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Year-round monitoring of antibiotic-resistant bacteria in pristine uppermost stream and estimation of pollution sources

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Studies on the conditions and pollution routes of antibiotic-resistant bacteria (ARB) in rivers can help provide countermeasures against the spread of ARB. This study focused on the pristine uppermost stream of a river, where *Escherichia coli* (*E. coli*) and enterococci were detected, although the stream flows through a pristine forest catchment. Antibiotic resistance of *E. coli* and enterococci isolated from the river water, riverbed sediment, and feces of waterside animals, such as birds and Mustelidae, were investigated throughout the year in the pristine uppermost sites. Antibiotic resistance was present in 1.4% (7/494) of the *E. coli* strains and 3.0% (24/812) of the enterococcal strains, and was low throughout the year. Although antibiotic resistance of bacteria isolated from feces was not detected in this watershed, the prevalence of multidrug-resistant *E. coli* was 0.4% (1/246) and 0.6% (1/172) in river water and riverbed sediment samples, respectively were observed. The presence of extended-spectrum β-lactamase (ESBL)-producing *E. coli* was confirmed in river water samples, and genomic analysis revealed that the samples possessed the *CTX-M-15* group. Multidrug-resistant strains and ESBL-producing strains were classified as phylogroups B1 and A, respectively, which are *E. coli* phenotypes isolated from wild animals. Pulsed-field gel electrophoresis revealed analysis targeting enterococci that strains isolated from river water and bird feces were in the same cluster with 100% similarity. Therefore, bird feces are a source of enterococci in the uppermost stream of the river. Because multidrug-resistant bacteria and ESBL-producing bacteria were present in the pristine uppermost stream of the pristine river, urgent elucidation of the spreading routes of ARB is important.

KEYWORDS

Escherichia coli, enterococci, antibiotic resistance, antibiotic-resistant bacteria, multidrug-resistant bacteria, extended-spectrum β-lactamase, genomic analysis, pristine uppermost stream

1 Introduction

The emergence of antibiotic-resistant bacteria (ARB) is one of the most serious problems globally (O'Neill, 2014; Willyard, 2017; Antimicrobial Resistance Collaborators, 2022). The Group of Seven (G7) is promoting global efforts to address this important issue (G7 Health Ministers' Communiqué, 2022; World Health Organization, 2022b) and Centers for Disease Control and Prevention (CDC) annually update and publish information on ARB (World Health Organization, 2022a; Centers for Disease, 2019), warning of the seriousness of the problem. Currently, a wide variety of ARB have been detected, and the main sources of ARB include clinical institutions, livestock farms, and fish farms, where antibacterial drugs are frequently used. ARB are ubiquitous in other environments, such as the water environment (Zhang et al., 2015; Daniel et al., 2017; Osińska et al., 2020; Grenni, 2022). Strains resistant to clinically important antibiotics, such as cephalosporins, carbapenems, and vancomycin, have been detected in the water environments (Diwan et al., 2018; Givens et al., 2023). In recent years, ARB, and antibiotic resistance genes (ARGs) have been detected in natural environments and wild animals that are not directly affected by humans. Of the six Enterobacterales isolated from pristine freshwater (rivers) in Brazil, 52%–77% were antibiotic resistance (Lima-Bittencourt et al., 2007). ARGs ($1.7 \pm 1.0 \times 10^6$ copies/mL), predominantly those resistant to β-lactam and tetracycline, were detected in the ocean near Antarctica (Jang et al., 2022). In Uganda, 17% of the *Escherichia coli* (*E. coli*) strains isolated from wild gorillas were antibiotic resistant. Of these, 4.2% were resistant to antibiotics used for treating lung inflammation in livestock, such as ceftiofur (Rwego et al., 2008). According to a wildlife review, many Enterobacterales isolated from wild animals possessed plasmids encoding ARGs related to β-lactam antibiotics and colistin, causing concerns about the spread of antibiotic resistance in wild animals and the risk to public health (Dolejska and Papagiannitsis, 2018). Therefore, the global spread of ARB cannot be denied.

Information on rivers that transport and spread ARB over a wide area is crucial, but information on ARB in pristine rivers that are not directly affected by human areas remains scarce. Furthermore, it is difficult to identify the appearance of ARB in pristine environments and the specific source and route of contamination. Although limited information is available, 18% of *E. coli* detected in a pristine upstream site of the Kaeda River in Miyazaki, Japan, where the catchment area was forest, were ARB, including multidrug-resistant strains (Nishimura et al., 2021). In addition, headwaters are surrounded by forests, which have a rich ecosystem and are home to many wild animals. Studies from other countries suggest that the source of ARB load in headwater areas is likely to be wild animals (birds, mammals, etc.) that use waterside areas (Bonnedahl and Järhult, 2014; Rogers et al., 2018; García et al., 2020; Yuan et al., 2021). However, there is a lack of information on whether the development of antibiotic resistance in headwaters is human-introduced or naturally occurring. When focusing on wild animals as a source, direct contact with wild animals to perform study is difficult and risky. Thus, monitoring ARB in headwaters surrounded by forests and identification of contamination routes using scientific methods can be important countermeasures against the spread of ARB. In addition, pristine environments are ideal environments for understanding the mechanisms and interactions of the early stages of evolution, acquisition, and transmission of drug resistance, and can accumulate new information related to drug resistance (Hwengwere et al., 2022).

Elucidating the actual presence and diffusion routes of ARB in pristine rivers is crucial for controlling the transport and diffusion of ARB. The purpose of this study is to determine the prevalence of ARB in pristine river. Then, the presence of clinically important ARB will be confirmed, and the pollution source will be estimated by genomic analysis. The survey focused on the pristine uppermost stream of the Kaeda River (Figure 1) to determine the prevalence of antibiotic-resistant *E. coli* (AR-*E. coli*) and antibiotic-resistant enterococci (AR-ENT) in the river water, riverbed sediment, and feces of waterside animals every 2 months for 1 year. Then, information on the detected ARB was organized, and their characteristics and presence of antibiotic-resistant strains, which are important in clinical institutions, were ascertained. Strains from river water, riverbed sediment, and waterside bird feces were analyzed by genomic analysis and pulsed-field gel electrophoresis (PFGE) to trace the source of ARB.

