Science of the Total Environment 829 (2022) 154556

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Uncovering antimicrobial resistance in three agricultural biogas plants using plant-based substrates

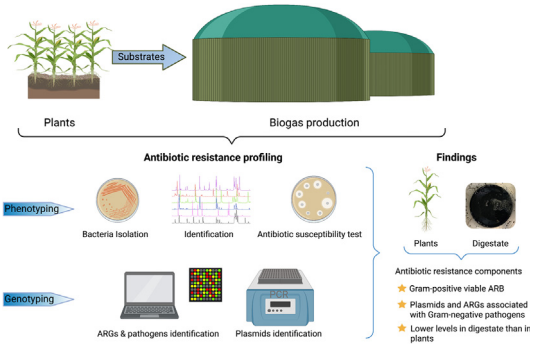
He Sun [a], Anna Schnürer [a,*], Bettina Müller [a], Bettina Mößnang [b], Michael Lebuhn [b], Oliwia Makarewicz [c]

[a] Department of Molecular Sciences, Swedish University of Agricultural Sciences, BioCenter, Box 7025, SE-750 07 Uppsala, Sweden
[b] Bavarian State Research Center for Agriculture, Central Department for Quality Assurance and Analytics, Lange Point 6, 85354 Freising, Germany
[c] Institute of Infectious Diseases and Infection Control, Jena University Hospital, Am Klinikum 1, 07747 Jena, Germany

HIGHLIGHTS

- Plant-based substrates for biogas production may contain AMR.
- ARB isolated from crops were mainly Gram-positive *Bacillus* spp.
- ARGs and plasmids associated with Gram-negative bacteria were detected in crops.
- Biogas digestate could pose a lower risk of spreading AMR than substrates per se.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 December 2021
Received in revised form 9 March 2022
Accepted 9 March 2022
Available online 16 March 2022

Editor: Huu Hao Ngo

Keywords:
Antimicrobial resistance
Agricultural biogas plant
Substrate
Digestate
Antibiotic-resistant bacteria
Antibiotic resistance genes

ABSTRACT

Antimicrobial resistance (AMR) is becoming an increasing global concern and the anaerobic digestion (AD) process represents a potential transmission route when digestates are used as fertilizing agents. AMR contaminants, e.g. antibiotic-resistant bacteria (ARB) and plasmid-mediated antibiotic resistance genes (ARGs) have been found in different substrates and AD systems, but not yet been investigated in plant-based substrates. AMR transfer from soils to vegetable microbiomes has been observed, and thus crop material potentially represents a so far neglected AMR load in agricultural AD processes, contributing to AMR spread. In order to test this hypothesis, this study examined the AMR situation throughout the process of three biogas plants using plant-based substrates only, or a mixture of plant-based and manure substrates. The evaluation included a combination of culture-independent and –dependent methods, i.e., identification of ARGs, plasmids, and pathogenic bacteria by DNA arrays, and phylogenetic classification of bacterial isolates and their phenotypic resistance pattern. To our knowledge, this is the first study on AMR in plant-based substrates and the corresponding biogas plant. The results showed that the bacterial community isolated from the investigated substrates and the AD processing facilities were mainly Gram-positive *Bacillus* spp. Apart from *Pantoea agglomerans*, no other Gram-negative species were found, either by bacteria culturing or by DNA typing array. In contrast, the presence of ARGs and plasmids clearly indicated the existence of Gram-negative pathogenic bacteria, in both substrate and AD process. Compared with substrates, digestates had lower levels of ARGs, plasmids, and culturable ARB. Thus, digestate could pose a lower risk of spreading AMR than substrates per se. In conclusion, plant-based substrates are associated with AMR, including culturable Gram-positive ARB and Gram-negative pathogenic bacteria-associated ARGs and plasmids. Thus, the AMR load from plant-based substrates should be taken into consideration in agricultural biogas processing.

