert, 2000). However, the result from Table S3 reveals that this organism is fully susceptible to antibiotic class penicillin (piperacillin), β-lactams combinations agents (ticarcillin-clavulanate) and fluoroquinolones (ciprofloxacin, levofloxacin and norfloxacin).

Additionally, *Alcaligenes* sp. also has been isolated successfully from the waterfall. *Alcaligenes* species is an opportunistic pathogen generally recovered from cystic fibrosis patients (Saiman *et al*., 2001). According to Huang's (2020) clinical study, the opportunistic infections caused by *Alcaligenes* in humans are often challenging to treat due to the organism's increased resistance to antibiotics such as aminoglycosides, anti-*pseudomonas* penicillin, carbapenems, cephalosporins and quinolones. Antibiotics are chosen based on the antibiotic sensitivity test performed on the patient, and ceftazidime and levofloxacin are the most commonly prescribed antibiotics in treating *A. faecalis* infections. Carbapenem is an appropriate antibiotic if the strain *A. faecalis* is an extended-spectrum beta-lactamase (ESBL). Huang (2020) also stated that imipenem, meropenem and ceftazidime are the three antibiotics with the highest sensitivity in treating *A. faecalis*. However, based on the findings, *A. faecalis* exhibits resistance toward ceftazidime and meropenem, as well as other antibiotic groups such as monobactams; aztreonam and aminoglycosides: gentamicin, and has a MAR index of 0.36, indicating that it was recovered from sources with a high risk of antibiotic-resistant contamination, as suggested by Kathleen *et al*. (2016).

Table 3 shows that all bacterial isolates had a susceptibility rate of 100% to the antibiotics cefepime, cefotaxime, ceftriaxone, ertapenem, amikacin, ciprofloxacin, levofloxacin, norfloxacin, nalidixic acid, trimethoprim-sulphamethoxazole, chloramphenicol and cephalothin. The results are consistent with the findings of Lihan *et al*. (2020). On the contrary, a high percentage of resistance to streptomycin (36.84%) and penicillin (36.36%) were observed among bacterial isolates. As shown in Table 4, the results revealed that the MAR index ranges from 0 to 0.50, with *Lysinibacillus telephonicus* exhibiting the highest resistance. This bacterium is resistant to five of the eleven antibiotics that have been tested. *L. telephonicus* are Gram-positive, motile rod-shaped, commonly isolated from soil and other environments, such as sea sediment, forest humus and cow manure (Rahi *et al*., 2017). In addition to being utilized as an alternative to agrochemicals and remediate heavy metal-contaminated environments, *Lysinibacillus* species are also recognized for their insecticidal action against various insects, particularly mosquitoes, which are carriers for several human diseases (Ahsan and Shimizu 2021).

In conclusion, increased antibiotic resistance is a certainty, as antibiotic-resistant genes are widespread in a vast array of environmental bacterial populations and are not limited to clinical settings. Thus, there is a drive to create more effective, standardized, and focused methods to produce accurate and rapid analyses.

CONCLUSION

Some bacterial species found at Sikog waterfall were coliforms or enteropathogenic species, indicating a human or animal waste source. It is important to continuously examine and monitor antimicrobial resistance trends in light of the fact that microbes are becoming more resistant to antibiotics. Regardless of age, local communities should be aware of the potential waterborne diseases associated with recreational water and precaution and prevention measures against disseminating waterborne disease-causing bacteria.

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SUPPLEMENTARY INFORMATION

Table S1: Antibiotic disk used in antibiotic susceptibility testing.

Antibiotic	Abbreviation	Concentration (µg)
Piperacillin	PRL	100
Ticarcillin-clavulanate	TIM	5
Cefepime	CEF	30
Ceftazidime	CAZ	30
Aztreonam	ATM	30
Imipenem	IPM	10
Meropenem	MEM	30
Gentamicin	CN	10
Ciprofloxacin	CIP	5
Levofloxacin	LEV	5
Norfloxacin	NOR	10
Sulphamethoxazole-trimethoprim	SXT	25
Penicillin	P	10 units
Erythromycin	E	15
Tetracycline	TE	30
Doxycycline	DO	30
Chloramphenicol	C	30
Rifampicin	RD	5
Ampicillin	AMP	10
Ceftriaxone	CRO	30
Ertapenem	ETP	10
Streptomycin	S	10
Nalidixic acid	NA	30
Cephalothin	KF	30
Amikacin	AK	10
Ofloxacin	OFX	5
Cefotaxime	CTX	30