In our previous study (Nishimura et al., 2021), we confirmed the presence of ARB in the upstream stream of the Kaeda River, and its prevalence was comparable between the upstream and downstream streams. However, we have not been able to elucidate the source of ARB in upstream streams, where deep in the forest and unaffected by humans. Therefore, this study was designed to focus on the uppermost reaches of the Kaeda River in the forest and the animals that use their banks. As the target bacteria, we focused on *E. coli* and enterococci. These bacteria, whose hosts are warm-blooded animals, including humans, livestock, and wild animals, are ubiquitous in aquatic environments (Farnleitner et al., 2010). They are also classified as fecal indicator bacteria and are important for the protection of public health (World Health Organization, 2022a; Ishii and Sadowsky, 2008). In addition, some important bacteria are resistant to antibiotics, and information is accumulated in the aquatic environment (Alonso et al., 2001; Baquero et al., 2008).

2 Materials and methods

2.1 Sampling sites and sample collection

Figure 1; Supplementary Table S1 show the sampling site map as land use data around the Kaeda River and the number of samples, respectively. The Kaeda River (channel length, 17.5 km; basin area, 53.8 km²) originates from headwaters dominated by forests in their catchment areas, flows through a deep valley with land with various uses, and drains into the Hyuganada (Pacific Ocean). In this study, two sites were selected as pristine uppermost streams not directly affected by human activities: 1) a headwaters site (S1: width, 0.3 m; depth, 0.2 m; flow rate, 2 m³/s) and 2) approximately 400 m below the headwaters site (S2: width, 4 m; depth, 0.4 m; flow rate, 13 m³/s). The catchments of S1 and S2 were exclusively forested and consisted of natural and planted forests, respectively. There is no anthropogenic impact from human activity in this study area. Sampling was conducted every 2 months for 1 year (31 August 2017–30 August 2018).

FIGURE 1
Sampling sites and major land use categories of pristine uppermost stream at the Kaeda River in southern Japan. S1, site1, river source; S2, site2, upstream.
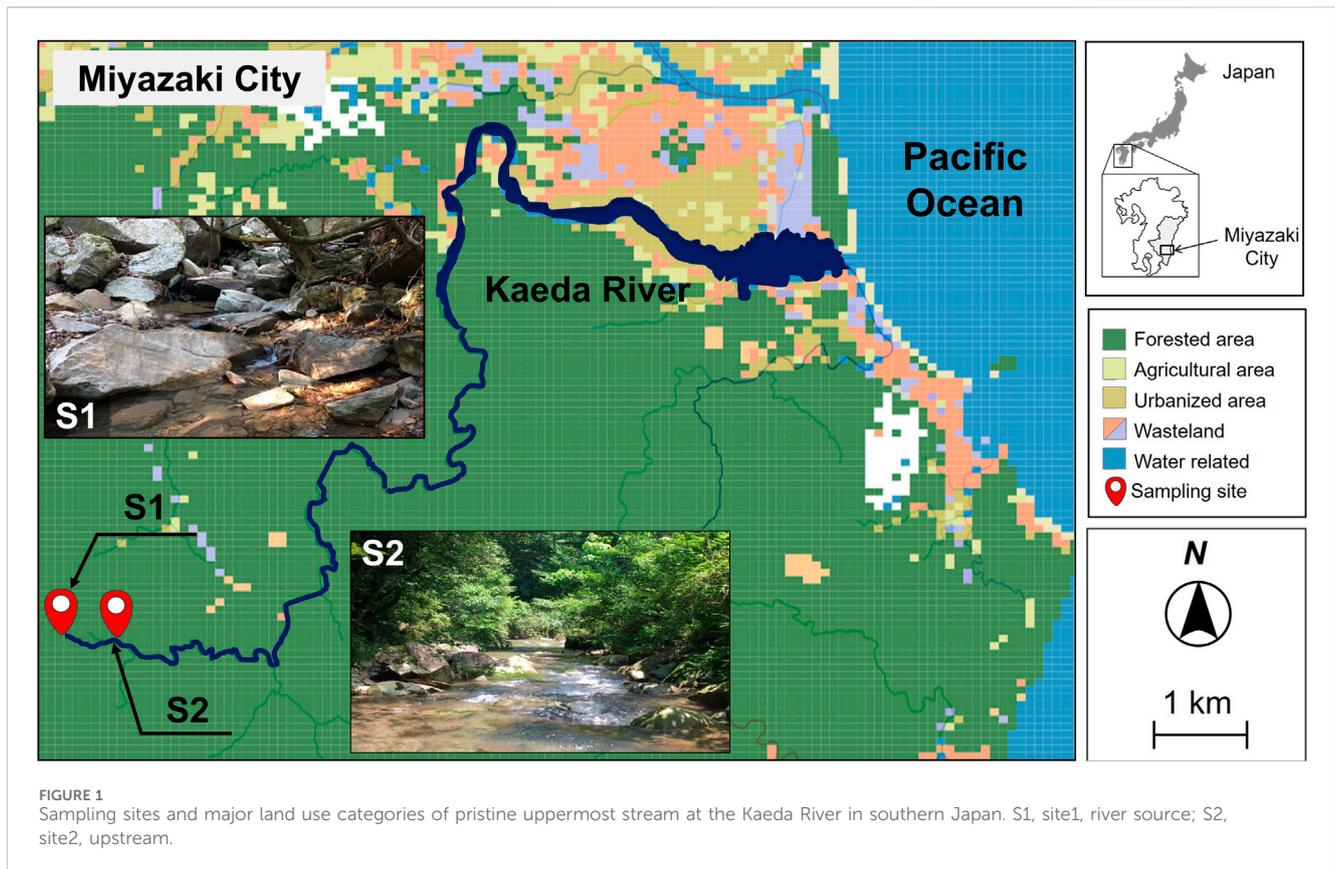
River water, riverbed sediment, and feces from wild animals that use the waterside were collected. River water samples were stored in sterile 5-L polyethylene bottles for each site. Riverbed sediments were collected by shoveling 2–5 cm of the surface layer from 3 m radius of the water sample points. The feces of wild animals (birds and Mustelidae) on waterside rocks situated within a 10 m radius of the water sample points were collected with a sterile cotton swab and placed into 15 mL sterilized polyethylene tubes.

