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Characterization of microbiome, resistome, mobilome, and virulome in anoxic and oxic wastewater treatment processes in Slovakia and Taiwan

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ABSTRACT

This study presents a comprehensive analysis of samples from urban wastewater treatment plants using anoxic/oxic processes in Slovakia and Taiwan, focusing on microbiome, resistome, mobilome, and virulome, which were analyzed using a shotgun metagenomic approach. Distinct characteristics were observed; in Taiwan, a higher abundance and diversity of antibiotic resistance genes were found in both influent and effluent samples, while there was a higher prevalence of mobile genetic elements and virulence factor genes in Slovakia. Variations were noted in microbial community structures; influent samples in Taiwan were reflected from fecal and hospital sources, and those in Slovakia were derived from environmental elements. At the genus level, the samples from Taiwan's sewage treatment plants were dominated by *Cloacibacterium* and *Bacteroides*, while *Acinetobacter* was predominant in samples from Slovakia. Despite similar antibiotic usage patterns, distinct wastewater characteristics and operational disparities influenced microbiome, resistome, mobilome, and virulome compositions, with limited reduction of most resistance genes by the studied anoxic/oxic processes. These findings underscore the importance of region-specific insights into microbial communities for understanding the dynamics of antimicrobial resistance and pathogenicity in urban wastewater treatment systems. Such insights may lay the groundwork for optimizing treatment processes and reducing the dissemination of antibiotic resistance and pathogenicity genes for safeguarding public health.

1. Introduction

Antimicrobial resistance (AMR), which is the ability of bacteria to resist antimicrobial agents, poses a substantial health threat globally, leading to increased morbidity and mortality rates and health-care expenses and reduced availability of effective antibiotics [1]. Recognizing the emergence and complex interplay of AMR across various media, — including humans, animals, plants, and the environment (e.g., soil, water, and air) — the World Health Organization proposed the One Health initiative. This initiative emphasizes the urgent need for comprehensive surveillance through internationally collaborative efforts, and highlights that proactive measures are essential to effectively address the increasing dissemination of AMR [2]. The increasing prevalence of AMR is exacerbated by the

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horizontal gene transfer of antibiotic resistance genes (ARGs) through mobile genetic elements (MGEs), a primary driver of the widespread dissemination of antibiotic resistance. MGEs can also transmit a wide array of virulence factor genes (VFGs), which can convert nonpathogenic bacteria into pathogens. Horizontal gene transfer is influenced by the presence of residual antibiotics in the local environment, which creates selective pressure, and interconnected physiochemical and biological conditions [3]. Analyses of the resistome, virulome, and mobilome provide valuable information on the composition and abundance of ARGs, VFGs, and MGEs, respectively, which can be used to assess the antibiotic resistance and pathogenicity potential of the environment.

Human and animal fecal waste harbor substantial amounts of antibiotic-resistant microorganisms [4,5], contributing to the development of antibiotic resistance. The application of human and animal waste as fertilizers results in the dispersion of AMR-carrying antibiotic-resistant bacteria into soil, groundwater, and crops [6,7]. Urban sewage, containing fecal waste with urban runoff, is directly released into aquatic environments or undergoes treatment in wastewater treatment plants (WWTPs) [8]. Urban WWTPs have become crucial reservoirs of the resistome and of antibiotic-resistant bacteria and facilitate the widespread dissemination of these bacteria [4,9]. Understanding the relationship and connectivity between resistomes of different origins is complicated by the complex interplay between various pollution sources [10], posing challenges in the implementation of effective mitigation strategies. WWTPs serve as hotspots for transmission, providing conditions conducive to the horizontal gene transfer of ARGs and facilitating the proliferation of antibiotic-resistant bacteria [9]. Effluent from WWTPs facilitates the transport of ARGs and antibiotic-resistant bacteria downstream, posing potential risks to aquatic ecosystems, sources of drinking water, and human health [4]. The complexities involved highlight the need for comprehensive strategies to prevent the transmission of AMR in wastewater and its potential effects on public health and the environment.

The abundance and distribution of specific ARGs are diverse across various ecological environments. For example, *bla*TEM and *qnrS* are frequently found in urban sewage treatment plants [11] and aquatic ecosystems [12], respectively. Several ARGs, such as *aac(6′)-Ib* and *vanA*, are abundant in hospital settings [13,14]. Furthermore, a high abundance of *aadA* is detected in farmland soil [15]. These findings collectively indicate the widespread distribution of ARGs across diverse environments; with their prevalence and persistence, they can serve as potential markers. Studies have reported that tetracycline resistance genes are more prevalent in WWTPs in Asia [16, 17] and that beta-lactam resistance genes are more commonly found in WWTPs in Europe [18]. The global surveys of WWTP resistomes have revealed common characteristics, notably the ubiquitous presence of multidrug resistance genes [19]. These genes confer resistance against a broad spectrum of antibiotics, indicating the pervasive occurrence of resistance in these treatment facilities.

Virulence factors play a crucial role in the colonization of hosts by microorganisms and subsequent disease mechanisms. Recent research has demonstrated the increased expression of VFGs in bacterial strains containing resistance genes [20]. These VFGs encode virulence factors that contribute to the microorganism's ability to infect host cells and induce diseases. Elevated VFG levels facilitate the transfer of MGEs containing these factors, potentially converting nonpathogenic recipient cells into pathogenic cells. Prior virulome-related research on environmental samples has concentrated on drinking water, natural aquatic environments, or specific bacterial strains, with minimal attention to WWTP effluents [21]. Previous studies have found that the concentration and abundance of ARGs, VFGs, and bacteria carrying these genes in rivers increased significantly after the discharge of sewage treatment plant effluents [20,22]. This finding highlights the potential environmental consequences of wastewater discharge and indicates the importance of determining the mechanisms by which hazardous substances are disseminated in natural ecosystems. WWTPs treat wastewater from diverse sources, such as hospitals, residences, and farms, but they have limited capabilities to eliminate ARGs and virulence factors. Many studies have consistently reported that the discharge of sewage treatment plant effluent significantly increases the detection frequency and abundance of ARGs and virulence factors in downstream rivers [20,23,24]. Therefore, exploring the complex associations between ARGs and virulence factors in effluent from WWTPs is essential.

Given the global issue of AMR and pathogenicity, substantial regional disparities should be acknowledged [4]. Disparities are attributed to antibiotic usage patterns, governance protocols, geographical location, environmental conditions, and sewage treatment methods [25]. Research also illustrates significant distinctions between influent and effluent from WWTPs. Influent wastewater typically contains elevated concentrations of resistance genes and pathogenicity factors from diverse sources such as households and health-care facilities [26,27]. Following sewage treatment, the levels of these risky genetic elements in effluent are typically reduced yet still detectable [26], posing potential risks to the environment and public health.

In WWTPs, activated sludge systems are commonly used to eliminate organic pollutants from wastewater. In past decades, the functions of WWTPs in many countries have been notably upgraded, with a focus on improving effluent quality by incorporating nitrogen removal processes. Several nitrification and denitrification-based methods designed for the simultaneous removal of organic carbon and nitrogenous pollutants typically involve cyclic anoxic and oxic (A/O) stages with unique flow, redox, and nutritional conditions. This distinct setup results in a unique microbiome structure and dynamics, influencing the dissemination of antibiotic resistance and pathogenicity, unlike the standard activated sludge treatment, which only involves an aerobic stage. Although recent studies have examined ARG diversity and spatiotemporal variations in activated sludge systems [28,29], our understanding of the resistome, virulome, and mobilome in WWTPs with COD and nutrient removal capabilities remains limited. Notably, a study comparing global antibiotic usage revealed similar daily doses per 1000 inhabitants per day in Taiwan and Slovakia [30]. However, the lack of an effective action plan for monitoring the spread of transmissible genetic material in the environment leads to an incomplete understanding of resistome, virulome, and mobilome in WWTPs. This study fills this knowledge gap by using the shotgun metagenomic approach to explore transmittable genes before and after the A/O processes in urban WWTPs in Taiwan and Slovakia, encompassing a total of six urban wastewater sources. Compared with traditional culture-dependent or targeted sequencing approaches, shotgun metagenomic analysis offers an impartial and rapid means of obtaining comprehensive genomic information from environmental samples. This method allows for an extensive investigation of microbial communities, functional potentials, and genetic risks present in the WWTP samples [15,31–33]. Using shotgun metagenomic, this study highlights the significance of regional

disparities in AMR and pathogenicity.