* Corresponding author.
E-mail address: anna.schnurer@slu.se (A. Schnürer).

http://dx.doi.org/10.1016/j.scitotenv.2022.154556

1. Introduction

Anaerobic digestion (AD) is a mature technology that shows high performance in valorization of organic wastes, provides great potential for green energy production, and plays an important role in transition towards a sustainable society (Kougias and Angelidaki, 2018; Lebuhn et al., 2014). In AD, different types of organic wastes, including crop residues, animal manure, by-products from the food and feed industry, and sludge from wastewater treatment plants (WWTP), are degraded and mainly converted to biogas (Schnürer and Jarvis, 2018). In some countries, such as Germany, biogas is also produced from dedicated energy crops, such as corn silage and grass silage (Daniel-Gromke et al., 2018). In addition to biogas, the AD process also results in a digestate that can be used as biofertilizer in crop cultivation due to its high content of valuable nutrients, thus maintaining agricultural productivity at lower environmental cost compared with conventional chemical fertilizers (Al Seadi et al., 2013). To ensure soil health and food safety, the quality of the digestate in terms of nutrient content and levels of chemical and biological contaminants, e.g., heavy metals, weed seeds, and pathogens, needs to be assured prior to use (Corden et al., 2019; Risberg et al., 2017). In addition to risks associated with these contaminants, several recent studies of biogas digestate show that it can contain components contributing to antimicrobial resistance (AMR) spread, i.e., antibiotic residues (ARs), antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) (Gurmessa et al., 2020; Kanger et al., 2020; Mitchell et al., 2013; Schauss et al., 2016). The presence of these components in digestate suggests a potential route of AMR spread, and application of digestate as fertilizer has been shown to cause accumulation and increased AMR level in soil (Lu et al., 2021).

Spread of AMR, especially of extended-spectrum β-lactamases (ESBLs) and carbapenemases in Enterobacteriaceae, has become an increasing worldwide problem. This means that effectiveness of antibiotics in the outpatient and inpatient sector is increasingly at risk, resulting in increased morbidity and mortality but also hospital stays of excessive length and costs (Stewardson et al., 2016). Bacteria can acquire resistance through horizontal gene transfer (HGT) by uptake of genetic material or through mutations, which gives them a survival advantage and thus enables them to spread in a selective environment (Mell and Redfield, 2014). Mobile genetic elements (MGEs), such as integrons or transposons, play a crucial role in this process, as they can variably integrate many resistance genes (Gillings et al., 2008) and remain extremely mobile, which together with plasmid conjugation/transformation favor gene transfer even between different bacterial species. Horizontal transfer of ARGs has been observed in the AD process (Wolters et al., 2014), illustrating that the biogas bacterial community can represent a reservoir pool of ARGs. This could contribute to increasing contamination of surface waters and crops, and ultimately colonization of wildlife, pets, and humans, when digestate is applied to soil (Ewers et al., 2012). In order to tackle the increasing problems with AMR, several countries have launched initiatives aiming to reduce veterinary application of antibiotics in animal husbandry and their effects in the nutrient chain and the environment, such as the DART and Strama initiatives in Germany and Sweden, respectively (Bundesministerium für Gesundheit et al., 2020; Goverment Offices of Sweden, 2016).