Water temperature and dissolved oxygen (DO) were determined using a fluorescent-type dissolved oxygen meter (HQ40d, Hach Company, COL., Tokyo, Japan) at the sampling site. A benchtop pH/water quality analyzer (LAQUA, HORIBA, Ltd., Kyoto, Japan) was used to measure pH and electrical conductivity. Turbidity was determined using a turbidity meter (SEP-PT-706D, Mitsubishi Chemical Co., Tokyo, Japan). All samples were transported back to the laboratory and used for experiments within 4 h of collection (Japanese Standard Association, 2016).

2.2 Counting and isolation of fecal indicator bacteria

The number of *E. coli* and enterococci, as well as other coliforms, was counted in each sample by the membrane filter method. Five liters of river water was collected from each sampling site. Bacterial counts varied greatly depending on the survey period, so the amount of passing water sample was set at three levels (10, 100, and 1,000 mL) for each survey and filtered through a 0.45 μm pore membrane filter (47 mm in diameter, mixed cellulose ester; Advantec, Tokyo, Japan). Membranes were placed on CHROMagar ECC agar plates (CHROMagar, Paris, France), and incubated at 37°C for 24 h. Subsequently, filters with appropriate flow volume for bacterial counting and isolation were selected and analyzed. On the filter, blue colonies were considered presumptive *E. coli* and mauve colonies were considered other presumptive coliform. Enterococci were counted using membrane-enterococci indoxyl-β-D-glucoside (mEI) agar plate (United States Environmental Protection Agency, 2014). The samples (10, 100, and 500 mL) were filtered through a membrane filter. Membranes were incubated on mEI agar plates at 41°C for 24 h. On the filter, blue colonies were considered presumptive enterococci. The number of bacteria in each sample was counted in three replicates and the mean was calculated as colony forming unit (CFU) per 100 mL. The detection limit of this method was 0.3 CFU/100 mL. Riverbed sediment samples (5 g) were mixed with 40 mL of sterilized physiological saline solution or phosphate-buffered saline (Boehm et al., 2009) and allowed to settle for 1 min. Supernatant liquid was filtered through a membrane filter. Then bacteria were isolated in a manner identical to that of water sample analysis. The detection limit of riverbed sediment samples was 7 CFU/100 g. Samples of wild animal feces (1 g) were mixed with 9 mL of sterilized physiological saline solution or phosphate-buffered saline and allowed to settle for 1 min. Then, 0.1–0.001 mL of the supernatant was filtered through a membrane filter. Bacterial counts of feces were not measured, only strains were isolated. Thirty single colonies were randomly isolated from CHROMagar ECC and mEI agar plates for each sample at

S1 and S2. The plates were streaked twice on Brain Heart Infusion agar (BD, New Jersey, United States) or Todd–Hewitt agar (Becton, Dickinson, NJ, United States) to isolate *E. coli* and enterococci, respectively. When less than 30 isolates were available, all single colonies were isolated. The plates were incubated for 24 h at 37°C. For *E. coli*-positive strains and enterococci-positive strains, max 30 strains were isolated from each sample at S1 and S2. Supplementary Tables S2, S3 show the number of *E. coli*-positive strains or enterococci-positive strains isolated from each sample at S1 and S2.

2.3 Identification of *E. coli* and enterococci by MALDI-TOF MS

For *E. coli*-positive (n = 567) and enterococci-positive (n = 839) strains isolated from all the samples, the identification of bacterial species was performed using MALDI-TOF MS (Fenselau and Demirev, 2001; Christner et al., 2014; Suzuki et al., 2018). All samples were analyzed using an Autoflex III TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, United States). Measurements were performed using flexControl 3.0 software (Bruker Daltonics, Billerica, MA, United States) for database construction and validation. The software settings were based on Suzuki et al. (2018). The instrument was calibrated using a Bruker bacterial test standard (part no. 8255343, Bruker Daltonics, Billerica, MA, United States). Recorded mass spectra were processed with the MALDI Biotyper Compass microbial identification system (Bruker Daltonics, Billerica, MA, United States) using standard settings. The MALDI Biotyper output score had a range of 0.00–3.00, and *E. coli* and enterococci identification scores were ≥ 2.00.

2.4 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on strains identified as *E. coli* (n = 494) and enterococci (n = 813). The minimum inhibitory concentration (MIC) of each antibiotic was determined using the agar dilution method, according to the guidelines of Clinical Laboratory Standards Institute (CLSI) (Clinical Laboratory Standards Institute, 2017). Twelve antibiotics that are important in the resistance of *E. coli* and enterococci were used. The preculture, dissolution and dilution of antibiotics, and test procedure were based on Nishimura et al. (2021) for *E. coli* and Nishiyama et al. (2017) for enterococci. According to the recommendations of CLSI, 12 antibiotics were tested using plates containing 2-fold dilutions of antibiotics with five graded concentrations. MIC breakpoints for resistance were based on CLSI (CLSI, 2012) criteria. Quality control was used for *E. coli* ATCC 25922 (*E. coli*) and enterococci ATCC 29212 (*Enterococcus faecalis*).

2.5 Genomic analysis

Genomic analysis was performed on strains determined as ESBL-producing *E. coli* (n = 1) and multidrug-resistant *E. coli* (n = 1). In advance, the double-disk synergy test was performed

for the ESBL-producing *E. coli* strain (Clinical Laboratory Standards Institute M100-S26). Genomic DNA was purified from 1 mL overnight culture of *E. coli* strains using DNeasy Blood and Tissue Kit (Qiagen). Libraries were prepared using Lotus DNA Library Prep Kit (Integrated DNA Technologies, Coralville, IA, United States) and NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) (New England BioLabs Japan, Tokyo, Japan) and sequenced on an Illumina HiseqX Ten platform (Illumina, San Diego, CA, United States) to generate 151-bp paired-end reads. Genome assembly was performed using Platanus_b v1. 3.2 (Kajitani et al., 2020) with default parameters. Assembly quality was assessed using CheckM v1. 2.0 (Parks et al., 2015). Phylogroup and sequence type were determined by ClermonTyping v20.06 (Beghain et al., 2018) and srst2 v2.0 (Inouye et al., 2014), respectively. ARGs were identified by ABRicate v0.9.8 (https://github.com/tseemann/abricate) using the ARG-ANNOT database (Gupta et al., 2014) with default parameters. Mutations for quinolone resistance and colistin resistance were analyzed using AMRFinderPlus 3.11.14 (Feldgarden et al., 2021) with default settings.