2. Materials and methods

2.1. Study area and sample collection

We selected urban WWTPs with A/O-based microbial treatment of carbonaceous and nitrogenous pollutants in different regions of Taiwan: Northern (ZN), Central (WS), and Southern (FS). A total of 12 samples were collected in July 2021 and March–April 2022: precisely six samples each of influent sewage and treated effluent. In Slovakia, we conducted sampling at urban WWTPs in Liptovský Mikuláš (SVLI), Kysucké Nové Mesto (SVKY), and Komárno (SVKO) in June or September 2021; these samples could be used to observe regional changes in the diversity and richness of the resistome. Table S1 provides specific informative details of different sewage treatment plants by geographic location, as well as of secondary treatment processes, water quality parameters, and possible origins of wastewater. The influent and effluent samples were collected at the points before and after the microbial treatment facilities. Grab samples of 500 mL were taken four times using a stainless-steel sampling device with a time interval of 5–10 min and then poured into 2-L sterile plastic containers and transported under refrigerated conditions at 4 °C to maintain their integrity. To minimize interference from debris, prefiltering was conducted through a 150-μm membrane, followed by filtration through a 0.22-μm sterilized mixed cellulose ester membrane (ME 24/21 ST, Diameter 47 mm, Whatman, USA) using a Millipore filtration apparatus (Merck Millipore, USA). This standardized filtration protocol was consistently applied at both sampling locations in Taiwan and Slovakia. The filter membranes were freeze-dried and stored at −20 °C until further processing. The Slovakian samples were shipped to Tainan, Taiwan by air express for downstream analysis.

2.2. DNA extraction and quantitative polymerase chain reaction analysis

Samples of influent and effluent underwent bead-based DNA extraction with DNeasy Powersoil Pro Kits (Qiagen, Carlsbad, CA, USA). DNA was purified and eluted following the manufacturer's instructions, with all procedures performed by the same well-trained technician to avoid experimental bias during the DNA extraction step. The quality of the final DNA extract was spectrometrically verified, and the DNA extract was stored at −20 °C until analysis. Quantitative polymerase chain reaction (qPCR) was used to quantify specific MGEs, class 1 integron gene (*intI1*), and ARGs, including beta-lactam (*bla*TEM), sulfonamide (*sul1*), tetracycline (*tetM*), and macrolide (*ermB*), as well as the 16S rRNA gene of the total bacteria. qPCR was performed with triplicate tubes per sample on a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA), and the primer sets and operational annealing temperatures in Table S2 were applied. This resulted in copy number data in terms of average and standard deviation for each gene target in a sample. The copy numbers of targeted ARGs and *intI1* genes were normalized to the 16S rRNA genes of the total bacteria to determine their relative abundances. Reference DNA fragments were prepared for qPCR as described previously [34].

2.3. Metagenomic sequencing and microbiome analysis

Genomic DNA was sequenced on an Illumina HiSeq platform with a 150-bp paired-end kit. Raw reads were filtered using FastQC (v0.11.2) to ensure sequencing quality and trimmed using Trimmomatic (v0.39) with default settings to remove adapter sequences [35]. The cleaned sequence data were de novo assembled using MEGAHIT (v1.2.9) [36] with default parameters to obtain reads over 500 bp. The prokaryotic16S rRNA gene per sample was estimated using ARGs_OAP (v3.2) [37,38]. For all datasets, the taxonomic profiling of the reads was conducted using MetaPhlAn3 with the very-sensitive algorithm of Bowtie2 [39].

2.4. Analysis of resistome, virulome, and mobilome

The open reading frame was predicted using prodigal [40]. The predicted sequences were then compared against the comprehensive antibiotic resistance database (CARD) by using the RGI with "perfect and strict hits only" to predict ARGs [41]. The predicted ARGs were divided into classes based on the antibiotic type to which they confer resistance. The ARGs universally present across all the samples were referred to as the core resistome. The ARG alpha-diversity metrics, including Sobs, Shannon, and Chao1, were analyzed using Past4 software. VFGs were identified by querying the nucleotide sequences of open reading frames against the Virulence Factor Database (VFDB, http://www.mgc.ac.cn/VFs/) [42]. Alignment was performed using DIAMOND [43] with the criteria of identity >80 %, coverage >70 %, and *e* value $< 10^{-5}$ [22,42]. To further classify MGEs, a database of bacterial MGE hallmark genes, namely mobileOG-db [44], was used to assign element class labels of transposable elements (defined as sequences derived from ISfinder [45]), integrative elements (integrases and transposases that are not in ISfinder and that do not encode conjugation machinery), and conjugative elements (reads with hits to conjugation machinery). Reads were annotated using DIAMOND [43] with the criteria of identity >90 %, coverage >90 %, and *e* value $< 10^{-5}$. The METAXA2 tool [46] was used to detect the abundance of 16S rRNA gene-related reads, which were subsequently used to normalize the relative concentrations of ARGs, VFGs, and MGEs in each sample.

2.5. Statistical analysis

To assess dissimilarity between microbial community groups and resistomes across various sites, we employed nonmetric multidimensional scaling (NMDS) with the Bray–Curtis distance. To evaluate the influence of geographical location and treatment status on

microbial community structure, we performed a two-way analysis of similarities (ANOSIM). The ANOSIM was conducted with 9999 permutations, using location (Taiwan vs. Slovakia) and treatment status (influent vs. effluent) as factors. The NMDS, Student's *t-test*, and ANOSIM were performed using the Past4 software. Furthermore, STAMP (v4.13) [47] was used to conduct differential abundance analysis and generate heatmaps for ARGs and ARG classes in effluent and influent samples.