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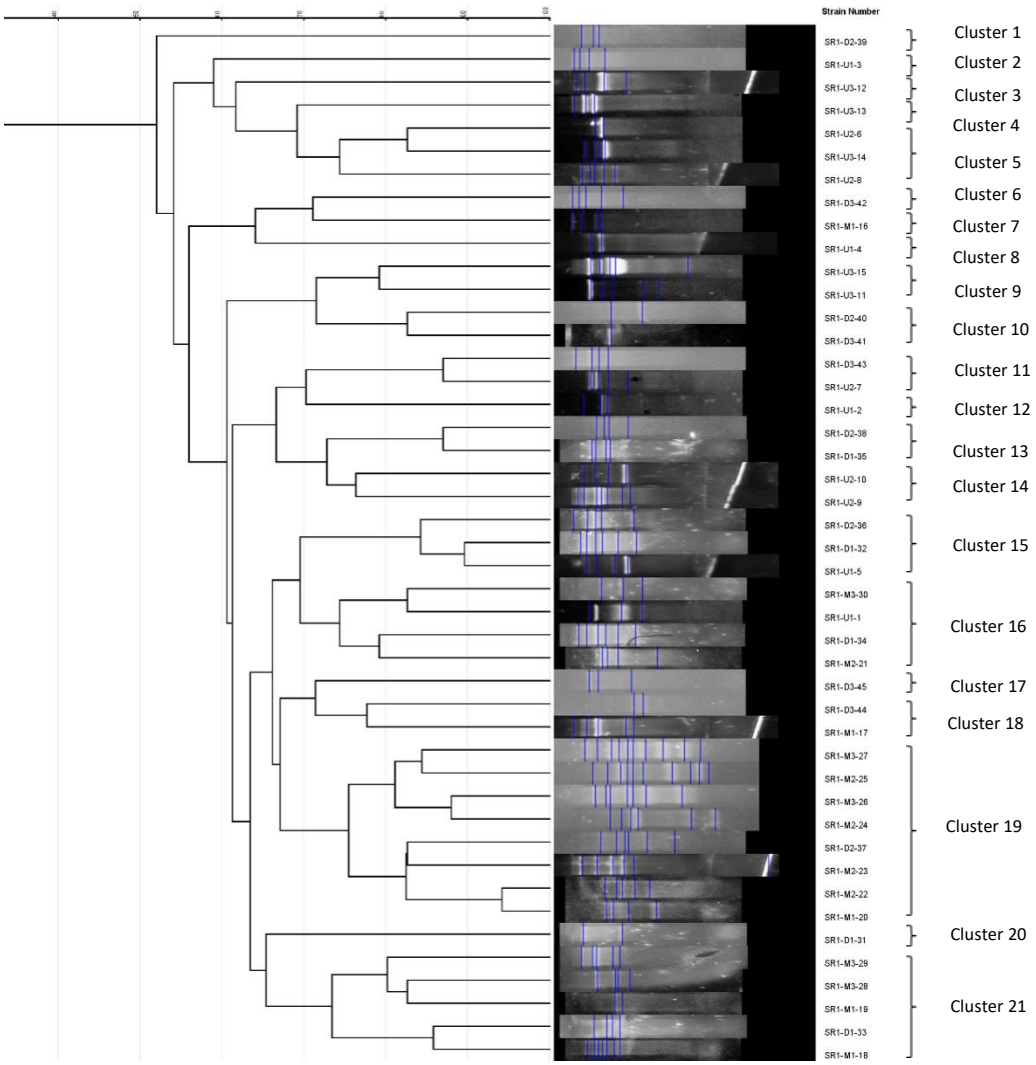