2.6 Analysis of PFGE typing for *E. coli* and enterococci

In total, *E. coli* (n = 30) and enterococci (n = 29) strains were randomly selected from river water, riverbed sediment, and bird feces from the October 2017 (autumn) survey for genotyping using PFGE. Traces of wild animals at the study site were considered important for examining the bacterial load in the surface waters. Therefore, the analysis focused on October 2017, when the highest number of bird fecal samples were collected at the study site. For *E. coli* genotyping, PFGE was performed according to the standardized PulseNet protocol for PFGE provided by CDC (Centers for Disease Control, 2017). For enterococci genotyping, PFGE was performed using CHEF Bacterial Genomic DNA Plug Kit (Bio-Rad, Hercules, California, United States), according to the manufacturer's protocol, with slight modifications. A lambda DNA ladder (range 48.5–873 kb; Lonza, Rockland, ME, United States) was used as a size marker. The details of analysis are shown in Supplementary Text S1.

Band-based PFGE patterns were clustered using Gene Profiler software (Scanalytics, Buckinghamshire, United Kingdom). Levels of similarity between fingerprints were expressed as Dice coefficient. PFGE patterns were clustered using the unweighted pair group method with arithmetic mean. PFGE patterns with 100% similarity were considered identical genotypes.

3 Results and discussion

3.1 Water quality of the pristine uppermost stream in the Kaeda River

Supplementary Table S4 shows the water quality of the pristine uppermost stream in the Kaeda River. Water temperatures at S1 (22.8°C) and S2 (24.7°C) were highest in August (summer) and ranged from 6.6°C to 20.4°C in other surveys (spring, autumn, and

FIGURE 2
Changes in the numbers of *Escherichia coli* (*E. coli*) and enterococci at the S1 and S2 over a 1-year period. Bacteria counts of river water at S1 **(A)**, at S2 **(B)**. Bacteria counts of riverbed sediment at S1 **(C)**, at S2 **(D)**.
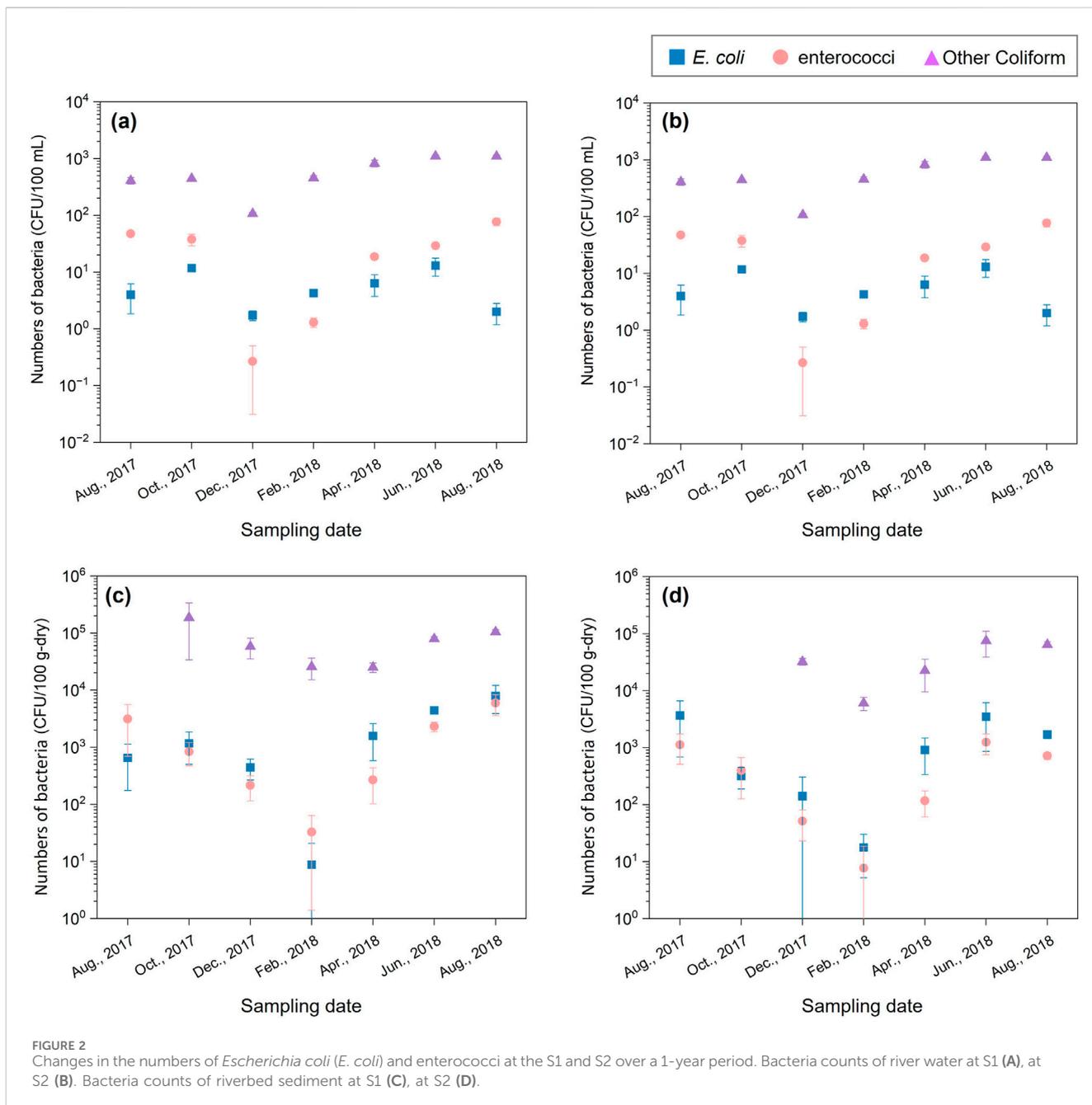
winter). The difference in water temperature between S1 and S2 was 1°C–2°C. DO was the highest in winter (10.9 mg/L, near the saturation concentration) and decreased in summer. pH ranged from 6.0 to 6.9, slightly lower than neutral. Electrical conductivity differed between S1 and S2 (47.9 and 58.9 µS/cm, respectively), presumably due to increased mineral components due to the flow processes. Turbidity was extremely low across all surveys, with mean values of 0.3 turbidity units at both sites, and the sample water was extremely clear. Analysis of DO, pH, and turbidity, the water quality parameters of river water, showed no significant differences in water quality between S1 and S2 ($p > 0.05$), resulting in the water quality in both stations being similar. It is unlikely that the process of flow downstream in these two points would directly affect bacterial inactivation.