3. Results and discussion

3.1. Differences in microbiome composition

The compositions of microbial communities can provide considerable insights into the dissemination of genetic materials. In this study, we first examined the composition in microbial communities between Taiwan and Slovakia by detecting 666–2112 16S rRNA gene sequences using metagenomic sequencing. The NMDS analysis with the Bray–Curtis distance revealed that in WWTPs in Slovakia, microbial communities in effluent samples exhibited greater similarity to their influent counterparts. Conversely, in Taiwan, influent samples exhibited a distant distribution in the ordination space but displayed a closer proximity in the microbial composition compared with their effluent counterparts (Fig. S1). To quantitatively assess the influence of geographical location and wastewater state (influent vs. effluent) on microbial community structure, we performed a two-way ANOSIM analysis. The results revealed significant effects of both factors (location: $R = 0.238$, $p = 0.0038$; influent/effluent: $R = 0.759$, $p = 0.0002$; permutations = 9999), with the influent/effluent factor exerting a stronger influence on community structure than geographical location. Taxonomic analysis assigned the detected microbial communities to eight phyla and revealed notable distinctions in the microbial community structures of influent samples (Fig. S2). At the phylum level, Bacteroidota was the most abundant (28.6%–62.8 % of the sequences) in the influent samples of WWTPs in Taiwan, followed by Pseudomonadota and Bacillota (up to 22.1 % ± 21.5 % and 21.3 % ± 8.9 %, respectively). The microbial community structure in Slovakia was slightly different and was dominated by Pseudomonadota (abundance ranging from 53.7 % to 91.0 %), Actinomycetota (11.3 % ± 7.9 %), and Bacillota (3.7 % ± 3.6 %). Specifically, at the genus level, *Cloacibacterium* (17.2%–39.2 %) and *Bacteroides* (5.8%–24.1 %) were dominant in influent samples from WWTPs in Taiwan, whereas *Acinetobacter* (18.5%–82.3 %) was dominant in influent samples in Slovakia. This divergence indicates that the microbial composition identified in influent samples is a critical indicator, reflecting the distinct domestic wastewater sources in each region (Table S1). Notably, the high prevalence of Pseudomonadota in Slovakia suggests that the microbiota in urban wastewater might be derived from environmental elements, such as vegetables, fruits, and soil [48]. By contrast, sewage wastewater in Taiwan had a higher abundance of Bacteroidota and Actinomycetota, which reflect contributions from fecal samples, and hospital effluents [48,49], respectively. The observed differences in microbial compositions indicate regional variations in the sources and constituents of domestic wastewater, which may be attributed to factors such as lifestyle, geographical characteristics, and operational disparities of sewer systems between Taiwan and Slovakia [50–52].

While A/O-based processes constitute the core treatment mechanism, the microbial diversity of effluent samples from Taiwan was higher than that of samples from Slovakia. In addition, effluent samples had distinct microbial compositions. Bacteroidota was the most abundant in the effluent samples in all WWTPs in Taiwan. The phylum Bacillota was predominant in the ZN_E_2022 (7.8 %), WS_E_2021 (11.0 %), and WS_E_2022 (48.4 %) effluent samples, and Actinomycetota was dominant only in the ZN_E_2022 (65.9 %) and WS_E_2021 (46.8 %) effluent samples. Pseudomonadota was dominant in the microbial communities in effluent samples in Slovakia, accounting for more than 91 % of the total microbial community. Variations in the types and concentrations of pollutants, including industrial discharge or agricultural runoff, and operational practices of the treatment facilities influence the composition of microbial communities in biological treatment systems [53]. Even in a single WWTP, such as the WS site in Taiwan, dissimilarities were found for effluent samples, with the detection of additional abundant genera including *Bifidobacterium*, *Collinsella*, *Trichococcus*, *Ruminococcus*, *Prevotella*, and *Arcobacter*. This finding revealed the marked variations in microbial communities in effluent, raising concerns regarding their environmental and health consequences. Specifically, these differences indicate disparities in the wastewater treatment processes and their operation and performance. While wastewater state was acknowledged to contribute to variability

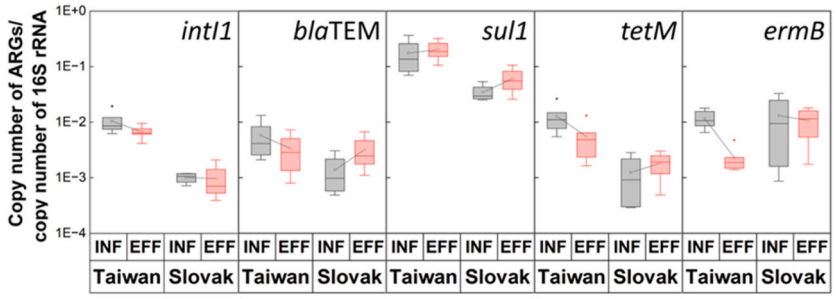

Fig. 1. Relative concentrations of ARGs (*bla*TEM, *sul1*, *tetM*, and *ermB*) and MGE (*intI1*) in influent and effluent samples from WWTPs in Slovakia and Taiwan. Relative concentrations are expressed as gene copies per 16S rRNA gene. Copy numbers of genes were determined using quantitative polymerase chain reaction analysis.

significantly, we cannot directly correlate water quality parameters with the microbial community structure due to the insufficient sample size and effluent data collection in the current study.

3.2. Quantification of specific ARGs and MGEs using qPCR

According to the annual drug usage reports from the Taiwan National Health Insurance Administration [https://www.nhi.gov.tw/ch/cp-2297-94173-2514-1.html], the most prescribed antibiotics from 2014 to 2021 were beta-lactams, followed by tetracyclines, macrolides, and sulfonamides. This usage pattern is similar to that in Europe [54,55]. To investigate the load of ARGs and MGEs in samples, we analyzed four common ARGs in WWTPs, namely *bla*TEM for beta-lactams, *tetM* for tetracyclines, *sul1* for sulfonamide, and *ermB* for macrolides, and the class 1 integron (*intl1*) gene by using qPCR. To facilitate a standardized evaluation of ARG abundance, the relative concentrations were obtained by normalizing the ARG copies to the 16S rRNA genes of the total bacteria (Fig. 1). Although the samples were characterized by temperature variations of around 10 °C, the results of our qPCR data revealed that temperature variations of samples did not result in statistically significant differences in the abundance of these five genes in samples from Taiwan. The sulfonamide-resisting *sul1*, with a level of approximately 10^{-1} ARG copies per 16S rRNA gene, had the highest relative concentration. The *sul1* concentrations in the influent and effluent from WWTPs in Taiwan were, on average, 1.75–3.44 times higher than those in Slovakia. The notable variation in the prevalence of *sul1* denotes potential differences in ARG concentrations between Slovakia and Taiwan. Notably, *sul1* concentrations were consistently approximately 1–2 orders of magnitude higher than other genes, ranging between 10^{-2} and 10^{-3} ARG copies per 16S rRNA gene; this finding implies the significant prevalence of *sul1* at the WWTP sites. High *sul1* levels were also reported in WWTPs in Beijing [56] and Europe [57–60], which levels were 3–4 orders of magnitude higher than in North American countries [61,62]. The *sul1* has been reported as a genetic marker of sites of anthropogenic activities, such as WWTPs [63]. The high prevalence may be attributed to its long-term utilization for both human and livestock populations, its association with MGEs [64], and the persistence of sulfonamide residues in wastewater [65]. Notably, although beta-lactam antibiotics are considerably prescribed and are easily degradable in aqueous environments [66], the resistance potential to *bla*TEM appeared to be comparable to *tetM* and *ermB* in this study.

The relative concentrations of the MGE *intl1*, a primary carrier of ARGs commonly found in WWTPs, were notably higher in Taiwan than in Slovakia. Additionally, the relative concentrations of *sul1*, *bla*TEM, and *tetM* in sewage samples in Taiwan were higher than those in Slovakia, although the result was not statistically significant ($p > 0.05$). The ARG concentrations in Taiwan in our study were very similar to the patterns observed in previous studies in Europe [23]. Although comparable levels of the monitored ARGs were detected in the effluent samples, the results revealed the potentially higher mobility and loads of ARGs in sewer systems in Taiwan. We observed slightly lower concentrations of *intl1*, *bla*TEM, *tetM*, and *ermB* in effluent samples than in influent samples in Taiwan, although these differences were nonsignificant ($p > 0.05$), with an exception for the *ermB* case ($p = 0.002$). The lack of significant changes in ARG levels implies that A/O treatment processes do not substantially reduce ARG levels [67]. In addition, the temperature variation confounding with other factors, such as geographic location, wastewater characteristics, and the operation of the microbial treatment process, can collaboratively influence the microbiome structure and dynamics, making it difficult to associate ARG profiles and environmental factors in this study.