Figure S1: Dendrogram based on Dice similarity method with a tolerance value of 5.0 using UPGMA linkage of $(GTG)_5$ PCR fingerprints. Cluster 1: SR1-D2-39; Cluster 2: SR1-U1-3; Cluster 3: SR1-U3-12; Cluster 4: SR1-U3-13; Cluster 5: SR1-U2-6, SR1-U3-14, SR1-U2-8; Cluster 6: SR1-D3-42; Cluster 7: SR1-M1-16, Cluster 8: SR1-U1-4; Cluster 9: SR1-U3-15, SR1-U3-11; Cluster 10: SR1-D2-40, SR1-D3-41; Cluster 11: SR1-D3-43, SR1-U2-7; Cluster 12: SR1-U1-2; Cluster 13: SR1-D2-38, SR1-D1-35; Cluster 14: SR1-U2-10, SR1-U2-9; Cluster 15: SR1-D2-36, SR1-D1-32, SR1-U1-5; Cluster 16: SR1-M3-30, SR1-U1-1, SR1-D1-34, SR1-M2-21; Cluster 17: SR1-D3-45; Cluster 18: SR1-D3-44, SR1-M1-17; Cluster 19: SR1-M3-27, SR1-M2-25, SR1-M3-26, SR1-M2-24, SR1-D2-37, SR1-M2-23, SR1-M2-22, SR1-M1-20; Cluster 20: SR1-D1-31; Cluster 21: SR1-M3-29, SR1-M3-28, SR1-M1-19, SR1-D1-33, SR1-M1-18.

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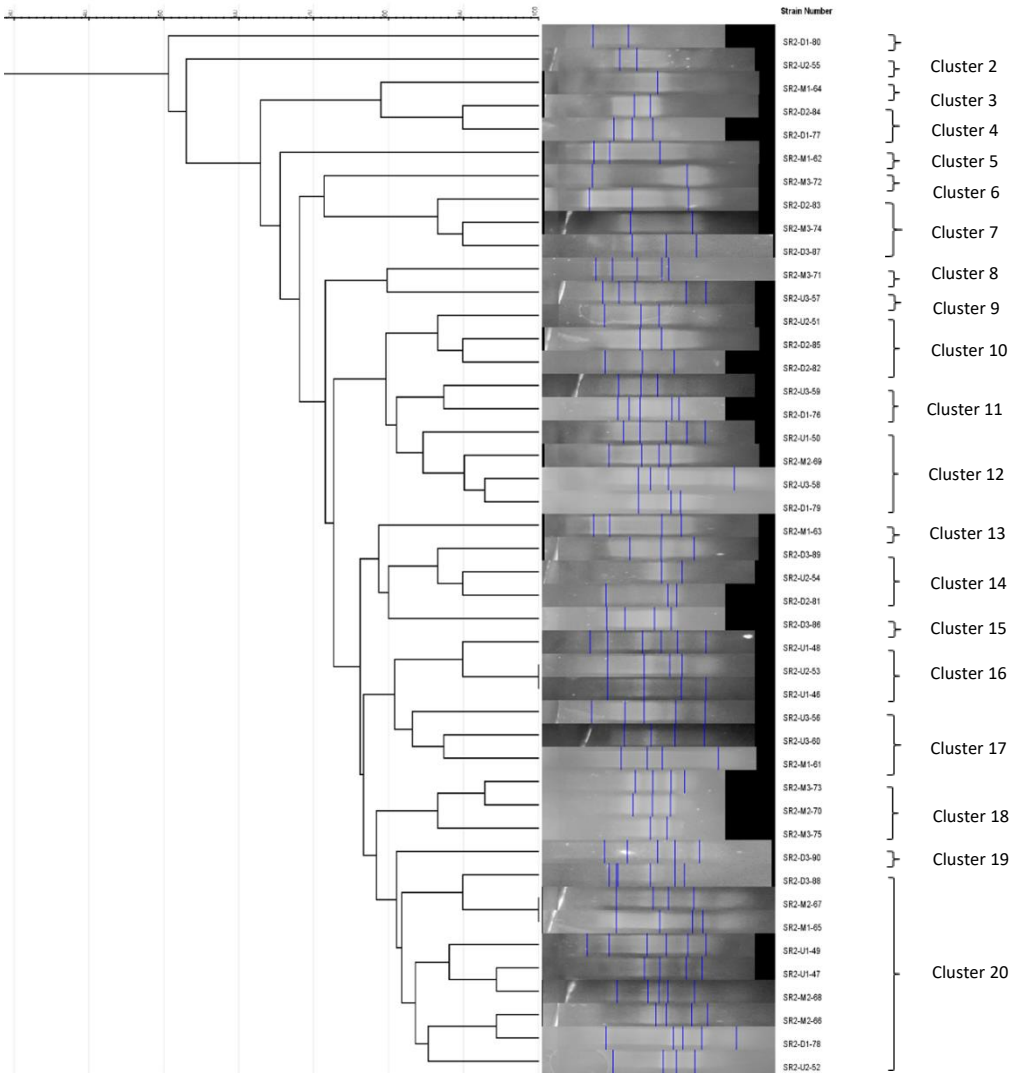