3.2 Bacteria counts of in river water and riverbed sediment

Figure 2 shows changes in the numbers of *E. coli*, enterococci, and other coliforms at S1 and S2 in 1 year. In river water at S1 and S2, *E. coli* count was low throughout the year (range: $1.7 ± 0.3$–$20.0 ± 3.3$ CFU/100 mL). In summer (August 2017 and 2018), enterococci count was higher than *E. coli* count, ranging from $47.3 ± 2.6$ to $101.3 ± 14.6$ CFU/100 mL. In winter (February 2018), enterococci count decreased to <0.3 CFU/100 mL and increased again in summer. Thus, seasonal variation in *E. coli* and enterococci count differed. Other coliform count showed seasonal variation similar to enterococci count, ranging from $9.2 × 10^1 ± 20.8$ to $1.9 × 10^4 ± 24.9$ CFU/100 mL. By contrast, the seasonal variation of *E. coli*

FIGURE 3
Identification rate of enterococci isolates collected from each sample by MALDI-TOF MS based on the MALDI biotyper software database.
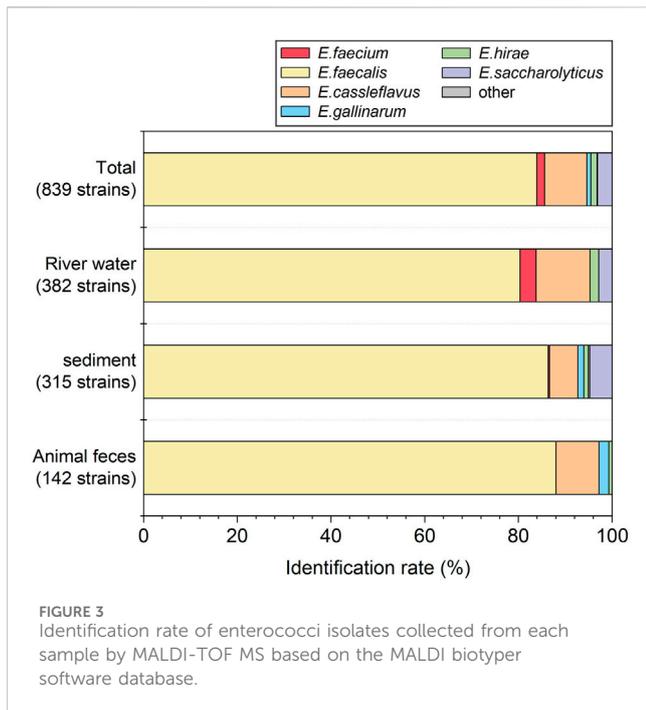
and enterococci counts in the riverbed sediment from both S1 and S2 were similar. The numbers of *E. coli* and enterococci were high in summer ($6.5 \times 10^2 \pm 4.7 \times 10^2$–$7.9 \times 10^3 \pm 4.0 \times 10^3$ CFU/100 g) and decreased in winter (7.7 ± 10.9 to $7.9 \times 10^3 \pm 4.0 \times 10^3$ CFU/100 g). However, bacterial counts were not significantly different between summer and winter (*E. coli*: $p > 0.05$, enterococci: $p > 0.05$).

Escherichia coli and enterococci were detected throughout the year, although the stream flows through a pristine forest catchment. The sampling site are not a human living area. Seasonal variations in bacterial counts in river water and riverbed sediment are associated with the activity of wild animals. Some species of wild animals are more active in summer, leading to a potentially higher impact of fecal coliform bacteria in the water due to waste deposition. Therefore, bacteria in the river may have been loaded by wild animals (Hansen et al., 2020; Afolabi et al., 2023). In addition to wild animals, other environmental factors, such as rainfall, water temperature, and decomposition of organic matter also significantly influence bacterial counts.

3.3 Identification rate of each positive strain

In total, 567 strains were isolated as *E. coli*-positive strains from all samples, and 494 (87.1%) were identified as *E. coli* after MALDI-TOF MS. The identification rate of *E. coli* was 88.4% (252/285) in river water, 88.8% (175/197) in riverbed sediment, and 78.8% (67/85) in wild animal feces. The major bacterial species of the pseudo-positive strains detected in this study were *Escherichia marmotae*, *Citrobacter freundii*, and *Serratia fonticola*.

In total, 839 strains were isolated as enterococci-positive strains from all samples, and 813 (96.9%) were identified as enterococci (Figure 3). Of the 813 strains identified as enterococci, 83.9% (704/839) were *E. faecalis*, 9.1% (76/839) were *E. cassleflavus*, 1.7% (14/839) were *E. faecium*, 1.3% (11/839) were *E. hirae*, 0.8% (7/839) were *E.*

gallinarum, and 0.1% (1/839) were *E. saccharolyticus*. The identification rate of enterococci for river water, riverbed sediment, and wild animal feces was 97.1% (371/382), 95.2% (300/315), and 100.0% (142/142) strains, respectively. *E. faecalis* was predominant in all samples (80.4%–88.0%). *E. faecium* was slightly detected (0.3%–3.4%). *E. faecalis* and *E. faecium* were frequently isolated in the clinical institutions as the major caUnited Statestive species of nosocomial infection (*E. faecalis*: 80%–90%, *E. faecium*: 5%–15%) (Ruoff et al., 1990; Gordon et al., 1992). The detection of *E. faecalis* and *E. faecium* in the pristine uppermost sites of the Kaeda River is a serious threat to public health, and it is important to investigate their antibiotic resistance.