Many studies have investigated the AD-associated AMR situation for agricultural wastes, mostly focusing on animal manures. So far, antibiotic-resistant Gram-negative pathogenic bacteria, such as *Escherichia/Shigella* (Schauss et al., 2016), *Enterococcus* (Glaeser et al., 2016), and *Acinetobacter* (Pulami et al., 2020), but also Gram-positive pathogens, such as *Clostridium perfringens* (Derongs et al., 2020), have been found in digestate from farm-scale biogas plants processing animal manures. Moreover, ARGs encoding for resistance to various classes of antibiotic agents and transferable plasmids have been found in different biogas plants using animal manure as substrate (Luo et al., 2017; Wolters et al., 2016a, 2014). However, the AMR situation in other agricultural sources, such as plant-based substrates, is still not clear. It has been found that AMR can transfer from soils to vegetable microbiomes (Zhang et al., 2019), suggesting that plant-based substrates potentially represent a so far neglected AMR load in agricultural AD processes, contributing to AMR spread. In general, AD-associated AMR studies to date have been conducted using either culture-independent methods, e.g., metagenomics and DNA arrays (Luo et al., 2017; Wallace et al., 2018; Zhang et al., 2015), or culture-dependent methods, e.g., ARB cultivation and characterization (Beneragama et al., 2013; Resende et al., 2014; Schauss et al., 2016; Sun et al., 2020). Both methods have their merits but also limitations in revealing the actual AMR situation, such as challenges in identification of unknown genes, complexity of gene expression, and non-culturable bacteria (Del Mar Lleò et al., 2003; Enne et al., 2006; Brandt et al., 2017; Zandri et al., 2012). Therefore, a combination of culture-independent and culture-dependent methods is necessary and can improve understanding of the actual AD-associated AMR situation and potential risks.

It is clear that AD digestate represents a potential AMR transmission route, but research in plant-derived digestates is so far quite limited and such work is needed to evaluate the risk of AMR spread. Therefore, the aim of the present study was to shed light on AMR situation in agricultural biogas plants using plant-based substrates alone or combined with manure, throughout the entire AD process, i.e., from substrates to primary/intermediate digestate and to final digestate. This aim was pursued using a combination of culture-independent and culture-dependent methods, including identification of ARGs, plasmids, and Gram-negative pathogenic bacteria by DNA arrays, and phylogenetic classification of cultured bacterial isolates and their phenotypic resistance to different antibiotics. To our knowledge, this is the first study on AMR in plant-based substrates and in the corresponding biogas production process.

2. Materials and methods

2.1. Samples and biogas plants operation

Fifteen samples, consisting of seven substrate samples and eight digestate samples, were taken from three farm-scale biogas plants in Germany. Details of samples and operating parameters of the biogas plants are shown in Table 1. At the time of sampling, biogas plant A (BPA) was using corn silage, grass silage, triticale silage, and sugar beet with average dry matter content of 58, 16, 18, and 8%, respectively, at an organic loading rate (OLR) of 1.5 kg volatile solids (VS) m^{-3} day^{-1}. This plant has two primary fermenters (900 m^3 each) in parallel and two secondary fermenters in series (1250 m^3 each), operating at 42 °C and 40 °C, respectively. Biogas plant B (BPB) was using corn-cob-mix (38%), corn silage (30%), grass silage (4%), sugar beet (24%), and cereal grains (4%) as substrate, at an OLR of 3.1 kg VS m^{-3} day^{-1}. This plant has two hydrolysis tanks (each 70 m^3) in series, followed by a main fermenter (1400 m^3) and two fixed-bed post reactors (each 60 m^3) in series, operating at 30 °C, 44 °C and 42 °C, respectively. Biogas plant C (BPC) was using corn silage (38%), poultry manure (28%), sugar beet (24%), and pig manure (10%) as substrate, at an OLR of 3.8 kg VS m^{-3} day^{-1}. The plant has one primary (1400 m^3) and one secondary fermenter (1400 m^3), operating at 48 °C and 45 °C, respectively. The total hydraulic retention time, including all reactors, was 215, 193, and 105 days in BPA, BPB, and BPC, respectively.

2.2. Bacterial isolation, identification and phylogeny

Plates of Todd Hewitt agar medium (THA; 30 g Todd Hewitt, 15 g agar, 1 L deionized water) were prepared. This medium was selected as it allows growth of most pathogenic microorganisms (MacFaddin, 1985). For each substrate and digestate sample, 1 g was suspended in 10 mL sterile saline (0.9% NaCl), mixed, and subjected to 10-fold serial dilution to 10^{-7}. Then 100 μL aliquots of each dilution were streaked on non-selective THA plates and the plates were incubated aerobically in darkness at 37 °C. After overnight incubation, colony-forming units (CFU) were counted for plates containing between 30 and 300 CFU. Morphologically different colonies from these plates were selected for subcultivation, identification, and antimicrobial susceptibility testing. Bacterial isolates were

sent to Bruker Daltonik (Bremen, Germany) for identification using ultraflex MALDI-TOF/TOF mass spectrometry with duplicate tests.