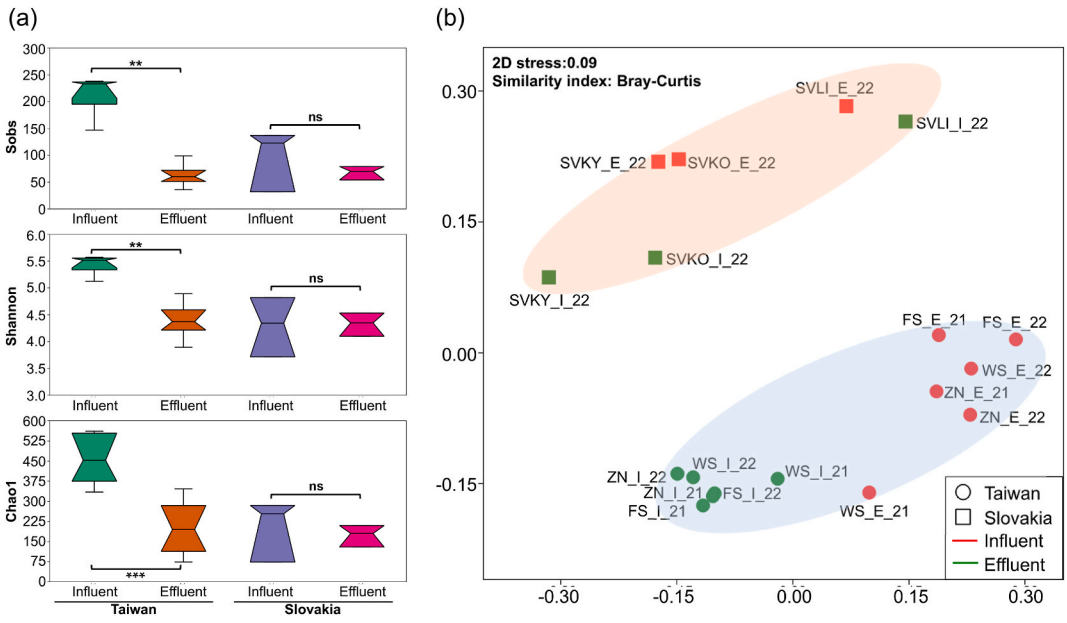

Fig. 2. (a) Analysis of alpha-diversity metrics for ARGs, including Sobs (species observed), Shannon, and Chao1 indices, and (b) nonmetric multidimensional scaling ordination for ARGs with Bray–Curtis dissimilarity matrices.

3.3. Diversity of ARGs

Metagenomic sequencing detected 32–238 and 36–99 types of ARGs in influent and effluent samples per site, respectively. WWTP sites in Taiwan exhibited more ARG types, with 147–238 and 36–99 types in influent and effluent, respectively, than did the sites in Slovakia (32–137 and 54–70 in influent and effluent, respectively) (Table S3). Presumably, sewage wastewater in Taiwan may contain a broader spectrum of organic compounds, likely resulting from industrial emissions or a high density of hospitals and clinics and favoring the proliferation of diverse ARGs with the evolution of resistance mechanisms of microorganisms [68].

To further examine the diversity of ARGs, we analyzed the Sobs, Shannon, and Chao1 indices. Significant differences were found in the three alpha-diversity measures between influent and effluent samples in Taiwan ($p < 0.001$); this result suggests that the A/O treatment systems in Taiwan significantly reduced ARG diversity. However, a reduction was not observed for sites in Slovakia (Fig. 2 (a)). In contrast to minimal differences between influent and effluent samples from WWTPs in Slovakia, the substantial reduction in ARG diversity between influent and effluent samples from WWTPs in Taiwan was noteworthy. Although wastewater treatment processes are similar, the characteristics of influent wastewater and possibly operational conditions [59,69], likely played a key role in contributing to the observed differences in ARG diversity between Taiwan and Slovakia. Furthermore, Fig. 2(b) illustrates the results of the NMDS analysis with the Bray–Curtis distance for dissimilarity matrices of the detected ARGs. On the basis of the results, samples were clustered into distinct groups corresponding to Taiwan and Slovakia. This clustering highlights the substantial differences in the distribution of ARGs between Slovakia and Taiwan. Specifically, in Taiwan, the NMDS analysis results even discerned ARGs between influent and effluent samples, except for the effluent sample WS_E_21, which indicates a compositional difference in inlet and outlet ARGs. Similar to substantial variations in alpha-diversity (Fig. 2(a)), the distribution of ARGs in Slovakia exhibited a notable pattern, in which influent samples were distributed at a distance but close to their corresponding effluent counterparts in the ordination space. This observation suggests, to some extent, a consistent ARG composition throughout the treatment process. Given the significant reduction in ARG diversity in WWTPs in Taiwan (Fig. 2(a)), future studies should investigate specific treatment strategies, influent organic profiles, and environmental and operational conditions. A comprehensive understanding of these factors is valuable for optimizing wastewater treatment processes to develop targeted interventions for mitigating antibiotic resistance in the context of wastewater treatment.

3.4. Ranking of ARG abundance in terms of category

To further elucidate the variations in ARGs, we examined their relative concentrations and normalized the ARG reads to 16S rRNA

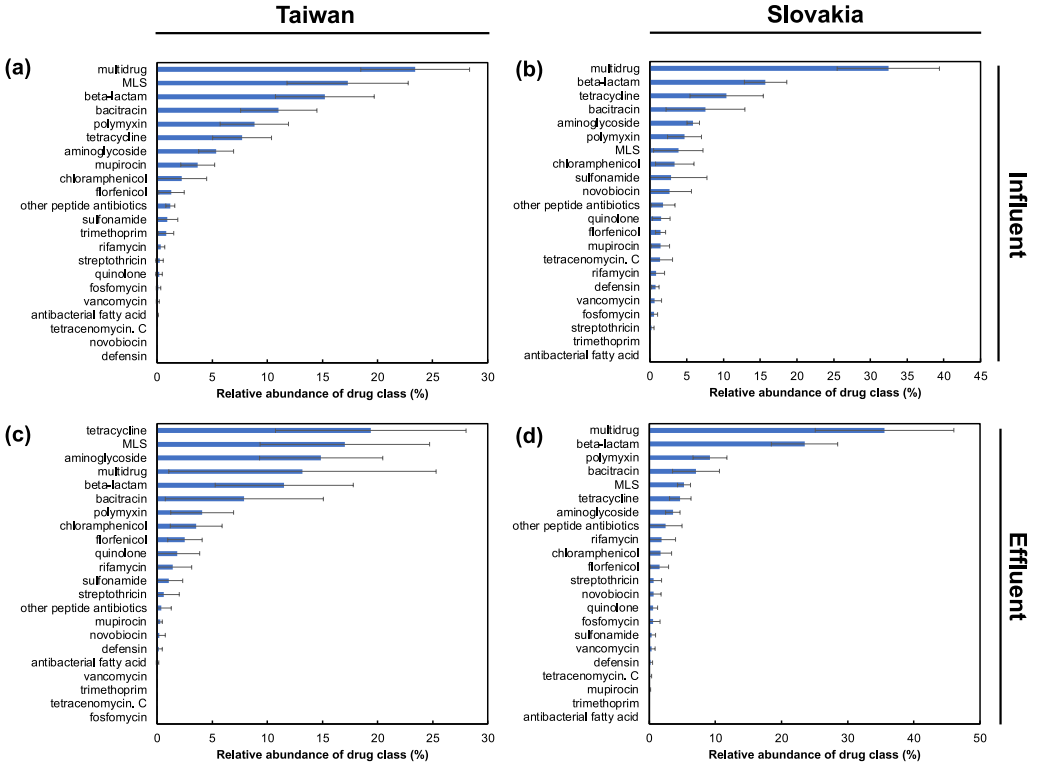

Fig. 3. Abundance ranking of ARGs in drug classes for influent samples from WWTPs in (a) Taiwan and (b) Slovakia and effluent samples from WWTPs in (c) Taiwan and (d) Slovakia. Bar and error represent average and standard variation of relative concentrations, respectively.

