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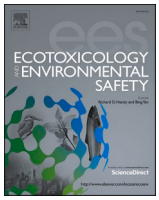

Assessing phenotypic and genotypic antibiotic resistance in bacillus-related bacteria isolated from biogas digestates

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ABSTRACT

Antibiotic resistance poses a significant public health challenge, with biogas digestate, a byproduct of anaerobic digestion (AD), presenting potential risks when applied as a biofertilizer. Understanding the actual resistance levels in digestate is crucial for its safe application. While many studies have investigated antibiotic resistance in AD processes using culture-independent molecular methods, these approaches are limited by their reliance on reference databases and inability to account for gene expression, leading to potential inaccuracies in resistance assessment. This study addresses these limitations by combining culture-independent whole-genome sequencing (WGS) with culture-dependent phenotypic testing to provide a more accurate understanding of antibiotic resistance in digestate. We investigated the phenotypic and genotypic resistance profiles of 18 antibiotic-resistant bacteria (ARB) isolated from digestates produced from food waste and animal manure. Resistance was assessed using WGS and Estrip testing across 12 antibiotics from multiple classes. This is the first study to directly compare phenotypic and genotypic resistance in bacteria isolated from digestate, revealing significant discrepancies between the two methods. Approximately 30 % of resistance levels were misinterpreted when relying solely on culture-independent methods, with both over- and underestimation observed. These findings highlight the necessity of integrating both methods for reliable resistance assessments. Additionally, our WGS analysis indicated low potential for transferability of detected ARGs among the isolated ARB, suggesting a limited risk of environmental dissemination. This study provides new insights into antibiotic resistance in digestate and underscores the importance of integrating methodological approaches to achieve accurate evaluations of resistance risks.

1. Introduction

Anaerobic digestion (AD) is a well-established technology for converting organic wastes, such as animal manure, food waste, and sludge from wastewater treatment plants (WWTP), into biogas and digestate (Nwokolo et al., 2020). This process generates green energy and a biofertilizer, contributing to sustainable development (Obaideen et al., 2022). Digestate is a commonly used fertilizer on farmland due to its high nutrient content (Al Seadi et al., 2013), facilitating nutrient recycling between urban and rural areas. However, it is crucial to avoid introducing chemical and biological contaminants into the environment through its use. Antibiotic resistance components, such as antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), are frequently detected in AD substrates (Qian et al., 2018; Wichmann et al., 2014). These components can survive the AD process to some extent and end up in the digestate (Beneragama et al., 2013; Mitchell et al., 2013; Resende et al., 2014; Visca et al., 2021; Zou et al., 2020). Additionally, mobile genetic element (MGEs), which can transfer ARGs, are often present alongside resistance components (Zou et al., 2020). This residual resistance in digestate poses a risk of spread of antibiotic resistance when applied as biofertilizer (Beneragama et al., 2013; Schauss et al., 2016). Antibiotic resistance is a significant public health challenge, with antimicrobial-resistant bacterial infections causing an estimated 4.95 million deaths globally (Murray et al., 2022).

Previous studies have investigated variations in antibiotic resistance throughout the AD process and the resistance level in the final digestate (Beneragama et al., 2013; Schauss et al., 2016; Sun et al., 2020a; Zou et al., 2020). These studies have mainly employed either

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culture-independent (Sun et al., 2019a; Zhang et al., 2015) or culture-dependent methods (Schauss et al., 2016; Sun et al., 2020a). Culture-independent methods typically involve extracting DNA directly from AD samples andusing one of the following main techniques: a) polymerase chain reaction (PCR) and quantitative PCR (qPCR), targeting specific genes of relevance (Sun et al., 2019b; Zou et al., 2020); b) DNA chips, such as ArrayMate Reader (Braun et al., 2014) and WaferGen SmartChip (Wang et al., 2014); and c) metagenomic analysis (Zhang et al., 2019, 2015). Among these methods, PCR and qPCR are perhaps the most straightforward and simplest to use in identification/quantification of ARGs and MGEs, as there is no need for subsequent bioinformatics work. However, these methods are restricted by the primers used, which are not capable of revealing a comprehensive resistance profile. The DNA chips represent high-throughput PCR/qPCR methods with hundreds of genes identifier integrated, substantially increasing the capacity for identification/quantification of genes, but still cannot cover all characterized gene variants. Metagenomic analysis is powerful in comprehensive identification/quantification of ARGs and MGEs in environmental samples, including AD samples, but requires bioinformatics skills for subsequent analysis of sequencing data. Notably, the culture-independent methods are database-dependent (metagenomic analysis) or capacity-dependent (PCR/qPCR and DNA chips). Considering the substantial numbers of unknown genes and uncharacterized mechanisms conferring antibiotic resistance in multiple environments (Brandt et al., 2017), the culture-independent methods may overlook existing resistance because of novel/unidentified genes. Culture-independent methods may also overestimate the resistance situation for identified ARGs because of the complexity of ARG expression, *e.g.*, non-expression and weak expression of genes (Chen et al., 2003; Enne et al., 2006). Thus, although culture-independent methods represent a significant technological advancement and provide an effective overview of antibiotic resistance in AD processes, the real situation may still be masked due to these methodological limitations.

Compared with culture-independent methods, culture-dependent methods have been less widely used to obtain information about antibiotic resistance in AD systems, possibly not only due to a lack of research focus but also likely because of the extensive laboratory work involved. Culture-dependent methods involve isolation and cultivation of bacteria from substrate or digestate, followed by antibiotic susceptibility testing (AST) (Schauss et al., 2016; Sun et al., 2020a). This method is reliable as it reveals direct phenotypic resistance. However, culturing often underestimates the diversity of ARB, since only a fraction of environmental bacteria can be cultivated and the species retrieved will also depend on the nutrient medium and evironmnetal conditions used (Del Mar Lleò et al., 2003; Yin et al., 2023; Zandri et al., 2012). Thus, culture-dependent methods also have limitations in revealing the full antibiotic resistance situation prevailing in AD processes.

Antibiotic-resistant bacteria isolated from biogas digestate have seldom been investigated for both genotypic and phenotypic resistance. Zou et al. (2020) isolated tetracycline-resistant bacteria from pig manure digestate with selective agar plates, but found no selected *tet* genes (*tet*A, *tet*O, *tet*X) in gene-specific PCR of most of the isolated strains, affiliating to genera such as *Escherichia*, *Enterococcus*, *etc*. Pulami et al. (2020) conducted phenotypic AST and shotgun whole-genome sequencing for six strains of *Acinetobacter baumannii* and found that all six strains were susceptible to ciprofloxacin and tetracyclines, even though the relevant resistance genes, *abe*M and *ade*IJK, were present in the genome. The results from these two studies illustrate some discrepancies between the methods. However, as only a few comparative studies have been done, consistency of results between phenotypic and genotypic resistance remains unclear. Therefore, more direct comparisons of genotypic and phenotypic antibiotic resistance are needed to further understand these observed differences.