To construct a 16S rRNA-based phylogenetic tree for the retrieved isolates, the type strain sequence of each identified species was taken from the SILVA database (SILVA 138 SSU tax silva T). The sequences obtained were aligned using Uni-pro UGENE v33.0. The aligned file was processed by W-IQ-TREE for construction of phylogenetic trees using the maximum likelihood method (Nguyen et al., 2015).

2.3. DNA extraction

The digestate samples and poultry manure substrate were used directly for DNA extraction, without pre-treatment. The plant-based substrate samples were added to sterile saline solution (0.9% NaCl), vortexed, and treated in an ultrasonic water bath for 15 min, after which aliquots (0.2 mL) of the liquid were used for extraction. Genomic DNA was extracted with the FastDNA Spin kit for soil (Qbiogene, Illkrich, France) and purified with AMPure XP beads (Beckman Coulter, Inc. Brea, CA, USA), according to manufacturer's protocol. DNA concentrations were determined using the NanoQuant Plate and the Infinity Pro2000 Plate reader (both Tecan, Männedorf, Switzerland), as recommended by the supplier. For samples giving a DNA concentration less than 100 μg mL^{-1}, the DNA solution was concentrated using a SpeedVac centrifuge (Eppendorf, Hamburg, Germany) at room temperature for 30 min at 1400 rpm.

2.4. Molecular genotyping by DNA array

To detect ARGs in each substrate and digestate sample, microarray genotyping was performed using the CARB-Detect AS-2 on the ArrayMate Reader (both Alere Technologies, Waltham, USA) as described in a previous study (Braun et al., 2014). In brief, 62 different β-lactamase variants, 56 other resistance and virulence determinants, and 13 genus- and species-specific genes differentiating the Enterobacteriaceae *Escherichia coli* (distinguishing the enteroinvasive *E. coli* (EIEC)), *Klebsiella pneumoniae*, *Enterobacter* spp., *Citrobacter freundii/braakiia*, *Salmonella* spp., and *Shigella* spp., as well as the non-fermenters *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, were analyzed.

2.5. Plasmid incompatibility grouping

Plasmid incompatibility (Inc) grouping was based on a published method (Carattoli et al., 2005) including 18 primer pairs targeting the following replicons: FIA, FIB, FIC, HI1, HI2, I1, L/M, N, P, W, T, A/C, K, B/O, X, Y, FrepB, and FIIS. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining using the G-Box (Synoptics Ltd., Cambridge, UK).

2.6. Antimicrobial susceptibility test for isolates

An antimicrobial susceptibility test (AST) was conducted on all isolates obtained (Subdivision 2.2) using agar diffusion assays according to EUCAST guidelines (EUCAST, 2019), with ceftazidime (CAZ, 10 μg $disc^{-1}$), meropenem (MEM, 10 μg $disc^{-1}$), vancomycin (VAN, 5 μg $disc^{-1}$), colistin (CST, 10 μg $disc^{-1}$), ciprofloxacin (CIP, 5 μg $disc^{-1}$), tetracycline (TET, 30 μg $disc^{-1}$), and gentamicin (GEN, 10 μg $disc^{-1}$). The plates were incubated overnight at 37 °C, and the inhibition zone diameter was assessed and compared with the breakpoints of minimum inhibitory concentration recommended by the EUCAST guidelines (EUCAST, for 2020, https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents/) and the European Food and Safety Authorities (EFSA) (EFSA panel, 2012) for *Bacillus* spp., *Enterococci*, or *Staphylococci* where applicable, and categorized accordingly as resistant (R) or sensitive (S).