Figure S2: Dendrogram based on Dice similarity method with a tolerance value of 5.0 using UPGMA linkage of $(GTG)_5$ PCR fingerprints. Cluster 1: SR2-D1-80; Cluster 2: SR2-U2-55; Cluster 3: SR2-M1-64; Cluster 4: SR2-D2-84, SR2-D1-77; Cluster 5: SR2-M1-62; Cluster 6: SR2-M3-72; Cluster 7: SR2-D2-83, SR2-M3-74, SR2-D3-87; Cluster 8: SR2-M3-71; Cluster 9: SR2-U3-57; Cluster 10: SR2-U2-51, SR2-D2-85, SR2-D2-82; Cluster 11: SR2-U3-59, SR2-D1-76; Cluster 12: SR2-U1-50, SR2-U3-58, SR2-M2-69, SR2-D1-79; Cluster 13: SR2-M1-63; Cluster 14: SR2-D3-89, SR2-U2-54, SR2-D2-81; Cluster 15: SR2-D3-86; Cluster 16: SR2-U1-48, SR2-U2-53, SR2-U1-46; Cluster 17: SR2-U3-56, SR2-U3-60, SR2-M1-61; Cluster 18: SR2-M3-73, SR2-M2-70, SR2-M3-75; Cluster 19: SR2-D3-90; Cluster 20: SR2-D3-88, SR2-M2-67, SR2-M1-65, SR2-U1-49, SR2-U1-47, SR2-M2-68, SR2-D1-78, SR2-M2-66, SR2-U2-52.

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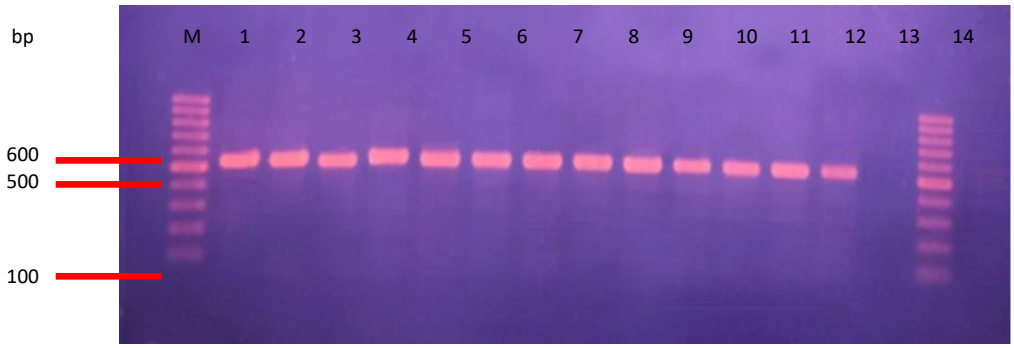

Figure S3a: Lane 1: SR1-D2-39; Lane 2: SR1-U1-3; Lane 3: SR1-U3-12; Lane 4: SR1-U3-13; Lane 5: SR1-U2-6, Lane 6: SR1-D3-42; Lane 7: SR1-M1-16; Lane 8: SR1-U1-4, Lane 9: SR1-U3-15, Lane 10: SR1-D2-40, Lane 11: SR1-D3-43, Lane 12: SR1-U1-2; Lane 13: SR1-D2-38; Lane 14: Negative control.

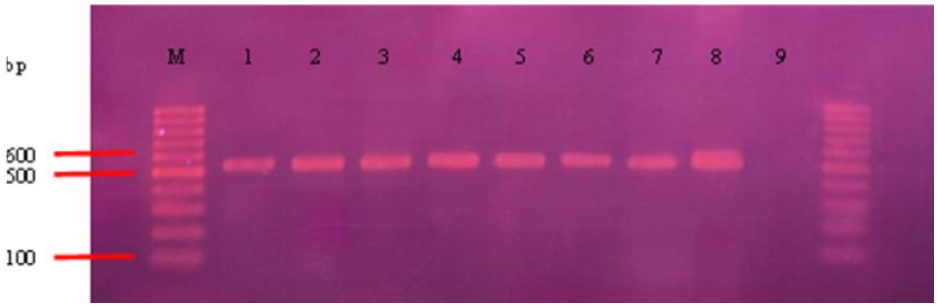

Figure S3b: Lane 1: SR1-U2-10; Lane 2: SR1-D2-36; Lane 3: SR1-M3-30; Lane 4: SR1-D3-45; Lane 5: SR1-D3-44; Lane 6: SR1-M3-27, Lane 7: SR1-D1-31; Lane 8: SR1-M3-29, Lane 9: Negative control.