3.4 Detection and seasonal variation of ARB in the pristine uppermost stream

The MIC values of the identified strains of *E. coli* (n = 494) and enterococci (n = 813) were tested for 8 and 9 antibiotics, respectively. One strain of enterococci was excluded because it did not grow. The antibiotic resistance rates of *E. coli* and enterococci strains isolated from all samples were 1.4% (7/494) and 3.0% (24/812), which were very low throughout the year (Figure 4). In river water, *E. coli* was highest in spring 4.8% (1/21) and enterococci was highest in winter 4.8%–9.6% (December 2017–February 2018). The riverbed sediment samples had a similar trend of antibiotic resistance as river water samples. Figure 4 shows the prevalence of ARB was very low throughout the year. The resistance rate of river water and riverbed sediment was slightly higher in spring than in summer and did not significantly correlate with the results of bacterial count ($p > 0.05$). In addition, ARB were not detected in the feces of wild animals.

In our previous survey, the antibiotic resistance rate of *E. coli* in the Kaeda River was 46% in the summer (July 2016) at S2 (Nishimura et al., 2021). By contrast, this value was significantly lower in this study. Although abrupt changes in the value can only be discussed by assumption, comparing this study with our previously reported study (Nishimura et al., 2021), the surrounding environment at the same site was altered. Parts of the artificial forest and riparian forest were cleared, and the number of trees covering the river was reduced. Although quantitative data are not available, the amount of light incident on the river increased. Therefore, the possibility that ecological changes or other factors affected the water cannot be excluded. By contrast, the resistance rate of AR-*E. coli* was 0.0%–2.5% upstream of the Tama River, which flows through Tokyo, Japan (Terada et al., 2012). We found that ARB were dispersed at a low rate in the pristine uppermost stream in Japan.

ARB were not detected in wild animal feces. This result contrasts with other study reporting detection of ARB in feces of wildlife (Ardiles-Villegas et al., 2011; Khare et al., 2020; Agga et al., 2021). Moreover, there are a few studies that indicate wildlife can also have an impact on ARB by spreading antibiotic resistance (Dolejska and Literak, 2019; Ramey and ahistrom, 2020; Laborda et al., 2022). In addition, several patterns of ARB occurrence in pristine environments have been considered in addition to the effects of wild animals. For example, plants are primary producers in pristine environments, and plants possess a diverse resistome (Berendonk et al., 2015; Chen et al., 2019; Obermeier et al., 2021). These
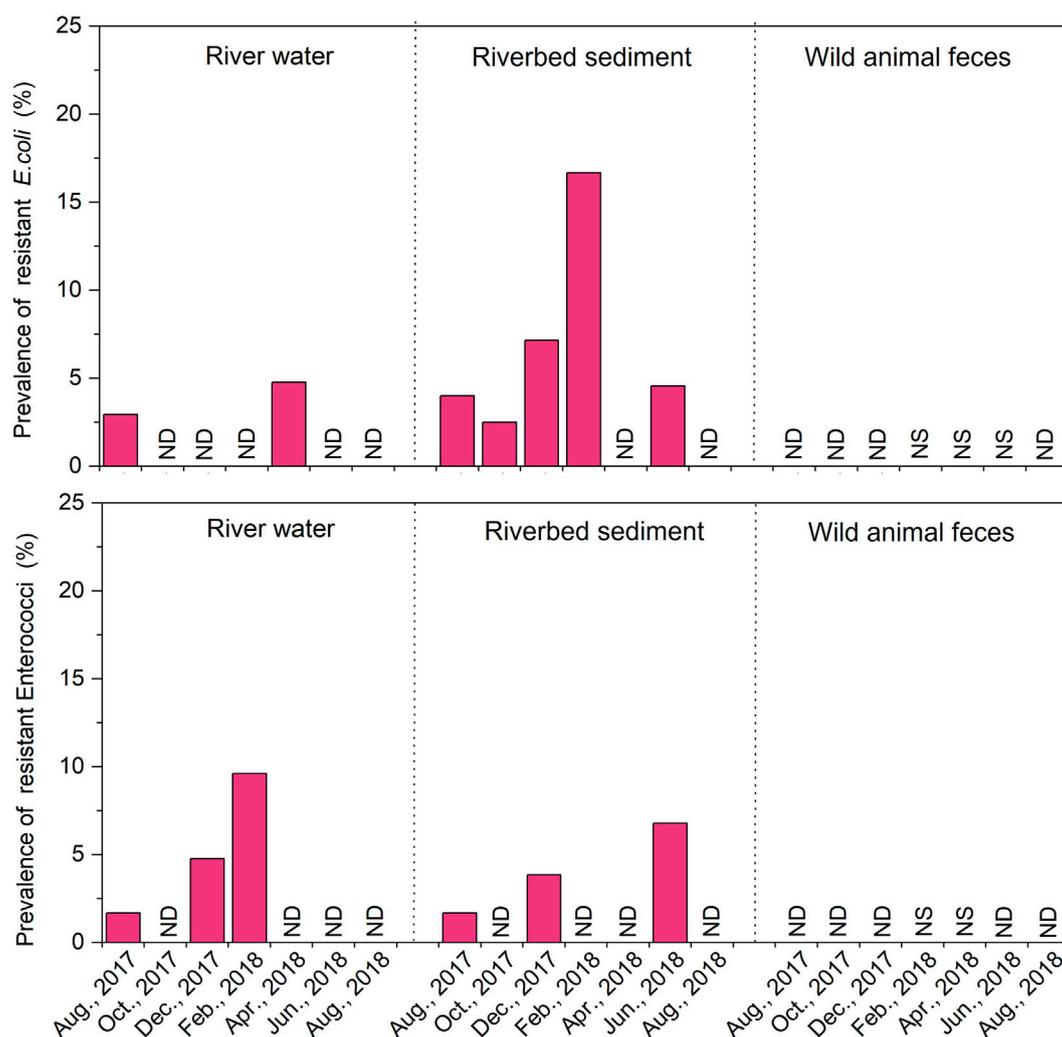
FIGURE 4
Changes in the prevalence of antibiotic resistant to one or more agents for *Escherichia coli* (*E. coli*) and enterococci strains isolated from the river water, riverbed sediment and wild animal feces samples over a 1-year period. No Date (ND), No Sample (NS).

resistomes may contribute to the antibiotic resistance of the bacteria in pristine environments. Thus, the occurrence of ARB in pristine rivers may need to consider both warm-blooded animal loading and natural occurrence (Allen et al., 2010; Hwengwere et al., 2022). Notably, ARB were detected in the uppermost pristine stream. When conducting surveys in such niche fields, it is important and prospects of this research to consider the occurrence of antibiotic resistance from multiple perspectives.