In addition to antibiotic resistance pattern *per se*, determining the transferability of resistance is critically important in terms of understanding risk and preventing the spread of antibiotic resistance in the environment. Acquisition of foreign genes through horizontal gene transfer (HGT) is one of the most important drivers of bacterial evolution, and it is frequently responsible for the development of antibiotic resistance (Munita and Arias, 2016). MGEs such as plasmids and integrons are used as vehicles for transferring ARGs during HGT (Munita and Arias, 2016). Antibiotic-resistant pathogens such as *Escherichia* spp. and *Pseudomonas* spp. identified in biogas digestate are direct health threats (Schauss et al., 2016). Resistance transfer via HGT between such pathogens has been widely studied because of their clinical importance (Nagachinta and Chen, 2008). However, the transferability of resistance carried by *Bacillus* and closely-related genera, *e.g.*, *Paenibacillus* and *Lysinibacillus*, has not been investigated, even though they represent an abundant community in biogas reactors (Schauss et al., 2016; Tao et al., 2020) and are part of the dominant ARB community (Sun et al., 2020a; Zou et al., 2020). These ARB have been found to exhibit resistance to a variety of antibiotic classes, including β-lactams, tetracyclines, and macrolides (Sun et al., 2020a). Investigation of ARG transferability for these ARB would improve understanding of the HGT situation within biogas reactors and the risk of resistance spreading to the environment when digestate is applied as fertilizer.

This study aimed to investigate antibiotic resistance in bacteria isolated from biogas digestates, by comparing genotypic and phenotypic resistance and exploring potential resistance transferability as a secondary outcome. Specifically, 18 strains of ARB, primarily belonging to the genera *Bacillus*, *Paenibacillus*, and *Lysinibacillus*, were isolated from digestates derived from dairy manure and food waste. Whole-genome sequencing (WGS) and AST were conducted to determine genotypic and phenotypic resistance patterns, respectively. To our knowledge, this is the first study to directly compare phenotypic and genotypic resistance in bacteria from biogas digestate across comprehensive antibiotic classes. Resistance transferability for the ARB strains was assessed based on WGS analysis by examining the presence of plasmids, integrons, transposons, and insertion sequences.

2. Materials and methods

2.1. Samples

In previous work, we isolated bacterial species from digestates originating from animal manure (AM) and food waste (FW). Details of the isolation procedure and antibiotic resistance pattern for these isolates can be found in Sun et al. (2020a). Briefly, AST was conducted for the isolates using E-TEST according to M100 performance standards (CLSI, 2019). The minimal inhibitory concentration (MIC) values for each isolate tested were compared with the MIC breakpoints stated in CLSI guidelines (CLSI, 2019, 2018, 2016). Based on the AST results, isolates were phenotypically categorized as resistant (R), intermediate resistant (I), or sensitive (S). A total of 18 strains of ARB (nine each of AM and FW) were selected for WGS analysis (Table 1), based on representativeness of phylogeny and resistance pattern. The selected ARB were revived from a glycerol (25 % v/v) stock frozen at −80 ℃ and cultivated in Mueller Hinton broth (MHB, Becton Dickinson) at 37 ℃.

2.2. Long-read DNA sequencing

2.2.1. DNA preparation

Long fragmented genomic DNA was extracted using NucleoBond kit (Macharey Nagal) and purified using AMPure magnetic beads (Beckman Coulter) according to the protocol described in a previous publication (Sun et al., 2020b). The DNA concentration was quantified by Qubit (Thermo Fisher Scientific) and the fragment length was visualized by agarose gel electrophoresis.

2.2.2. Sequencing

The SQK-LKS109 and EXP-NBD104 kits (Oxford Nanopore Technologies) were used for library preparation, according to the

Table 1
Isolates selected for whole-genome sequencing from digestates originating from animal manure (AM) and food waste (FW), and their phenotypic antibiotic resistance pattern (Sun et al., 2020a).

No	Species[a]	Digestate	AMP[b]	CAZ	MEM	VAN	CIP	RIF	CHL	CLI	ERY	TET	GEN	SXT
2	*Bacillus zhangzhouensis*	AM												
3	*Bacillus licheniformis*	AM												
6	*Bacillus aerius*	AM												
7	*Bacillus albus*	AM												
10	*Bacillus paramycoides*	AM												
13	*Brevibacillus parabrevis*	AM												
14	*Lysinibacillus sphaericus*	AM												
15	*Lysinibacillus fusiformis*	AM												
21	*Bacillus zhangzhouensis*	AM												
1	*Bacillus oleronius*	FW												
2	*Bacillus subtilis*	FW												
4	*Bacillus cereus*	FW												
9	*Brevibacillus agri*	FW												
11	*Lysinibacillus sphaericus*	FW												
12	*Lysinibacillus macroides*	FW												
14	*Paenibacillus lactis*	FW												
15	*Paenibacillus cookii*	FW												
16	*Stenotrophomonas rhizophila*	FW												

[a]Identification of the isolates was based on 16S rRNA similarity. [b]Antibiotic susceptibility testing (AST) was conducted with the antibiotics ampicillin (AMP), ceftazidime (CAZ), meropenem (MEM), vancomycin (VAN), ciprofloxacin (CIP), rifampicin (RIF), chloramphenicol (CHL), clindamycin (CLI), erythromycin (ERY), tetracycline (TET), gentamicin (GEN) and sulfamethoxazole/trimethoprim (SXT). White, light red and dark red cells represent sensitive, intermediate resistant, and resistant, respectively.

manufacturer's instructions. Long-read sequencing was performed using a MinION device (Oxford Nanopore Technologies) for 72 h at a bias voltage of −180 mV, with a FLO-MIN106 flow cell. Refuelling using a "Refuel-Mix" was performed after the first 18 h of sequencing.