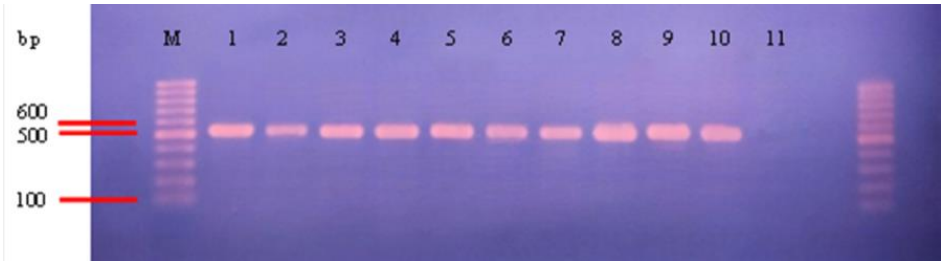

Figure S3c: Lane 1: SR2-D1-80; Lane 2: SR2-U2-55; Lane 3: SR2-M1-64, Lane 4: SR2-D2-84; Lane 5: SR2-M1-62; Lane 6: SR2-M3-72; Lane 7: SR2-D2-83; Lane 8: SR2-M3-71; Lane 9: SR2-U3-57; Lane 10: SR2-U2-51; Lane 11: Negative control.

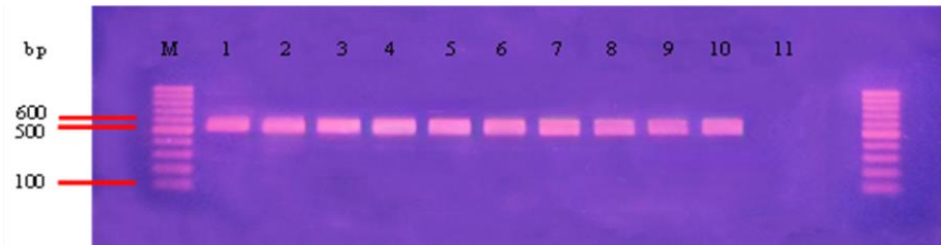

Figure S3d: Lane 1: SR2-U3-59; Lane 2: SR2-U1-50; Lane 3: SR2-M1-63; Lane 4: SR2-D3-89; Lane 5: SR2-D3-86; Lane 6: SR2-U1-48; Lane 7: SR2-U3-56; Lane 8: SR2-M3-73; Lane 9: SR2-D3-90; Lane 10: SR2-D3-88; Lane 11: Negative control.

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Table S2: Antibiotic susceptibility profile of environmental bacteria based on *Enterobacteriaceae* (CLSI, 2021).

Isolate	Bacteria species	Antibiotic Susceptibility Profiles																
		AMP	PRL	TIM	ATM	ETP	IPM	MEM	S	TE	DO	LEV	NA	NOR	C	CAZ	CN	AK
SR1-U3-15	*Citrobacter farmeri*	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S
SR1-U3-13	*Enterobacter chengduensis*	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S
SR1-U3-12	*Enterobacter mori*	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S
SR2-M3-71	*Enterobacter mori*	S	S	S	S	S	R	S	I	S	S	S	S	S	S	S	S	S
SR1-M1-16	*Enterobacter cloacae*	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
SR2-M1-62	*Enterobacter cloacae*	I	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S
SR2-M1-63	*Enterobacter asburiae*	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S
SR2-U3-56	*Enterobacter soli*	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
SR1-M3-29	*Kosakonia oryzae*	R	S	S	S	S	S	I	R	S	S	S	S	S	S	S	S	S
SR1-D2-40	*Kosakonia sacchari*	R	I	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S
SR2-U2-55	*Kosakonia oryzendophytica*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SR2-U3-57	*Kosakonia oryzendophytica*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SR2-D3-89	*Klebsiella pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	R	S	S	S	S	S
SR2-U1-48	*Escherichia coli*	I	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
SR2-M3-72	*Escherichia coli*	I	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
SR2-U1-49	*Escherichia coli*	I	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
SR1-D2-36	*Serratia marcescens*	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SR2-D1-80	*Serratia marcescens*	R	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S
SR2-M1-64	*Cronobacter sakazakii*	R	S	S	S	S	S	S	I	S	I	S	S	S	S	S	S	S

Table S3: Antibiotic susceptibility profile of environmental bacteria based on *Pseudomonas aeruginosa* (CLSI, 2021).