3.5 Characteristics of ARB

3.5.1 Resistance to various classes of antibiotics

Supplementary Table S5 shows the antibiotic resistance rates of *E. coli* isolated from all samples. Ampicillin (ABPC)-resistant strains (0.4%–1.7%), cefazolin (CEZ)-resistant strains (0.8%–1.7%), and oxytetracycline (OTC)-resistant strains (0.4%–1.2%) were detected in river water and riverbed sediment. The antibiotic resistance rates were similar between the two sample types.

Additionally, ciprofloxacin (CPFX)-resistant strains were detected in riverbed sediment.

Supplementary Table S6 shows the antibiotic resistance rates of enterococci isolated from all samples. CPFX-resistant strains (1.9%; 7/371) were detected in river water. OTC-resistant (2.0%; 6/300) and minocycline-resistant (2.0%; 6/300) strains were detected in riverbed sediment. Among the AR-ENT detected, antibiotic-resistant *E. faecium*, an important enterococci species in clinical institutions, was not observed.

Notably, *E. coli* and enterococci resistant to semisynthetic or synthetic antibiotics were detected in each sample. Bacteria resistant to semisynthetic or synthetic antibiotics have been detected in pristine environments (Lenart-Boroń et al., 2022; Sajjad et al., 2023). This result confirms similar trends to previous studies.

3.5.2 Comparison of antibiotic-resistant profiles

Tables 1, 2 show the antibiotic resistance profiles of AR-*E. coli* and AR-ENT. In this study, strains resistant to ≥3 antibiotics were defined as multidrug-resistant strains. The resistance combinations

TABLE 1 Profiles of antibiotic-resistant *Escherichia coli* (AR-*E. coli*).

Antibiotic resistance profiles	Number of AR-*E. coli*		
	River water (n = 246)	Riverbed sediment (n = 172)	Animal feces (n = 67)
ABPC-CEZ-CTX-OTC	1 (0.4%)	—	—
ABPC-CPFX-OTC	—	1 (0.6%)	—
ABPC-CEZ	—	2 (1.2%)	—
CEZ	1 (0.4%)	1 (0.6%)	—
OTC	—	1 (0.6%)	—

(ABPC), Ampicillin; (CEZ), Cefazolin; (CTX), Cefotaxime; (CPFX), Ciprofloxacin; (OTC), Oxytetracycline.

TABLE 2 Profiles of antibiotic-resistant enterococci (AR-ENT).

Antibiotic resistance profiles	Number of AR-ENT		
	River water (n = 370)	Riverbed sediment (n = 300)	Animal feces (n = 142)
OTC-MINO	—	6 (2.0%)	—
CPFX	7 (1.9%)	—	—

(OTC), Oxytetracycline; (MINO), Minocycline; (CPFX), Ciprofloxacin.

of the 494 *E. coli* strains were profiled and compared between sample types (Table 1), revealing 2 and 4 patterns in river water and riverbed sediment, respectively. The major resistance patterns of river water strains were ABPC-CEZ-CTX-OTC (0.4%, 1/246) and CEZ (0.4%, 1/246), and the presence of multidrug-resistant *E. coli* was confirmed. In addition, the presence of multidrug-resistant *E. coli* was confirmed in riverbed sediment (profile: ABPC-CPFX-OTC; 0.6%, 1/172). Multidrug-resistant strains of enterococci were not identified (Table 2). Notably, multidrug-resistant *E. coli* (profile: ABPC-CEZ-CTX-OTC) detected in river water may be an important ESBL-producing strain in clinical institutions. In recent years, the rate of dissemination of ESBL-producing bacteria has been accelerating worldwide, making the control of their spread a significant challenge (Kawamura et al., 2017; Husna et al., 2023). The presence of ESBL-producing genes encoded on Enterobacteriaceae plasmids deserves is an issue of particular concern (Pana and Zaoutis, 2018). These genes can spread and disseminate across bacterial species, raising concerns about transmission (Stadler et al., 2018). Globally, the presence of ESBL-producing bacteria and associated genes has been confirmed in pristine environments (Hernandez et al., 2012), raising concerns about their dissemination. The presence of ESBL-producing bacteria in the pristine headwaters of rivers is highly significant information, and the spread and transportation of ARB in river water or downstream processes must be avoided. Therefore, our future challenge is to elucidate the mechanisms of antibiotic resistance in bacteria isolated pristine environment and to accumulate information on the horizontal transmission of antibiotic resistance.

3.5.3 Genomic analysis of multidrug-resistant *E. coli* and ESBL-producing *E. coli*

Genomic analysis of ESBL-producing *E. coli* (1 strain) and multidrug-resistant *E. coli* (1 strain) isolated from the Kaeda River was used to estimate the possession of ARGs and hosts.

Table 3 shows the results of genomic analysis of ESBL-producing *E. coli* (ID:KS_R23, profile: ABPC-CEZ-CTX-OTC) isolated from river water and multidrug-resistant *E. coli* (ID: KS_S24, profile: ABPC-CPFX-OTC) isolated from riverbed sediment.