2.2.3. Sequence processing

Raw nanopore sequencing data were basecalled and demultiplexed using guppy (v. 4.0.15–1) and filtered by filtlong (v. 0.2.0). Genome reconstruction was performed using flye (v.2.8) and subsequently polished using racon (v. 1.4.13) and medaka (v. 1.0.3). Read mapping for polishing was performed using minmap2 (v. 2.17). The final sequence quality was ensured, with 99 % of the genomes covered by at least 40 reads. Whole-genome sequences have been submitted to NCBI, under accession numbers CP079720-CP079723 and CP139094-CP139120 in BioProject PRJNA745572.

2.3. Genome analysis

2.3.1. Phylogenetic identification and relationships

Sourmash (v. 4.8.2) was used for identification of genomes, using the database GTDB R08-RS214 all genomes (LCA, k-mer = 31) (Brown and Irber, 2016). Phylogenetic relationships between the genomes were compared pair-wise and displayed as a phylogenetic tree using Sourmash.

2.3.2. Identification of antibiotic resistance genes and mobile genetic elements

Antibiotic resistance genes (ARGs) were annotated for WGS by ABRicate (Seemann T, ABRicate, Github https://github.com/tseemann/abricate) with a minimum DNA sequence identify of 80 % and a minimum coverage of 80 %. Annotation was performed using a combination of three databases: NCBI, CARD, and ARG-ANNOT (all updated on August 31, 2023). Bacteria were classified as genotypic-resistant or genotypic-sensitive based on presence of identified ARGs.

For mobile genetic elements (MGEs), plasmids were annotated by ABRicate using the PlasmidFinder database (updated on August 31, 2023). Integrons were detected by IntegronFinder 2.0 (Néron et al., 2022) on the Galaxy Pasteur webserver (assessed on September 4, 2023). Transposons and insertion sequences (ISs) were detected using MobileElementFinder (v. 1.1.2) (Johansson et al., 2021). The distances between identified ARGs and transposons or ISs within genomes were analyzed using Geneious Prime (v. 2024.0.5). The transferability of identified ARGs depends on the presence of these MGEs and the genomic proximity of ARGs to MGEs.

3. Results & discussion

3.1. Phylogenetic identification

Whole-genome sequencing of species previously identified through phylogenetic analysis based on 16S rRNA gene sequences revealed some discrepancies between the methods. The taxonomy was altered for 10 out of the 18 strains, while it remained the same for the remaining eight (Table 2). Among the strains showing a different classification, isolate FW12 could not be identified to species level. Isolate FW1, initially classified as *Bacillus oleronius*, was reclassified as *Heyndrickxia oleronia* (Gupta et al., 2020). For the remaining strains, the genus-level identification was consistent with the 16S rRNA gene classification, but changed at the species level. For instance, AM2 and FW16, which were initially identified as *Bacillus zhangzhouensis* and *Stenotrophomonas rhizophila*, respectively, through 16S analysis, were instead identified as *Bacillus pumilus* and *Stenotrophomonas maltophilia*, respectively, using WGS. The phylogenetic relationships based on WGS for all selected isolates are shown in Fig. 1. Three groups displayed close phylogenetic relationships: AM2 and AM21, both identified as *B. pumilus*; FW11 and AM14, both identified as *L. sphaericus*; and a group of *Bacillus* species including *B. cereus*, *B. anthracis*, and *B. paranthracis*.

Bacteria in the genus *Bacillus* and closely related genera, such as *Paenibacillus* and *Lysinibacillus*, exhibit high similarity in terms of their 16S rRNA gene sequences, so it can be challenging to precisely identify them based solely on 16S rRNA gene similarity (Blackwood et al., 2004; Giffel et al., 1997). Our extended WGS analysis revealed that three isolates were known pathogens, namely *B. anthracis*, *B. paranthracis*, and *S. maltophilia*. Thus, these results underscore the importance of WGS in correct identification of bacteria and improve understanding of the risk associated with applying digestate to soil in terms of pathogens.

3.2. Identification of antibiotic resistance genes

Antibiotic resistance genes were identified for the selected isolates based on WGS including both chromosome and extra-chromosomal DNA (Supplementary Table S1). In brief, the ARGs identified in the different strains encoded resistance to a broad range of antibiotic classes, including all phenotypic resistances evaluated, as well as other classes such as streptothricin and fosfomycin (Table 3, Supplementary Table S1). Most of the ARGs were identified in chromosomal sequences, apart from a tetracycline (TET) resistance gene, *tet*L, which was identified in a plasmid sequence from FW1 *H. oleronia* (Sun et al., 2023).

Table 2
Phylogenetic identification of selected bacterial isolates from animal waste (AM) and food waste (FW) based on 16S rRNA and whole-genome sequence (WGS) similarity comparison. Inconsistencies in identification between the analyses are marked in bold.

AM	16S rRNA	WGS FW		16S rRNA	WGS
2	***B. zhangzhouensis***	***B. pumilus***	1	***B. oleronius***	***H. oleronia***
3	***B. licheniformis***	***B. paralicheniformis***	2	*B. subtilis*	*B. subtilis*
6	***B. aerius***	***B. altitudinis***	4	*B. cereus*	*B. cereus*
7	***B. albus***	***B. anthracis***	9	*BreviB. agri*	*BreviB. agri*
10	***B. paramycoides***	***B. paranthracis***	11	*LysiniB. sphaericus*	*LysiniB. sphaericus*
13	*BreviB. parabrevis*	*BreviB. Parabrevis*	12	***LysiniB. macroides***	***LysiniB. sp.***
14	*LysiniB. sphaericus*	*LysiniB. sphaericus*	14	*PaeniB. lactis*	*PaeniB. lactis*
15	***LysiniB. fusiformis***	***LysiniB. capsici***	15	*PaeniB. cookii*	*PaeniB. cookii*
21	***B. zhangzhouensis***	***B. pumilus***	16	***S. rhizophila***	***S. maltophilia***