gene read counts. The data were presented as the proportion of drug classes to which ARGs belong for each sample. The shotgun metagenomic results revealed that influent from WWTPs in Taiwan had the highest content of multiple drugs (23.4 %), followed by macrolide-lincosamide-streptogramin (MLS), beta-lactam, bacitracin, polymyxin, tetracycline, and aminoglycosides, each with an abundance of >5 % (Fig. 3 (a)). The distribution of the resistance potential to the drug classes was consistent with the high usage patterns of beta-lactam, MLS, and tetracyclines in Taiwan. The distribution profile for influent in Slovakia was slightly different. The highest content was found for multiple drugs (32.5 %) in Slovakia, with abundance far higher than that in Taiwan, followed by beta-lactam, tetracycline, bacitracin, and aminoglycosides (Fig. 3 (b)). The considerable resistance potential in the multidrug, beta-lactam, and tetracycline categories was found in this study and in WWTPs globally [19], emphasizing their high prevalence. Nevertheless, notable disparities in the abundance of resistance genes in many drug classes, including aminoglycoside, beta-lactam, chloramphenicol, defensin, fosfomycin, multidrug, novobiocin, other peptide antibiotics, polymyxin, quinolone, rifamycin, and tetracycline, were found in urban wastewater in this study. These variations are likely attributable to distinct antibiotic usage patterns and potential sources of contamination.

In effluent samples derived from WWTPs in Taiwan, the relative abundance of resistance genes exhibited a descending order in drug classes as follows: tetracycline (19.4 %), MLS, aminoglycoside, multidrug, and beta-lactam (Fig. 3 (c)). With slight differences, the

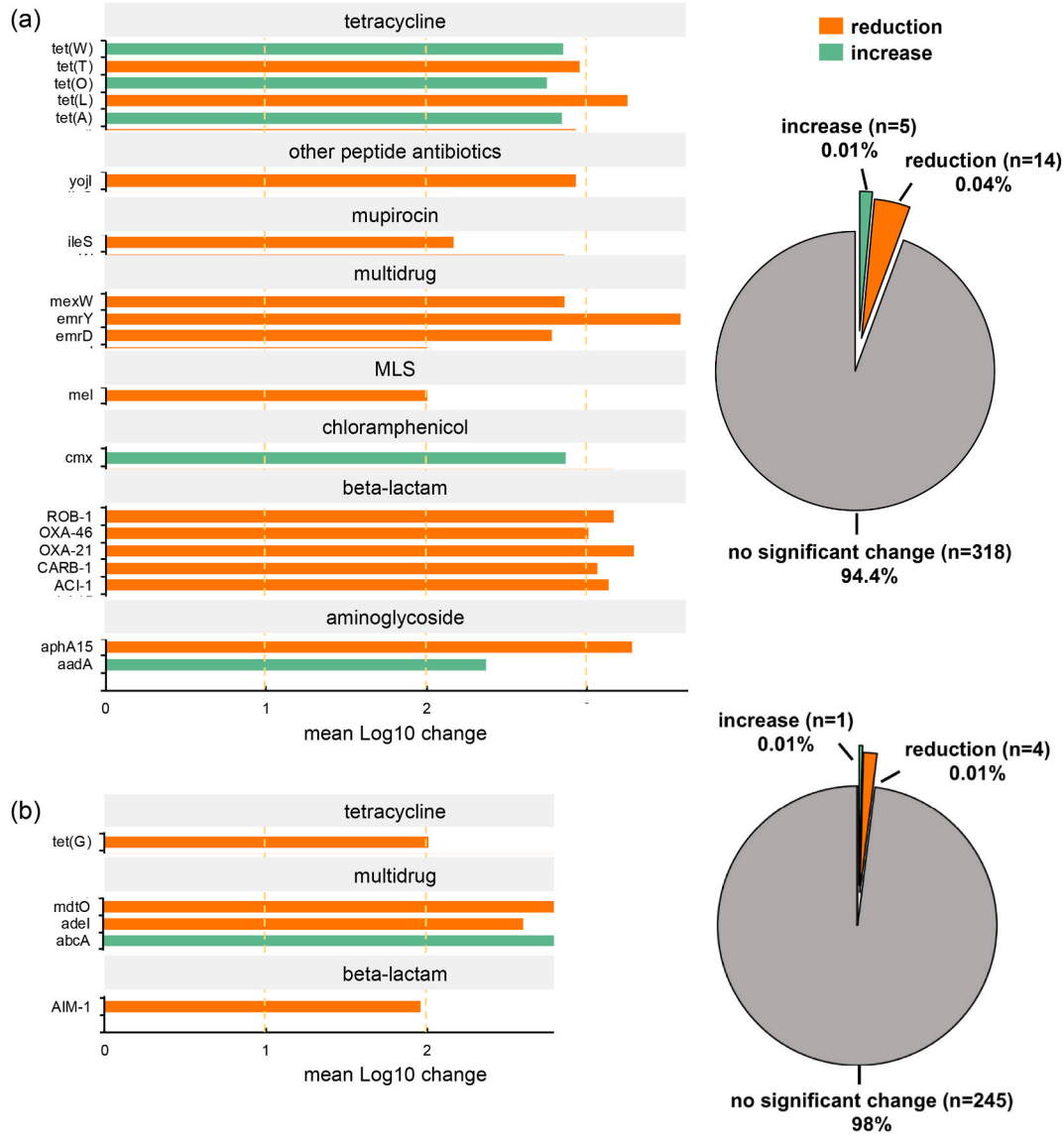

Fig. 4. In (a) Taiwan and (b) Slovakia, specific ARGs with significant changes in gene copy per 16S rRNA gene between influent and effluent ($p < 0.05$). ARGs are categorized based on their resistance to specific antibiotic classes. Bars represent significant differences in ARG copies per 16S rRNA gene (log-10 scale). Green bar distinguishes genes with increased proportions, and orange bar indicates genes with decreased proportions in effluent. Pie chart depicts proportion of ARGs with significantly changes relative to all ARGs detected.

abundance of resistance genes in the effluent from WWTPs in Slovakia in descending order in drug classes was as follows: multidrug (35.6 %), beta-lactam, polymyxins, bacitracin, and MLS (Fig. 3 (d)). In a Swiss study, the resistance genes in the drug categories of sulfonamide, beta-lactam, and aminoglycoside were prominent in effluent samples from WWTPs [70]. In effluent samples from WWTPs in Hong Kong, absolute quantification discerned temporal shifts in resistance groups, particularly revealing ARG types associated with frequently used antibiotics, such as quinolones and sulfonamides [71]. In effluent samples from WWTPs in Colombia, MLS and beta-lactam resistance–related genes were mainly identified, and these genes (e.g., *acrB*, *adeG*, and *mexD*) confer multidrug resistance [72]. These studies and our study reveal the distinct ARGs in different regions, indicating the complex and global nature of ARG dissemination and the need for a nuanced understanding of resistomes for tailoring effective mitigation strategies for each region.