3. Results and discussion

3.1. Identification of isolates

In total, 46 morphologically different colonies (16 from the substrates, 30 from the digestates) were picked out from THA plates and subcultured for taxonomic identification. The identification revealed 17 different species, with eight and 11 species (two shared species) from the substrates and digestates, respectively (Fig. 1 and Table S1). The score values of identification using ultraflex MALDI-TOF/TOF mass spectrometry for each isolate are shown in Table S2. The majority of the isolates belonged to the genus *Bacillus* (eight species) and the closely related genera *Paenibacillus* and *Lysinibacillus* (five species) (Fig. 2). Other Gram-positive isolates affiliated with the genera *Staphylococcus*, *Enterococcus*, and *Streptococcus*. *Bacillus licheniformis* and *Lysinibacillus fusiformis* were frequently found in both grass and corn silage substrates, while *Bacillus cereus* was present only in the grass silage substrates. This is in line with previous findings that these species are commonly associated with plant silage (Driehuis et al., 2018). *Bacillus cereus* is a food-borne pathogen with certain strains shown to cause harm in humans and animals (Kotiranta et al., 2000), while *B. licheniformis* can cause abortion and mastitis in cattle (Nieminen et al., 2007). *Lysinibacillus fusiformis* has not been characterized as pathogen, but certain strains play a role in aerobic deterioration of corn stalk silage (Liu et al., 2013). *Staphylococcus lentus* was isolated from the poultry manure used by plant BPC, operating with a mix of poultry manure and corn silage. This bacterium is commonly isolated from poultry and

Table 1
Summary of samples analyzed.

No.	Biogas plant	OLR[a], kg (VS[b]) m^{-3} day^{-1}	Sampling source	Operating temperature, °C	Digester volume, m^3	HRT[c], d	NH_3[d], mg L^{-1}	Category
1	A	1.5	Grass silage (16%)	–	–	–	–	Substrate
2			Corn silage (58%)	–	–	–	–	Substrate
3			Primary fermenter 1	42	900	78	84	Digestate
4			Primary fermenter 2	42	900	78	84	Digestate
5			Secondary fermenter	40	1250	59	158	Digestate
6	B	3.1	Corn silage (30%)	–	–	–	–	Substrate
7			Corn-cob mix (38%)	–	–	–	–	Substrate
8			Grass silage (4%)	–	–	–	–	Substrate
9			Hydrolysis	30	140	40	1	Digestate
10			Fermenter	44	1400	150	903	Digestate
11			Fixed bed	42	120	3	930	Digestate
12	C	3.8	Corn silage (38%)	–	–	–	–	Substrate
13			Poultry manure (28%)	–	–	–	–	Substrate
14			Primary fermenter	48	1400	51	1088	Digestate
15			Secondary fermenter	45	1400	54	823	Digestate

[a] Organic loading rate.
[b] Volatile solids.
[c] Hydraulic retention time.
[d] Free ammonia level calculated according to Calli et al. (2005).

respective food products (Huber et al., 2011) and certain strains can infect humans and colonize animal skin (Hay and Sherris, 2020). Only one Gram-negative species was isolated from corn-cob mix: *Pantoea agglomerans*, a plant-colonizing bacterium with low pathogenic potential to humans (Dutkiewicz et al., 2016). However, *P. agglomerans* has been isolated from the human bloodstream, abscesses, etc., and shown to cause infections following penetrating trauma by vegetation (Cruz et al., 2007).

Bacterial species isolated from substrates and digestates were mostly not the same, although *B. licheniformis* and *B. oleronius* were found both in substrates and digestates (Fig. 1). Several species (*S. lentus*, *P. agglomerans*, *B. cereus*, *B. circulans*, *L. fusiformis*, and *P. amylolyticus*) were isolated only from substrates and not from digestates, which might indicate some inactivation during the AD process. In a previous study using selective isolation of *Staphylococcus* from biogas plants, a similar trend was seen, with many isolates from the substrate and none from the digestate (Glaeser et al., 2016).