3.2.1. Antibiotic resistance genes in bacillus and closely-related genera

3.2.1.1. Beta-lactams resistance genes. The β-lactamase genes identified, such as BPU-1, *bla*P, *bla*1, *etc.*, were all found in the *Bacillus* spp., but not in other genera (Table 3). The BPU-1 gene, previously identified in *B. pumilus*, is responsible for conferring resistance to penicillins (*e.g.*, ampicillin (AMP) (Toth et al., 2016). In the present study, BPU-1 was detected in the chromosomes of *B. pumilus* (AM2 and AM21) and *B. altitudinis* (AM6). However, only AM21 exhibited resistance to AMP, while AM2 and AM6 did not. The BPU-1 gene has been proven to markedly enhance AMP resistance when cloned from *B. pumilus* and expressed in *E. coli* (Toth et al., 2016). However, it is worth noting that four strains of *B. pumilus* isolated from diverse sources, *e.g.*, food and the normal flora in gastropods, have all been found to be sensitive to AMP (Branquinho et al., 2015). While genotype information regarding presence of BPU-1 for these strains is unavailable, it appears that the gene in *B. pumilus* may not always confer sufficient resistance to AMP. *Bla*P was identified in the isolate of *B. paralicheniformis* (AM3) in this study, showing resistance to both AMP and ceftazidime (CAZ). This is in line with previous identification of this β-lactamase gene in *B. paralicheniformis* (Othoum et al., 2018). *Bla*1 and *bla*2 are chromosomal-encoded beta-lactamase. *Bla*1 can hydrolyze penicillins, while *bla*2 has penicillin-, cephalosporin- and carbapenem-hydrolyzing abilities (Materon et al., 2003). These two genes are usually not sufficient to confer resistance to β-lactam agents, due to weak expression (Chen et al., 2003). *Bla*1 and *bla*2 have been found previously in *B. anthracis* (Materon et al., 2003). In a previous study, 11.5 % of 96 isolates of *B. anthracis* tested were resistant to penicillin G and amoxicillin, while all were susceptible to imipenem (belongs to carbapenem) (Cavallo et al., 2002). In line with these results, the *bla*1 and *bla*2 genes were identified in the isolates of *B. anthracis* (AM7) and *B. paranthracis* (AM10), and both isolates were resistant to AMP and CAZ and sensitive to meropenem (MEM). *Bc*I and *Bc*II are zinc metallo-beta-lactamases that hydrolyze a large number of penicillins and cephalosporins, and these genes have previously been found in *B. cereus* (Carfi et al., 1995). These genes were also identified in the isolate of *B. cereus* (FW4) in the present study and, in line with this, the isolate was also resistant to AMP (penicillins) and CAZ (cephalosporins).

3.2.1.2. Vancomycin, chloramphenicol, ciprofloxacin, and rifampicin resistance genes. In the genomes analyzed in the present study, *Van*ZF was the only gene present encoding for vancomycin (VAN) resistance (Fraimow et al., 2005). *Van*ZF was found in *B. cereus* (FW4), *B. anthracis* (AM7), and *B. paranthracis* (AM10), but despite this, all three isolates were sensitive to this antibiotic compound. However, these results are in line with previous findings that several different *B. cereus* strains (n = 15) carrying *van*ZF are phenotypically sensitive to VAN (Bianco et al., 2021). *Blt* and *bmr* are two multidrug efflux transporters previously found in *B. subtilis* (Ahmed et al., 1995; Neyfakh et al., 1991) and have been shown to cause efflux of a variety of antibiotic compounds such as chloramphenicol (CHL) and fluoroquinolones (*e.g.* ciprofloxacin (CIP)) (Ahmed et al., 1995). These two genes are regulated by the transcriptional activators BltR and BmrR, respectively, which require different inducers for expression (Ahmed et al., 1995; Neyfakh et al., 1991). *Blt* and *bmr* function by overexpression via intrachromosomal amplification or by expression from a plasmid vector (Ahmed et al., 1995). In the present study, these two genes were identified in the chromosome of *B. subtilis* (FW2), but the isolate was still sensitive to both CHL and CIP. Therefore, the genes were likely not mobile, and the susceptibility could have been caused by insufficient expression of *blt* and *bmr* (Ahmed et al., 1995). In the present study, CHL resistance was found to be linked to the gene *cat*86, a chromosome-encoded variant of the *cat* gene previously found in *B. pumilus* (Harwood et al., 1983). Both *B. pumilus* strains (AM2 and AM21) and *B. altitudinis* (AM6) were found to carry *cat*86, resulting in varying degrees of resistance, with *B. pumilus*

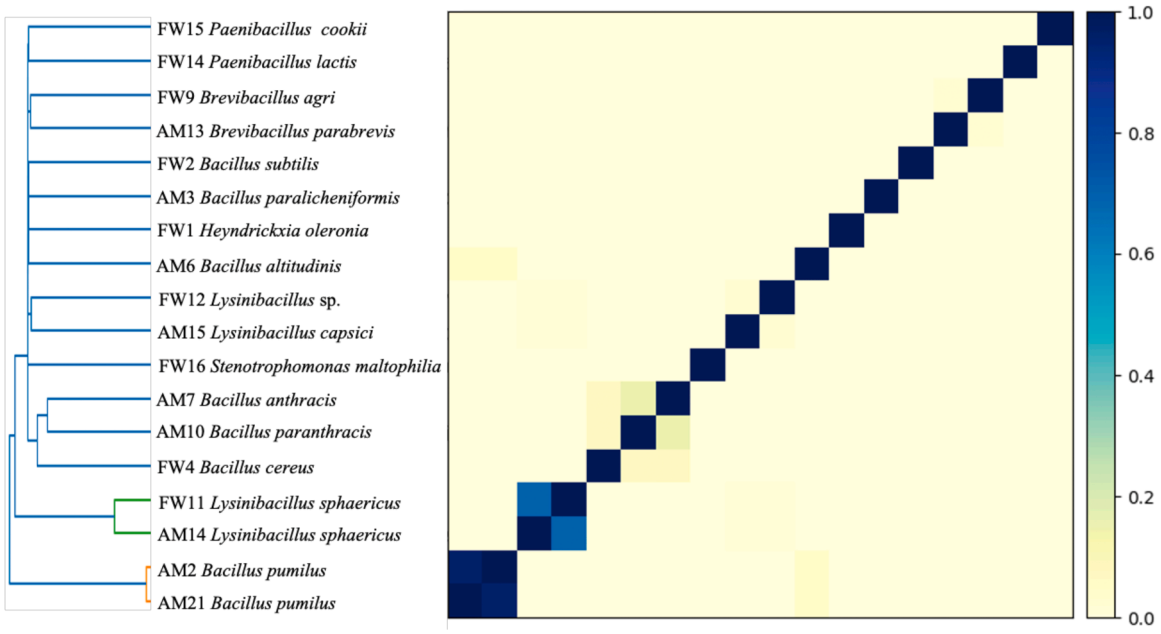

Fig. 1. Phylogenetic relationships between selected isolates based on pair-wise comparison of whole-genome sequences.

Table 3
Antibiotic resistance genes identified in whole-genome sequences of the selected isolates.