3.5. Evaluation of ARG removal efficacy

Because the diversity and abundance of ARGs were reduced after A/O treatment, we next evaluated the removal of specific ARGs. The relative concentrations of ARGs in the corresponding drug categories varied from 10^{-4} to 10^{-1} ARG copies per 16S rRNA gene (Fig. S3). Differential analysis of influent and effluent samples from WWTPs in Taiwan revealed notable differences in the relative concentrations of resistance genes in these drug categories. Specifically, the relative concentrations of genes conferring resistance to mupirocin, polymyxin, and trimethoprim were significantly higher in influent samples than in effluent samples. Conversely, the relative concentrations of resistance genes conferring resistance to aminoglycoside and tetracycline were higher in effluent samples than in influent samples. Notably, in influent samples from WWTPs in Slovakia, the relative concentrations of genes conferring resistance to aminoglycoside significantly increased compared with effluent samples ($p < 0.05$).

Further analysis of relative concentrations revealed significant differences ($p < 0.05$) of 19 and 5 among the 337 and 250 unique ARGs discovered in WWTPs in Taiwan and Slovakia, respectively. Consequently, the overall effect of A/O treatment on ARG concentration in both countries was modest, affecting only 0.1 % of the total ARGs detected (Fig. 4 (a) and 4 (b)). The most substantial shifts were observed for genes conferring resistance to tetracycline (n = 5) and beta-lactams (n = 5), followed by genes conferring resistance to multidrug agents (n = 3), aminoglycoside antibiotics (n = 2), other peptide antibiotics (n = 1), MLS (n = 1), mupirocin (1), and chloramphenicol (n = 1). For WWTPs in Taiwan, the results indicated a significant reduction by > 2 orders of magnitude for 14 ARGs in effluent samples, and the concentrations of five ARGs increased (Fig. 4 (a)). Among these ARGs, the relative concentrations of *emrY*, *OXA-21*, *aphA15*, and the tetracycline efflux protein *tetL* exhibited the most substantial reduction (3.24–3.58 log10 reduction). Conversely, the relative concentrations of genes *cmx*, ribosomal protective protein *tetW*, tetracycline efflux pump *tetA*, ribosomal protection protein *tetO*, and *aadA* exhibited the most notable increases (2.37–2.86 log10 increase). In WWTPs in Slovakia, the relative concentrations of most ARGs remained constant. The relative concentrations of four ARGs and one ARG significantly decreased and increased, respectively, in effluent samples. On average, the relative concentrations of the multidrug resistance efflux pump *mdtO* exhibited the most substantial decrease (3.31 log10), followed by *adel* (2.60 log10 decrease), the tetracycline efflux protein *tetG* (2.02 log10 decrease), and *AIM-1* (1.96 log10 decrease). The multidrug-resistant ABC transporter *abcA* exhibited a significant increase by more than three orders of magnitude (Fig. 4 (b)).

The observed variations in the relative concentrations of ARG between influent and effluent from WWTPs reveal the complex dynamics in the removal of ARGs during wastewater treatment processes. These variations have implications for public health and the environment. The notable increase in the relative concentrations of specific ARGs, including *tetW*, *tetO*, *tetA*, *cmx*, *aadA,* and *abcA*, in effluent samples indicates the risk of the introduction of these resistance genes into aquatic ecosystems. This can lead to the persistence of these genes in environmental matrices, serving as reservoirs for their acquisition by bacteria [73]. Consequently, this may increase resistance to tetracycline, chloramphenicol, aminoglycoside, and multidrug agents, potentially exacerbating the already present challenge for clinical treatment and environmental management [74].

We further compared results from qPCR and metagenomic analyses. The sul1 gene had the highest relative abundance among the tested ARGs. For *tetM* and *bla*TEM, while exact values differed between methods, both indicated similar trends in relative abundance between influent and effluent samples within each location. However, *ermB* showed divergent patterns (Table S4). These observations highlight the complexity of ARG dynamics and the importance of using multiple methodologies. Using both qPCR and metagenomics provides a more comprehensive understanding of the resistome, as each technique offers unique insights: qPCR delivers high sensitivity for specific gene detection, while metagenomics offers a broader, untargeted overview of the microbial community and resistome. Our investigation results indicate that WWTPs in Taiwan exhibit higher resistance potential than those in Slovakia. This comprehensive resistome analysis provides insights into the complexities of antibiotic resistance dynamics in urban wastewater and in WWTPs, emphasizing the need for tailored mitigation strategies and for continual monitoring to prevent the environmental dissemination of antibiotic resistance.

3.6. Identification of core resistome and genetic context

We examined 28 ARGs in eight drug classes that were consistently detected across all sites, designating them as the core resistome. These common ARGs conferred resistance against antibiotics from eight classes, with multidrug, bacitracin, and tetracycline being the most abundant; their relative concentrations ranged from 0.05×10^{-3} to 1.4×10^{-1} ARG copies per 16S rRNA gene in urban wastewater. The total concentrations of the core ARGs in each sample ranged from 3.4×10^{-3} to 4.9×10^{-2} ARG copies per 16S rRNA gene (Fig. 5 (a)). Due to the selective pressure induced by the co-occurrence of antibiotic residues and ARGs, the emergence of novel antimicrobial determinants is possible [75]. Given the high prevalence of core ARGs in effluent samples, their regular dissemination into the environment raises significant public health concerns.

Examining the core resistance genes, the concentrations of *aadA*, which confers resistance to aminoglycoside, were the highest (Fig. 5 (b) and 5 (c)). In Taiwan, the concentrations of the *mexW* ($p < 0.05$), which confers multidrug resistance, and the *ileS* ($p < 0.001$), which confers resistance against mupirocin, were higher in effluent than in influent, whereas the concentration of *tetW*, which confers resistance against tetracycline, was higher in influent than in effluent ($p < 0.01$; Fig. 5 (b)). Notably, the *mexW* and *ileS* (and *ugd*) were specific to Taiwan. The *mexW*, as part of the MexVW-OprM efflux pump complex in *Pseudomonas aeruginosa*, plays a role in the bacterium's resistance to various antibiotics [76]. The gene *ileS* encodes isoleucyl-tRNA synthetase and confers low-level resistance to mupirocin, used to treat bacterial skin infections, is mediated by mutations in the native *ileS* [77]. In Slovakia, significant differences were observed in the relative concentrations of only *bacA* between influent and effluent samples ($p < 0.05$; Fig. 5 (c)).

To elaborate on the dissemination potential of effluent samples, we thoroughly examined the genomic locations (plasmid or chromosomal) of the core ARGs. Notably, significantly higher concentrations of the core ARGs on chromosomes that conferred