KS_R23 harbored CTX-M-15 and tetA. KS_S24 harbored TEM-214 and tetB, mutations in perC (A56T, S80I) and gyrA (D87N, S83L). CTX-M can break down a wide range of beta-lactam antibiotics, including penicillins and cephalosporins (Canton et al., 2012). The gene tet provides resistance to tetracycline antibiotics by encoding the efflux pumps that discharges the antibiotics out of the bacterial cell (Li and Nikaido, 2009). These were resistant genes and mutations that could explain the results of susceptibility testing. ARGs associated with antibiotic resistance to macrolide antibiotics (*mph*B) and polypeptide antibiotics [*pmr*B (Y358N)], which were not tested in this study, were detected from KS_R23. This strain may exhibit resistance to these two antibiotics. ARGs associated with antibiotic resistance to aminoglycoside antibiotics [*aph*(3")-Ib, *aph*(3')-La, *aph*(6)-Id, *str*A, *str*B]; macrolide antibiotics [*pmr*B (Y358N)]; and sulfonamide antibiotics (*sul*2) were detected from KS_S24. This strain may exhibit resistance to these three antibiotics. Among the detected genes, the difference between the resistance genes that could explain the results of susceptibility testing and other genes was considered to be ARGs originally possessed by the bacteria and ARGs acquired through horizontal spread via mobile genetic factors such as plasmids. KS_R23 and KS_S24 were classified into phylogroups B1 and A, respectively. *E. coli* isolated from wild animals was mainly classified into phylogroups A and B1 (Johnson et al., 2017; Cristovao et al., 2017; Haenni et al., 2020). Therefore, ESBL-producing *E. coli* and multidrug-resistant *E. coli* may be derived from wild animals. However, ARB were not detected in the feces of wild animals in this study. The only wild animals targeted in this study were bird and Mustelidae that excrete fecal matter on riverbanks. It is possible that other wild animals may have loaded ESBL-producing bacteria. It is

TABLE 3 The results of genomic analysis of ESBL-producing *Escherichia coli* (ID:KS_R23) isolated from the river water and multidrug-resistant *Escherichia coli* (ID:KS_S24) isolated from riverbed sediment.

Sample ID		KS_R23	KS_S24
Antibiotic resistance profiles		ABPC-CEZ-CTX-OTC	ABPC-CPFX-OTC
Antibiotic resistant gene	Aminoglycoside	—	*aph(3″)-Ib, aph(3′)-Ia, aph(6)-Id*, StrA, StrB
	B-lactam	CTX-M-15	TEM-214
	Fluoroquinolone	*qnrS1*	*parC* (A56T, S80I), *gyrA* (D87N, S83 L)
	Macrolide	*mphB*	*mphB*
	Polymyxin	*pmrB*(Y358 N)	—
	Sulfonamide		*sul2*
	Tetracycline	*tetA*	*tetB*
Serotype		-:H37	O89:H10
phylogroup		B1	A
ST		58	744

(ABPC), Ampicillin; (CEZ), Cefazolin; (CTX), Cefotaxime; (CPFX), Ciprofloxacin; (OTC), Oxytetracycline.
The names of bacterial species and genes.

FIGURE 5
Dendrogram analysis based on the similarity of enterococci PFGE types. Enterococci strains isolated from the river water, riverbed sediment and of bird feces at S2 site. Bird 1, 2, 4: Enterococci strains isolated from bird 1, 2, 4.

possible that fecal matters from wild boars and deer live in the catchment may have been input into the river as surface water via forest surface soils with rainfall. Therefore, future items to be considered are feces of other major wild animals and surface water flows into the river.

3.6 Comparison of genotypic similarities between strains isolated from river water and bird feces by PFGE analysis

Based on genomic analysis, there was a high commonality of antibiotic resistance between strains obtained from river sample and bird feces. Therefore, PFGE analysis was used to compare genotypic similarities between strains isolated from river samples and bird feces. In a survey conducted on 27 October 2017, *E. coli* strains (n = 30) were randomly selected from river water, riverbed sediment, and bird feces collected at S2. A dendrogram based on the similarity of PFGE types of each *E. coli* strain is shown in Supplementary Figure S1. When the clusters were classified into groups with 100% similarity, PFGE types of *E. coli* were highly diverse, and no information on the source of the *E. coli* pollution. The 29 randomly selected enterococci strains from each sample were analyzed for the similarity of PFGE types for each strain (Figure 5). Three strains isolated from bird feces were excluded because PFGE band patterns could not be identified. When the clusters were classified into groups with 100% similarity, 4 clusters (A, B, C and D types) were identified, consisting of 2–4 strains each. Type A consisted of one strain isolated from river water and three strains isolated from bird feces strains. Thus, an identical enterococci clone was present in both the river water and bird feces. The results of enterococcal PFGE analysis suggest that bird feces is one of the sources of enterococci contamination of the pristine uppermost stream of the Kaeda River. In addition, the number of target strains needs to be increased to increase the significance of the PFGE analysis. This is an issue to be addressed in the future.

4 Conclusion

AR-*E. coli* and AR-ENT were investigated in river water, riverbed sediment, and feces of waterside animals, such as birds and Mustelidae, at pristine uppermost sites throughout the year. The resistance rates of *E. coli* and enterococci fluctuated at low levels (*E. coli*: 2.9%–17%, enterococci: 1.7%–9.6%). ESBL-producing *E. coli* (0.4%) with multidrug resistance (0.6%) were detected among the AR-*E. coli* detected in the river. Notably, multidrug-resistant bacteria and clinically important ESBL-producing bacteria were found in the pristine environment. This shows that rapid elucidation of the routes of ARB dissemination is important. Although we could not determine the sources of the detected ARB in this study, birds were one of the sources of bacterial contamination of the pristine uppermost stream of the Kaeda River. To elucidate the sources of ARB in pristine natural environments, conducting investigations from a multifaceted perspective (e.g., large wild animals, production by plants, climate change) and not limited to the influence of birds is necessary (Torres et al., 2020; Caliz et al., 2022).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

EN: Data curation, Investigation, Writing–original draft, Writing–review and editing. HX: Validation, Writing–original draft. ST: Validation, Writing–original draft. MN: Investigation, Writing–original draft. KN: Validation, Writing–original draft. YH: Investigation, Writing–original draft. YO: Investigation, Writing–original draft. YS: Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Writing–original draft, Writing–review and editing.

Conflict of interest

Author EN was employed by IDEA Consultants, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2024.1439174/full#supplementary-material

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