No.	Species	Digestate	AMP*	CAZ*	MEM*	VAN*	CIP*	RIF*	CHL*	CLI*	ERY*	TET*	GEN*	SXT*
2	*B. pumilus*	AM	BPU−1						cat86					
3	*B. paralicheniformis*	AM	blaP	blaP				rphC		ermD	ermD			
6	*B. altitudinis*	AM	BPU−1						cat86					
7	*B. anthracis*	AM	bla1	bla2	bla2	vanZF					mphL			
10	*B. paranthracis*	AM	bla1	bla2	bla2	vanZF								
13	*BreviB. parabrevis*	AM												
14	*LysiniB. sphaericus*	AM												
15	*LysiniB. capsici*	AM												
21	*B. pumilus*	AM	BPU−1						cat86					
1	*H. oleronia*	FW						rphC				tetL		dfrG
2	*B. subtilis*	FW					blt, bmr	rphB, rphC	blt, bmr	vmlR lmrB	mphK	ykkC, ykkD, tetL	aadK, ykkC, ykkD	
4	*B. cereus*	FW	BcI	BcI, BcII		vanZF								
9	*BreviB. agri*	FW												
11	*LysiniB. sphaericus*	FW												
12	*LysiniB.* sp.	FW								clbA, cfrB				
14	*PaeniB. lactis*	FW						rphB		cipA				
15	*PaeniB. cookii*	FW												
16	*S. maltophilia*	FW					smeD, smeE, smeF				mexP, mexQ, smeD, smeE, smeF	mexP, mexQ, smeD, smeE, smeF		smeD, smeE, smeF

* ampicillin (AMP), ceftazidime (CAZ), meropenem (MEM), vancomycin (VAN), ciprofloxacin (CIP), rifampicin (RIF), chloramphenicol (CHL), clindamycin (CLI), erythromycin (ERY), tetracycline (TET), gentamicin (GEN), and sulfamethoxazole/trimethoprim (SXT).*

strains exhibiting intermediate resistance and *B. altitudinis* displaying full resistance to CHL. *Rph*B and *rph*C are widely distributed rifampicin (RIF) phosphotransferases present in RIF-sensitive bacteria, such as *B. cereus* and *P. lactis* (Pawlowski et al., 2016; Spanogiannopoulos et al., 2014). In this study, *rph*B was identified in *B. subtilis* (FW2) and *P. lactis* (FW14), while *rph*C was detected in *B. paralicheniformis* (AM3), *B. oleronius* (FW1), and *B. subtilis* (FW2). All these isolates exhibited sensitivity to RIF (Table 1).

3.2.1.3. Erythromycin and clindamycin resistance genes. Erythromycin (ERY) and clindamycin (CLI) belong to the antibiotic classes macrolides and lincosamides, respectively. Due to overlapping binding sites, cross-resistance to macrolide, lincosamide, and streptogramin-B antibiotics (MLSB) is common. For instance, the *erm*D gene, which imparts resistance to MLSB, was initially discovered in *B. licheniformis*, with 10 of 15 natural isolates of *B. licheniformis* exhibiting MLSB antibiotic resistance (Docherty et al., 1981). In the present investigation, *B. paralicheniformis* (AM2) was found to carry this gene and displayed resistance to both ERY and CLI (Table 1). Additionally, *mph*L and *mph*K are chromosomally-encoded macrolide phosphotransferases (Pawlowski

et al., 2018; Wang et al., 2015). The *mph*L gene has previously been reported in the *B. cereus* group, including *B. anthracis* (Wang et al., 2015), while *mph*K has been identified in *B. subtilis* (Pawlowski et al., 2018). In the present study, *mph*L was detected in *B. anthracis* (AM7), which displayed intermediate resistance to ERY. Conversely, *mph*K was found in *B. subtilis* (FW2), which exhibited sensitivity to the antibiotic. The susceptibility of FW2 is likely due to the narrow substrate range of *mph*K, which cannot confer resistance to ERY but is effective for other antibiotics in the class of macrolides (Pawlowski et al., 2018). As for specific CLI resistance, *vml*R (Crowe-McAuliffe et al., 2018) and *lmr*B (Yoshida et al., 2004) have been identified previously in *B. subtilis*. Both genes were also detected in *B. subtilis* (FW2) in this study, and this isolate demonstrated an intermediate level of resistance to CLI (Table 1). CLI resistance can also be related to the presence of *cfr*B, *clb*A, and *cip*A, which are *cfr*-like genes encoding for multiple antibiotic resistance (Atkinson et al., 2013; Deshpande et al., 2015; Hansen et al., 2012). In the present study, *cfr*B and *clb*A were identified in *Lysinibacillus.* sp. (FW12), and *cip*A in *P. lactis* (FW14), showing intermediate resistance and resistance to CLI, respectively.

3.2.1.4. Tetracycline, gentamycin, and sulfamethoxazole/trimethoprim resistance genes. *Tet*L is a tetracycline efflux protein found in many species of Gram-negative and Gram-positive bacteria (Roberts, 2005). This gene was identified in a plasmid (pAMαl) from *H. oleronia* (FW1), which exhibits resistance to TET (Sun et al., 2023). In addition to *tet*L, the *ykk*C and *ykk*D genes have also been identified as contributors to resistance in *B. subtilis* (Jack et al., 2000). These two genes encode small multidrug resistance (SMR) antibiotic efflux pumps, conferring resistance not only to TET, but also to aminoglycoside (*e.g.*, gentamycin (GEN)) and phenicol antibiotics. In the present study, these two genes were detected in *B. subtilis* (FW2), but the isolate displayed only intermediate resistance to TET and was sensitive to GEN (Table 1). Previous studies have shown that these two genes can enhance resistance levels in *E. coli* to antibiotics, including TET and streptomycin, when they are co-expressed, but not when expressed individually as either *ykkC* or *ykkD* (Jack et al., 2000). Whether a similar mechanism regulates resistance in *B. subtilis* has not yet been evaluated. In contrast to the findings in the present study, a previous investigation of the phenotypic antibiotic susceptibility of various *Bacillus* strains revealed that 29 strains of *B. subtilis* isolated from Sudanese bread production were sensitive to TET (MIC_{90}=8 mg/L) and GEN (MIC_{90}=4 mg/L), but resistant to streptomycin (MIC_{50}=64 mg/L) (Adimpong et al., 2012). The variation in antibiotic susceptibility of *B. subtilis* between the studies may represent true differences, but is more likely to have arisen from different guidelines on the breakpoint values of minimum inhibitory concentration (MIC). In our study, the Clinical and Laboratory Standards Institute (CLSI) M45 (2018) was applied (Sun et al., 2020a) while Food Safety Authority (FSA) (2012) guidelines were used by Adimpong et al. (2012). The TET- and GEN-sensitive values in the FSA guideline correspond, respectively, to TET-intermediate resistance and GEN-sensitive ranges in the CLSI guideline. Considering these differences, these two studies actually produced consistent results regarding TET and GEN susceptibility for *B. subtilis*. Hence, the genes *ykk*C and *ykk*D identified in *B. subtilis* seem likely to confer weak resistance to TET, but do not affect the resistance level to GEN. Another GEN resistance gene identified in this study was *aad*K, a chromosomal-encoded aminoglycoside nucleotidyl transferase gene that has also been identified previously in *B. subtilis* (Noguchi et al., 1993). This gene contributes to low-level resistance to streptomycin, another antibiotic belonging to the aminoglycoside class (Noguchi et al., 1993). In the study by Adimpong et al. (2012), all 29 strains of *B. subtilis* were found to be sensitive to GEN, but resistant to streptomycin. Consistent with that, our investigation found *B. subtilis* (FW2) to be sensitive to GEN. *Dfr*G is a dihydrofolate reductase encoding resistance to trimethoprim (TMP) (Sekiguchi et al., 2005). This gene has previously been found in the chromosome of *H. oleronia* (FW1) and in the type strain *H. oleronia* DSM 9356 (Sun et al., 2023), suggesting it is chromosome-encoded. However, strain FW1 was not resistant to a combination of sulfamethoxazole (SUL)/trimethoprim (SXT) in the present study.