Among the bacteria isolated from digestate samples, most belonged to the genus *Bacillus*, with the phylogenetically related species *B. licheniformis* and *B. subtilis* most frequently found (Fig. 1). *Bacillus* species are aerobes or facultative anaerobes, spore-forming, and are commonly detected in biogas fermenters, where they participate in e.g., decomposition of fat and carbohydrates (Tao et al., 2020). Their presence in digestate might be explained by spore formation enabling them to survive under the anaerobic conditions. Interestingly, *P. polymyxa*, found in the digestate (secondary fermenter) from BPA, is known to produce the non-ribosomal peptide antibiotic polymyxin (Naghmouchi et al., 2012), which could potentially allow for selection of colistin-resistant bacteria.

3.2. Antibiotic resistance of the isolates

To gain insights into the AD-associated AMR situation, all isolates obtained from the substrates and digestates were tested for antimicrobial susceptibility. Seven clinically relevant antibiotics with varying mechanism of action from different antibiotic classes were used: a) CAZ (cephalosporins), MEM (carbapenems), and VAN (glycopeptides), targeting bacterial cell walls, b) CST (polymyxins) targeting cell membranes, c) CIP (fluoroquinolones) targeting nucleic acid, and d) TET (tetracyclines) and GEN (aminoglycosides) inhibiting ribosomal protein biosynthesis. In total, 46 bacterial isolates were tested, and 44 of these displayed resistance to at least one of the test antibiotics (Table 2). Thus, the majority (95.7%) of the bacterial isolates retrieved under the selected isolation conditions were apparently ARB, even though no selective pressure was applied during isolation. This is consistent with findings in previous studies, in which *Bacillus* species also dominated an ARB community isolated from digestates produced in AD of food waste and animal manure (Schauss et al., 2016; Sun et al., 2020). However, Gram-negative pathogenic bacteria have also been found in manure-based substrates and corresponding AD processes, e.g., vancomycin-resistant *Enterococcus* (Glaeser et al., 2016), *Acinetobacter* with intrinsic resistance to β-lactamases (BLs) (Pulami et al., 2020), *Escherichia coli* resistant to ampicillin and ampicillin-sulbactam (Resende et al., 2014), and the genera *Escherichia/Shigella*, *Proteus*, *Citrobacter*, and *Serratia* resistant to amoxicillin, tetracycline, and ceftiofur (Schauss et al., 2016). The differences in taxonomic profile of the ARB community isolated in different studies can likely be explained by different substrate sources and/or operational conditions, and by laboratory-related variations in the ARB cultivation.

Antibiotic resistance can be categorized into intrinsic resistance and acquired resistance. In contrast to potentially transferable acquired resistance, intrinsic resistance is typically not transferable and carries no risk of AMR spread (Cox and Wright, 2013). Of the forms of antibiotic resistance identified in the present study, CST and VAN resistance are generally caused by intrinsic resistance because of their specific effective spectrum, i.e. CST and VAN inhibits only for Gram-negative and Gram-positive bacteria, respectively. All the bacterial isolates identified in this study, except for *Pantoea agglomerans*, were Gram-positive, explaining the broad CST resistance and VAN sensitivity observed among the isolates. VAN resistance has been observed previously, for *Bacillus* sp. carrying the *van*A gene responsible for VAN resistance in Enterococci (Fontana et al., 1997), but in the present study all *Bacillus* isolates showed sensitivity towards this antibiotic and only the Gram-negative *P. agglomerans* was resistant. In our previous investigation of biogas digestate originating from food waste and manure, all *Bacillus* spp. isolated were also sensitive to VAN (Sun et al.,