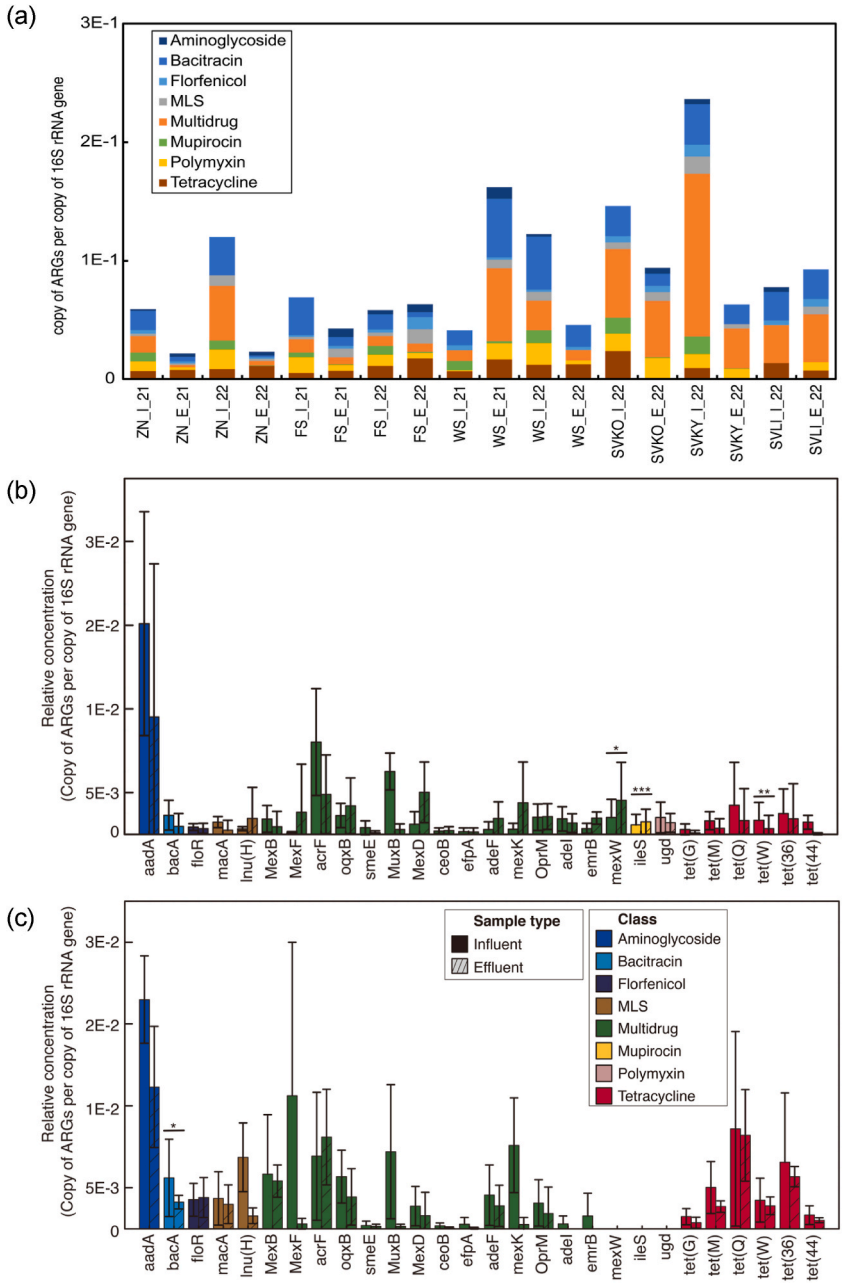

Fig. 5. Abundance of core ARGs across all sampling sites. (a) Relative concentrations of core ARGs categorized in corresponding drug classes. Relative concentrations of core ARGs in influent and effluent samples in (b) Taiwan and (c) Slovakia. Statistical significance was assessed using *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Bars and errors represent average values and standard deviations, respectively.

resistance to aminoglycosides, multidrug agents, and tetracycline were found in effluent from WWTPs in Taiwan ($p < 0.05$). Specifically, some core ARGs that conferred resistance to aminoglycoside, MLS, and tetracycline were located on chromosomes (Fig. S4). The predominant location of these core ARGs on chromosomes implies their low mobility, as noted in previous studies [78,79], and their frequent involvement in essential physiological processes in bacteria. Because very few multidrug genes are associated with plasmid transmission, this observation suggests that multidrug efflux pumps are evolutionarily ancient components encoded into bacterial species' chromosomes. Moreover, chromosomal ARGs, particularly those related to aminoglycosides, have been linked to the enhancement of the minimum inhibitory concentration (MIC) when compared with their plasmid counterparts; these chromosomal ARGs encoding drug-modifying enzymes have been observed to be transmissible among bacterial genera [80]. The potential persistence of these chromosomally located ARGs in the environment is further exacerbated by their higher MIC, and their involvement in efflux- or impermeable-mediated resistance renders them susceptible to mutation [81]. However, the mechanism of chromosomal ARG transmission requires elucidation, considering the phenomenon involving the released cell-free DNA from chromosomes being taken up by neighboring bacteria [82]. Identifying core resistome elements and their genetic characteristics in effluent samples can provide insights into the persistence and potential environmental effects of ARGs. Further research is warranted for elucidating the mechanisms of the dissemination of chromosomally located ARGs and their implications for antibiotic resistance in natural ecosystems.

3.7. Characterization of the metal resistance genes

Given the co-occurrence of heavy metals and antibiotics in wastewater, we investigated the co-occurrence of metal resistance genes (MRGs) and ARGs. Metagenomic analysis detected 193 subtypes of MRGs with resistance to mercury, copper, iron, lead, and zinc. These MRG subtypes exhibited resistance to multiple heavy metals, indicating the environmental implications of heavy metals in wastewater. A notable difference was observed in the levels of cobalt and magnesium between influent samples from Slovakia and Taiwan. The concentrations of MRGs were notably low in effluent from WWTPs in Taiwan. In WWTPs in Slovakia, particularly the SVKO and SVKY sites, higher MRG concentrations related to cobalt, copper, gold, iron, magnesium, and manganese were detected in effluent samples than in influent samples (Fig. S5). Concurrently, substantial amounts of MRGs related to arsenic, copper, iron, nickel, and zinc were found in effluent samples, and their abundance remained unchanged in influent samples.

When a gene serves dual functions as both an ARG and MRG, coselective pressure or cross-selection is common, especially when such genes are located on the same genetic element, such as a plasmid. This phenomenon is observed in multidrug efflux pumps capable of expelling both metals and antibiotics [83]. In our study, specific MRGs in influent samples from WWTPs in Taiwan exhibited significant correlations with gold-related MRGs, but this trend was not observed in samples from Slovakia. These results suggest potential variability in the dynamics of metal and ARG interactions across the two regions studied. While previous studies have demonstrated a positive correlation between the relative concentration of total MRGs and total ARGs in wastewater on a global scale [84], others have indicated variation in the selectivity of different metals for ARGs [25,85]. Therefore, more region-specific studies are needed to gain a comprehensive understanding of the complex interaction between metals and ARGs in wastewater microbiomes.

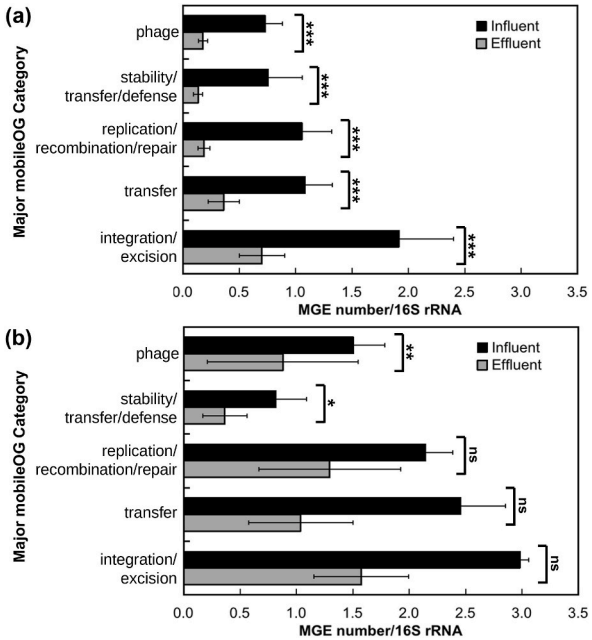

Fig. 6. Relative concentrations of MGEs in influent and effluent samples from WWTPs in (a) Taiwan and (b) Slovakia. Relative concentration is expressed as gene copy per 16S rRNA gene and categorized in major mobileOG groups. Bar and error represent average and standard deviations, respectively. p values are annotated as follows: ns (not significant) for $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3.8. MGE analysis