3.2.2. Antibiotic resistance genes in Stenotrophomonas maltophilia

Five genes (*mex*P, *mex*Q, *sme*D, *smeE*, and *sme*F) were identified in the chromosome of *S. maltophilia* (FW16) (Table 3). *Mex*P and *mex*Q encode components of the efflux pump *Mex*PQ-*opm*E, which consists of *mex*P (membrane fusion protein), *mex*Q (inner membrane transporter), and *opm*E (outer membrane channel) (Mima et al., 2005). When cloned in hypersensitive *P. aeruginosa*, *mex*PQ-*opm*E has been found to markedly elevate resistance levels to macrolides (*e.g.*, ERY, MIC 16–64 mg/L), and slightly increase resistance level to fluoroquinolones (*e.g.*, CIP, MIC 0.016–0.03 mg/L) and TET (MIC 0.5–1.0 mg/L) (Mima et al., 2005). In the present study, *S. maltophilia* (FW16) was found to be resistant to ERY, but sensitive to CIP and TET. The susceptibility to CIP and TET was likely due to inadequate resistance enhancement. The multidrug efflux complex *sme*DEF is the most well-studied system of resistance nodulation division (RND) in *S. maltophilia* (Gil-Gil et al., 2020). The system consists of *sme*D (membrane fusion protein), *sme*E (RND protein of the efflux complex), and *smeF* (outer membrane multidrug efflux protein). Inactivation of the *sme*DEF efflux complex increases the susceptibility to several antibiotics, such as CHL, TET, ERY, SXT, *etc.* (Gil-Gil et al., 2020). All these genes were found to be present in *S. maltophilia* (FW16), which may explain the intermediate resistance to CHL and resistance to ERY and SXT. However, TET resistance appeared not to be expressed in *S. maltophilia* (FW16), regardless of the presence of the five genes.

3.3. Comparison of genotypic and phenotypic resistance

Inconsistency in genotypic and phenotypic resistance was observed for all genera examined in this study, with the most frequent inconsistency in resistance observed for CAZ, CLI, and ERY (Fig. 2). Four specific scenarios emerged in comparison of genotypic and phenotypic resistance: (a) both resistant (n = 23); (b) genotypic resistant, but phenotypic sensitive (n = 17); (c) genotypic sensitive, but phenotypic resistant (n = 44); and (d) both sensitive (n = 132) (Fig. 2). Among the inconsistent scenarios found, scenario (b) was most likely caused by insufficient gene expression, such as β-lactamase BPU-1 in *B. pumilus* (AM2) and *B. altitudinis* (AM6), and *van*ZF in the *B. cereus* group (FW4, AM7, and AM10). It is difficult to distinguish the precise reason for scenario (c), as there is currently insufficient knowledge about all genetic variations resulting in reduced susceptibility for a given antibiotic compound (Ellington et al., 2017). Therefore, as previously suggested, it is plausible to assume that this inconsistency may have arisen from novel genes or through an unknown mechanism. (Brandt et al., 2017; Sun et al., 2022). Overall, consistency accounted for 71.8 % of all cases (n = 155, scenarios (a) and (d)) and inconsistency for 28.2 % (n = 61, scenarios (b) and (c)). Scenario (b) and (c) accounted for 27.9 % and 72.1 %, respectively, of the inconsistent cases. These results indicate that molecular analysis of ARGs may result in inaccurate predictions of phenotypic resistance for around 30 % of AD samples and that over 70 % of these inaccurate predictions would lead to underestimation of the true phenotypic resistant cases.

Intriguingly, resistance consistency appeared to be related to phylogeny, as similar consistency patterns were found for closely related bacteria (Fig. 2). For example, in the group AM7, AM10, and FW4, the genotypic and phenotypic resistance patterns for nine out of 12 antibiotics were found to be similar, with the only difference observed for CLI, ERY, and SXT. Moreover, in the groups FW11 and AM14, and AM2 and AM21, which are different strains of the same species (*L. sphaericus* and *B. pumilus*, respectively), the ARG patterns identified were similar for the strains of each species (Table 3). However, these isolates showed different resistance susceptibility. Thus, the resistance discrepancy