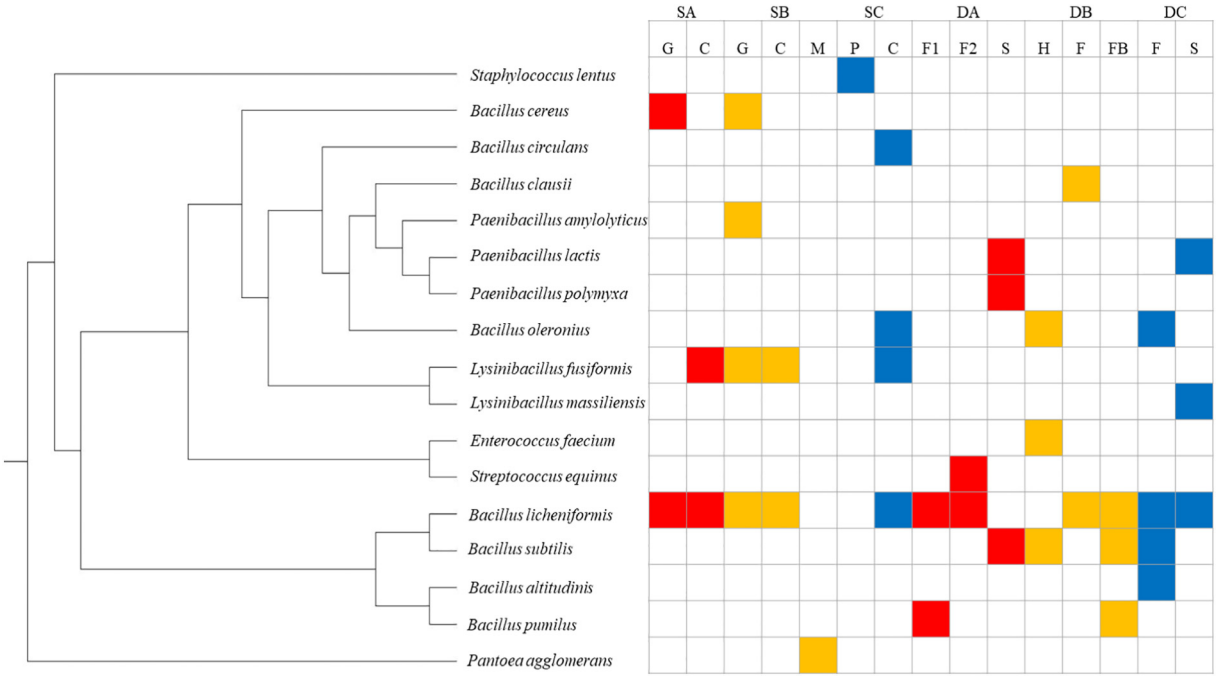


Fig. 1. Phylogenetic relationship of the species isolated and occurrence of their isolation from different sample sources. SA, SB, and SC represent substrate of biogas plant A, plant B, and plant C, respectively. DA, DB, and DC represent digestate from biogas plant A, plant B, and plant C, respectively. G represents grass silage, C corn silage, M corn-cob mix, and P poultry manure. F, F1, F2, and FB represent primary fermenter, primary fermenter1, primary fermenter2, and fixed bed, respectively. H and S represent hydrolysis and secondary fermenter, respectively. Red, yellow, and blue boxes represent occurrence of species isolation from biogas plant A, B, and C, respectively.