Isolate	Bacteria species	Antibiotic Susceptibility Profiles										
		PRL	TIM	FEP	CIP	ATM	IPM	CAZ	MEM	CN	LEV	NOR
SR2-D3-90	*Pseudomonas aerµginosa*	S	S	S	S	S	S	S	S	S	S	S
SR1-D3-42	*Pseudomonas aerµginosa*	S	S	S	S	S	S	S	S	S	S	S
SR1-M3-27	*Alcaligenes aquatilis*	S	S	S	S	R	S	R	R	R	S	S
SR1-D1-30	*Alcaligenes faecalis*	S	S	S	S	R	S	R	R	R	S	S

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Table S4: Antibiotic susceptibility profile of environmental bacteria based on *Staphylococcus aureus* (CLSI, 2021).

Isolate	Bacteria species	Antibiotic Susceptibility Profiles										
		P	CN	E	TE	DO	CIP	LEV	NOR	SXT	C	RD
SR1-U2-6	*Staphylococcus saprophyticus*	S	S	S	S	S	S	S	S	S	S	S
SR1-D3-43	*Bacillus pumilis*	S	S	S	S	S	S	S	S	S	S	S
SR1-U1-3	*Bacillus thuringiensis*	R	S	I	S	S	S	S	S	S	S	S
SR1-U1-2	*Bacillus cereus*	R	I	S	S	S	S	S	S	S	S	R
SR1-U2-10	*Lysinibacillus telephonicus*	R	S	R	R	R	-	S	S	S	S	R
SR1-U1-4	*Rossellomorea vietnamensis*	S	S	I	S	S	S	S	S	S	S	S
SR1-D2-39	*Rossellomorea marisflavi*	S	S	I	S	S	S	S	S	S	S	S
SR1-D3-45	*Rossellomorea aquimaris*	S	S	S	S	S	S	S	S	S	S	S
SR2-M3-75	*Rossellomorea aquimaris*	S	S	S	S	S	S	S	S	S	S	S
SR2-U3-59	*Rossellomorea aquimaris*	S	S	S	S	S	S	S	S	S	S	S
SR2-D3-86	*Staphylococcus saprophyticus*	R	S	S	S	S	S	S	S	S	S	R

Table S5: Antibiotic susceptibility profile of environmental bacteria based on *Aeromonas* sp. (CLSI, 2021).

Isolate	Bacteria species	Antibiotic Susceptibility Profiles										
		MEM	IPM	ATM	TE	CAZ	C	CN	AK	CIP	CRO	KF
SR1-D1-31	*Aeromonas aquatica*	S	S	S	S	S	S	S	S	S	S	S
SR2-U1-50	*Aeromonas encheleia*	S	R	S	S	S	S	S	S	S	S	S

Table S6: Antibiotic susceptibility profile of environmental bacteria based on *non*-Enterobacteriaceae (CLSI, 2021).

Isolate	Bacteria species	Antibiotic Susceptibility Profiles									
		DO	LEV	TE	PRL	CTX	ATM	IPM	MEM	CAZ	OFX
SR1-D2-38	*Chromobacterium* sp.	S	S	S	R	S	S	S	S	S	S
SR2-D2-84	*Kocuria palustris*	S	S	S	S	S	S	R	S	I	S

Table S7: Antibiotic susceptibility profile of environmental bacteria based on *Stenotrophomonas maltophilia* (CLSI, 2021).

Isolate	Bacteria species	Antibiotic Susceptibility Profiles	
		LEV	SXT
SR1-D3-44	*Stenotrophomonas acidaminiphila*	S	S
SR2-U2-51	*Stenotrophomonas maltophilia*	S	S
SR2-D2-83	*Stenotrophomonas terrae*	S	S

Note: Symbol "S": Susceptible, "I": Intermediate, "R": Resistant.

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