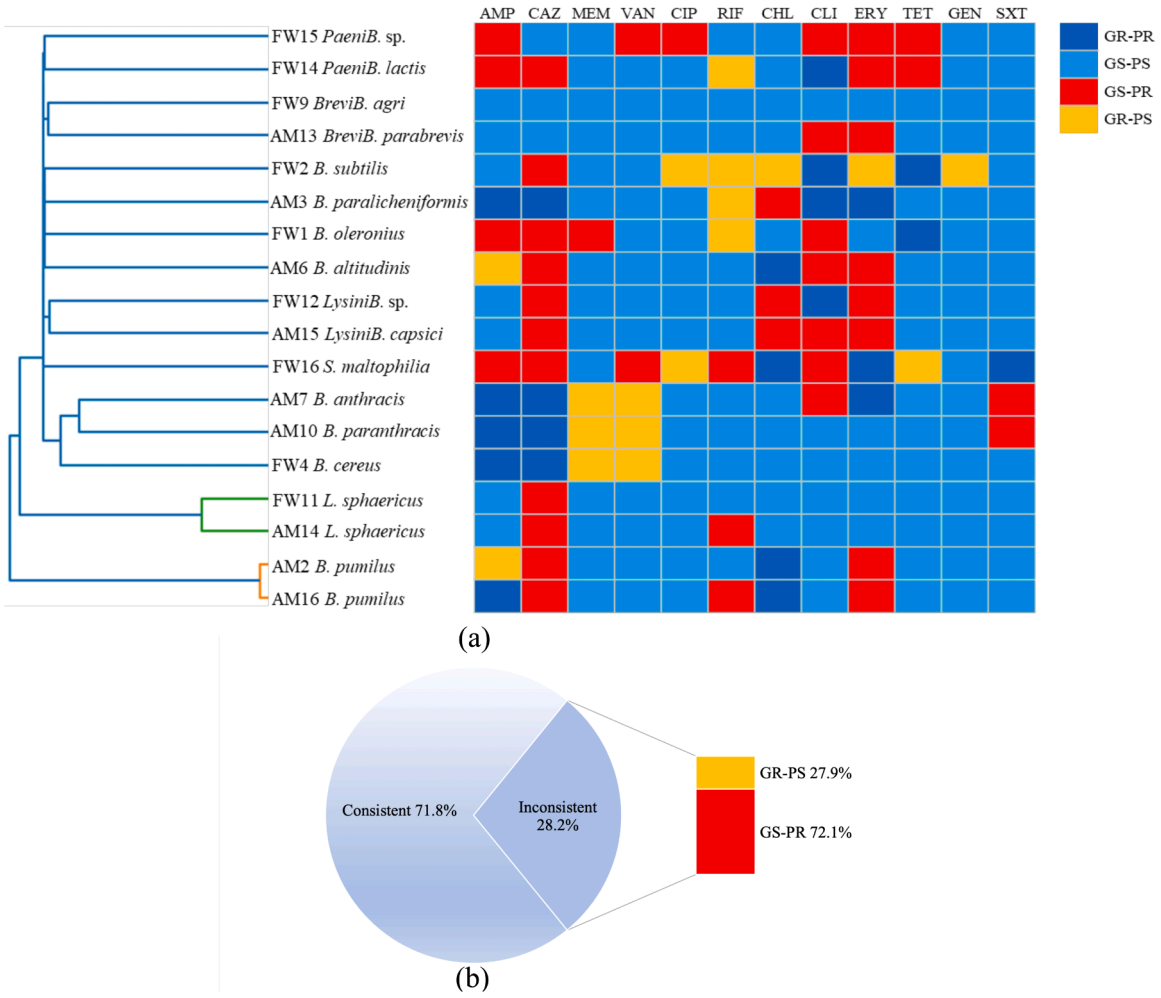

Fig. 2. (a) Comparison of genotypic and phenotypic resistance for selected isolates. GR and GS represent genotypic resistant and sensitive, respectively, based on identification of relative antibiotic resistance genes (ARGs). PR and PS represent phenotypic resistant (including intermediate resistant) and sensitive, respectively, based on antibiotic susceptibility test with antibiotics: ampicillin (AMP), ceftazidime (CAZ), meropenem (MEM), vancomycin (VAN), ciprofloxacin (CIP), rifampicin (RIF), chloramphenicol (CHL), clindamycin (CLI), erythromycin (ERY), tetracycline (TET), gentamicin (GEN), and sulfamethoxazole/trimethoprim (SXT). (b) Consistency of genotypic and phenotypic resistance.

between individual strains of the same species was attributable to variations in the phenotypic resistance.

3.4. Identification of mobile genetic elements

Extra-chromosomal DNA (ECD) were found in nine isolates affiliated to different species (Fig. 3). The number of ECD carried by these isolates varied from one to three. However, only one ECD was identified as a plasmid (pAMαl), which in an earlier study was found to be non-transferable in plasmid conjugation testing (Sun et al., 2023). Variation in the number of ECD was found among isolates of the same species. For instance, within the *B. pumilus* species, AM2 had one ECD, while AM21 had none. Similarly, in the case of *L. sphaericus*, AM 14 had one ECD, while FW11 had none. Notably, AM14 exhibited phenotypic resistance to RIF while FW11 did not. However, for AM14 no specific RIF resistance gene was identified. This suggests that the ECD carried by AM14 may play a role in conferring resistance to RIF. However, further research is needed to unravel the underlying genetic factors contributing to this.

No integrons were identified in the genomes of any of the bacteria analyzed. However, 14 isolates were found to harbor ISs on their chromosomes (Fig. 4). Among these, AM7 and AM10 were the only isolates with ISs on their ECD. Most of the predicted ISs are of *Bacillus* origin, with three from other origins, such as *Desulfitobacterium hafniense, Streptococcus iniae* and *Pseudomonas aeruginosa* (Table S2). For transposons, seven isolates carried putative composite transposons, and one unit transposon was identified in AM15 (Fig. 4 and Table S3). However, none of the identified ISs and transposons were flanking ARGs, with a closest distance of approximately 24 kb between the MGEs and ARGs. Therefore, these results indicate a limited possibility of transferring the identified ARGs with the detected MGEs. However, further experimental studies are required to validate and confirm the transferability of these ARGs.

4. Concluding remarks

Inconsistences emerged on comparing genotypic and phenotypic resistance for the dominant ARB isolated from digestates, with two main inconsistency scenarios: (i) ARGs identified, but isolates shown to be phenotypically sensitive, and (ii) ARGs absent, but isolates shown to be phenotypically resistant. The inconsistent cases, particularly the latter scenario, accounted for around 30 % of all cases studied. These results indicate that stand-alone molecular analysis of ARGs would result in non-negligible deviation in revealing the true antibiotic resistance level in AD environments. A combination of molecular and culture-dependent methods is therefore needed to fully reveal antibiotic resistance in AD processes. Nonetheless, the dominant ARB community isolated from AW and FW digestates appears to pose a limited risk of spread of antibiotic