The MGEs present in 18 metagenomes were systematically analyzed using the mobileOG database. A total of 67 minor mobileOG categories were identified and combined into five major groups, representing the fundamental divisions of MGE-associated molecular mechanisms. Similarities were observed in the profiles of the major MGE categories in influent samples. The mechanism associated with integration/excision was predominant across all samples, followed by mechanisms related to transfer, replication/recombination/repair, phage, and MGE-related biological processes linked to stability/transfer/defense (Fig. 6). In WWTPs in Taiwan, significant changes in MGE abundance were observed from influent to effluent samples ($p < 0.0001$; Fig. 6 (a)). Specifically, MGEs associated with the integration/excision mechanism exhibited a significant reduction of 1.22 MGEs per 16S rRNA. Additionally, MGEs linked to transfer, replication/recombination/repair, stability/transfer/defense, and phage mechanisms decreased by 0.55–0.87 MGEs per 16S rRNA. In Slovakia, significant decreases were only observed in phage and stability/transfer/defense–related MGEs from 1.5 to 0.82 MGEs per 16S rRNA in influent samples to 0.88 and 0.36 MGEs per 16S rRNA in effluent samples ($0.05 > p > 0.01$), respectively. The average abundance of MGEs related to the other three main categories decreased from influent to effluent samples, although these changes were not significant ($p > 0.05$; Fig. 6 (b)). The observed alterations in MGE abundance highlight the dynamic nature of MGEs in urban wastewater treatment systems. Reductions in specific MGE categories from influent to effluent samples may be influenced by treatment processes that selectively affect certain MGEs. Overall, our findings indicate noteworthy variations in the composition of MGEs between influent and effluent samples, which is consistent with the results of recent studies on urban WWTPs [32]. Although some studies have demonstrated the presence of different types of MGEs, such as integrons, transposons, and conjugative plasmids [69,86,87], comprehensive investigations into MGEs remain limited [31,33]. Our study provides a systematic analysis of various functional modules involved in the MGE life cycle, including integration (integration/excision), replication (replication/recombination/repair), transfer and defense of bacterial MGE (stability/transfer/defense), and other phage-related accessories (such as structural proteins, lysogenic machinery, and other viral factors). These findings provide valuable information that can be used to assess the horizontal gene transfer potential in WWTPs.

3.9. Assessment of virulence factors in WWTP effluent

This study investigated VFG profiles to gain a comprehensive understanding of the risks associated with bacterial pathogens in WWTP effluent. Although previous studies have demonstrated the presence of bacterial pathogens in WWTP effluent [88], assessing the risk of the emergence of specific virulence determinants is crucial given the increased pathogenicity of bacterial pathogens carrying VFGs. Regarding bacterial pathogenicity, a total of 1011 unique VFGs were identified in effluent samples by using the most recent VFDB 2022 database [42]. Based on the distribution of bacterial pathogens, the detected VFGs in all effluent samples were categorized into 13 groups. Notably, the mean abundance of VFG categories in effluent from WWTPs in Slovakia was generally higher than that in Taiwan (Fig. 7). This notable difference may be strongly linked to the microbial community compositions. Bacteria from the *Acinetobacter* genus, which are associated with bacteremia, pulmonary infections, meningitis, diarrhea, and life-threatening nosocomial infections [89], were predominant in samples in Slovakia (Fig. S2). Among the categories, VFGs related to immune modulation were the most abundant (0.17 ± 0.07 number of VF category/16S rRNA), followed by the regulation group (0.09 ± 0.05 number of VF category/16S rRNA), adherence group (0.09 ± 0.05 VFG species/16S rRNA), nutritional/metabolic factor group (VFG species/16S rRNA), and effector delivery system group (0.08 ± 0.03 VFG species/16S rRNA). Notably, VFGs assigned to the motility group identified in effluent samples can increase the motility of bacterial recipients in the environment, which is crucial for increasing the pathogenicity of bacteria in the bacterial infection process [90]. Because the spread of pathogenic traits and the dissemination of antibiotic resistance can be mediated by MGEs, the study results indicate the higher potential risk of AMR and pathogenicity in Slovakia than in Taiwan.

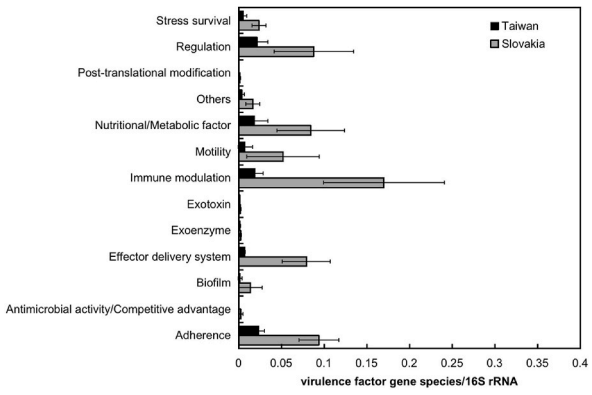

Fig. 7. Distribution and abundance of virulence factor genes in effluent samples from WWTPs in Taiwan and Slovakia. Abundance of each virulence factor gene was normalized per 16S rRNA gene and grouped in corresponding mechanism category.

4. Conclusions

This study conducted a comprehensive -omic analysis of samples collected from urban WWTPs with A/O processes as the primary treatment method in Taiwan and Slovakia, uncovering distinct microbiome, resistome, mobilome, and virulome profiles. Influent and effluent samples from WWTPs in Taiwan had a higher abundance and diversity of ARGs, which were also revealed by the evaluation of removal efficacy. However, the distribution of MGEs and VFGs exhibited the opposite trend. These findings highlight the influence of microbial community structures on the prevalence of AMR and pathogenicity in Taiwan and Slovakia despite their similar antibiotic usage patterns. Notably, our study contributes to understanding the microbiome, resistome, mobilome, and virulome in A/O treatment systems, addressing knowledge gaps about ARGs, MGEs, and VFGs. Establishing virulome profiles in WWTP effluents offers crucial insights into the potential environmental and health risks of treated wastewater. Importantly, our results suggest the necessity of region-specific insights into microbial communities for understanding the dynamics of AMR and pathogenicity in urban WWTPs and have implications for future management strategies for AMR and pathogenicity. By providing valuable information on the composition and abundance of these harmful transmittable genes, our findings can inform the optimization of treatment processes, mitigation of resistance dissemination, and protection of public health. However, further research is required to elucidate the transmission mechanisms of ARGs and VFGs as well as to assess the co-selection effects of heavy metals on ARGs. By incorporating microbial source tracking with metagenomic data, it would be valuable to specify the contributions of different sources (e.g., hospital wastewater, domestic wastewater, agriculture, and industrial wastewater) and targeted environmental factors, such as nutrient loads and operational parameters, to the microbiome composition in wastewater treatment plants. This would help clarify their precise role in shaping resistome and mobilome profiles. Understanding the functional roles, interactions, and origins of microbial communities with ARGs, MGEs, and VFGs in WWTPs is essential for developing effective mitigation strategies.

Environmental implication

Wastewater treatment plants are significant sources of hazardous genetic materials, including antibiotic resistance and virulence factor genes, posing potential threats to animal and human health. Our study conducted metagenomic analyses on samples collected from urban wastewater treatment plants in Taiwan and Slovakia, where anoxic and oxic processes are the primary treatment methods. We identified significant differences in the microbiome, resistome, mobilome, and virulome between these regions. These findings highlight the importance of understanding regional microbial communities to elucidate the dynamics of antimicrobial resistance and pathogenicity, facilitating the development of tailored mitigation strategies for each region.

Data availability statement

All data are available in the main text or the supplementary materials. All the sequenced datasets in this study are publicly available on the NCBI SRA database (SUB14264437).

CRediT authorship contribution statement

Wei-Yu Chen: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Chun-Pao Lee:** Investigation, Data curation. **Jelena Pavlović:** Resources, Data curation. **Domenico Pangallo:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Jer-Horng Wu:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38723.

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