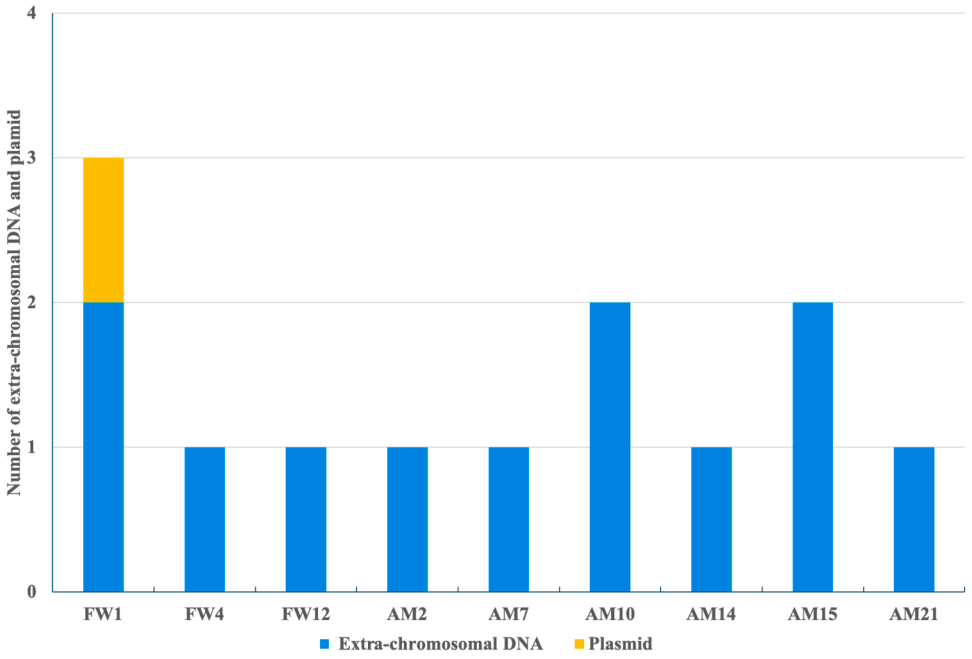

Fig. 3. Isolates with extra-chromosomal DNA and plasmids.

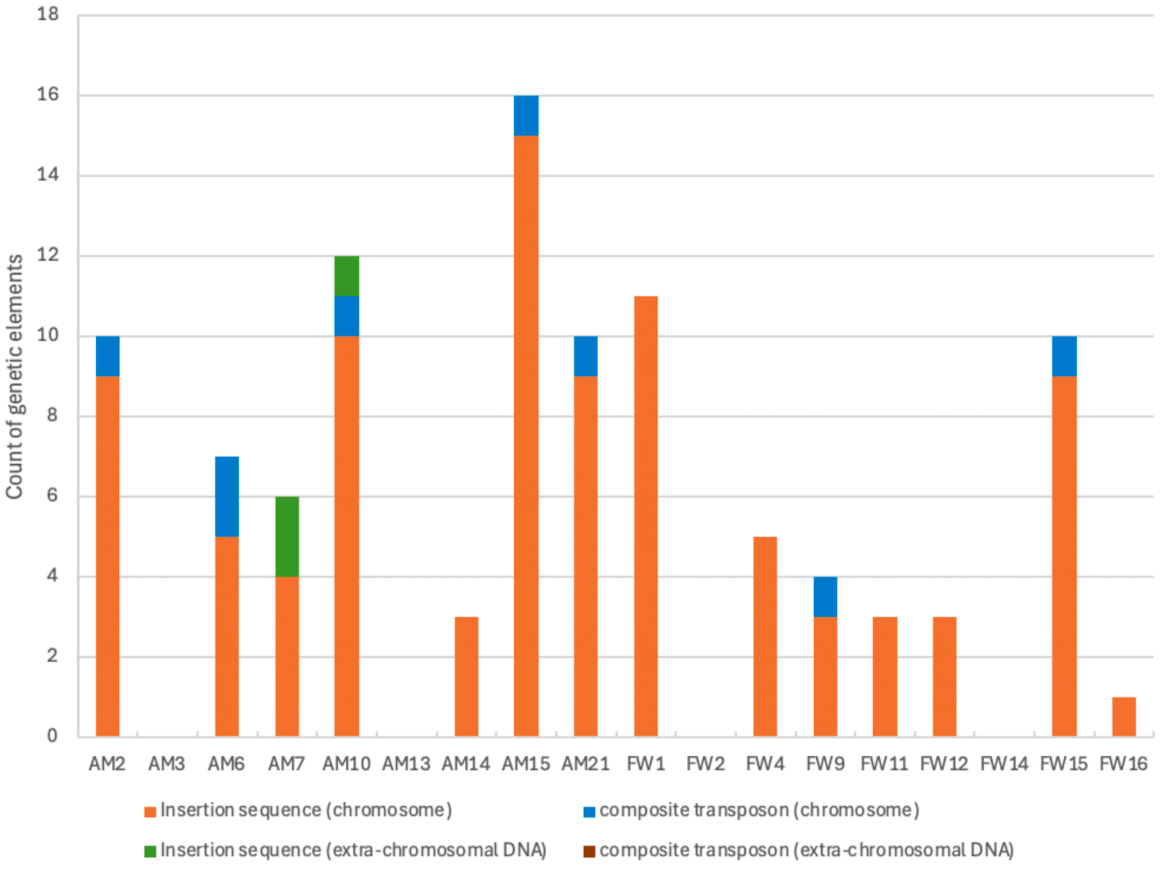

Fig. 4. Count of transposons and insertion sequences of selected isolates.

resistance due to low transferability of the ARGs identified. However, further experimental validation is required to confirm this observation.

In addition to employing a combination of culture-independent and culture-dependent methods to provide a more accurate assessment of antibiotic resistance levels in biogas digestate, complementary studies such as transcriptomics and proteomics could offer deeper insights into gene expression and functionality. Additionally, innovative techniques like ion chromatography, fluorescence-based methods, and nanoparticle-assisted detection (Muhammad et al., 2018, 2017) offer promising avenues for detecting ARG presence or activity. These

techniques may also help elucidate discrepancies between genotypic and phenotypic resistance, making them a valuable focus for future research. Moreover, advancements in bioinformatics have reduced reliance on existing databases. Tools such as fARGene (Berglund et al., 2019) and deepARG (Arango-Argoty et al., 2018) have been developed to identify uncharacterized resistance genes, even when their sequence similarity to known ARGs is low. However, the putative ARGs identified by these methods require further validation through experimental testing.

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CRediT authorship contribution statement

Schnürer Anna: Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Sun He:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Brandt Christian:** Writing – review & editing, Validation. **Levenfors Jolanta J.:** Validation, Supervision, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: He Sun reports financial support was provided by China Scholarship Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.117859.

Data availability

Data will be made available on request.

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