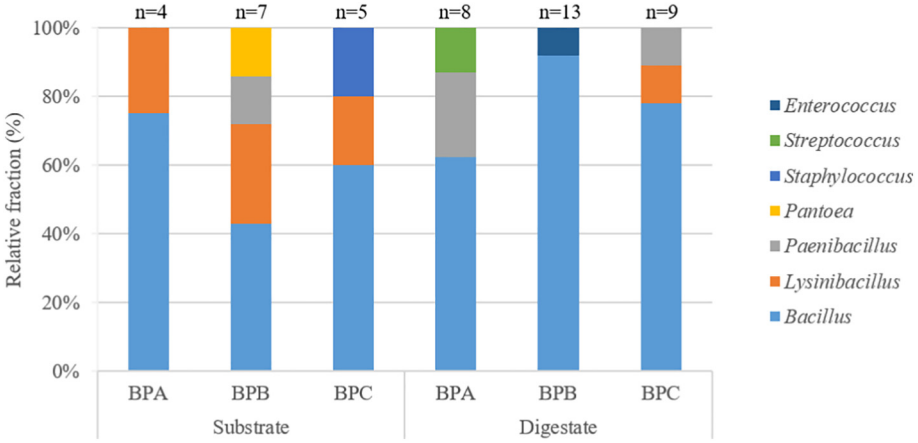


Fig. 2. Phylogenetic identification of isolates found in substrates and digestates from three biogas plants (BPA, BPB, and BPC). Numbers (n =) above bars indicate number of isolates investigated per sample source.

2020). Resistance other than to CST and VAN observed for the isolates represents possible acquired resistance, with many studies indicating transferability of possible relevant genes (Wishart et al., 2008). For example, TET resistance was observed for seven isolates in the present study, and such resistance is often acquired by HGT and frequently associated with MGEs (Grossman, 2016). Many mobile genes, e.g., *tet* (O) and *tet* (M), have been found in both Gram-negative and Gram-positive organisms (Roberts, 2011).

Comparing the presence of ARB in the different processing steps of the biogas plants, it was found that the resistance type in the final digestates was obviously less diverse than that in the substrates (Fig. 3). However, CAZ-, CST- and TET-resistant bacteria were still frequently found in the final digestates. These ARB were also present in the substrates, suggesting that they survived the AD process. Alternatively, these ARB might have taken over foreign genes or acquired resistance through gene mutations in the AD process. Other resistant bacteria, e.g., VAN-, CIP-, and GEN- resistant bacteria from substrates, and also MEM-resistant bacteria from early stage digestate, seemed to decrease throughout the process, indicating that the AD process might have an effect on removal of such resistance-associated ARB. This is in line with previous reports of decayed antibiotic resistance pattern in cultured ARB after 60 days of AD processing with cattle manure (Resende et al., 2014). However, it is inconsistent with results obtained in another study, where resistance patterns were similar for the isolates from input and output samples of 15 biogas plants treating animal manure and slurry (Schauss et al., 2016). The difference is likely caused by substrate variance, e.g., substrate sources and the resistance types they carry, but also by the operating conditions of different AD processes.

3.3. Detection of antibiotic resistance genes and Gram-negative bacteria

A DNA microarray was used to analyze the presence of ARGs for most relevant antibiotic classes, i.e., β-lactams (BLs), fluoroquinolones (only *qnr* efflux-pump related resistance), aminoglycosides, macrolides, trimethoprim, and sulfamethoxazole, virulence factors (mobile elements, efflux pumps and toxins), and also the presence of genes characteristically found only in certain Gram-negative bacteria (Braun et al., 2014). The results are shown in Table 3 and Fig. S1, where specific allelic variants are listed for genes of the identified BLs and the virulence factors. For the other classes of genes, presences are reported independent of the allelic or genetic variant. For genus-/species-specific genes, the corresponding bacterial taxonomy is listed if identified. For all substrate samples except poultry manure, the DNA concentration did not reach sufficient levels for identification of all the genes of interest, likely due to their physical characteristics complicating DNA extraction. Therefore, ARGs from these plant substrate samples could not be fully analyzed and may therefore be underestimated to a certain extent.

However, genes encoding different BLs were still detected in both plant and poultry manure substrates. In particular, these were the potential ESBLs, known as OXA-2, OXA-10, and OXA-18 and TEM and CME. In line with this result, the genes e.g., OXA-2, OXA-10, and TEM have been found in biogas plants processing animal manures (Luo et al., 2017; Schauss et al., 2015). Additionally, carbapenemases, such as OXA-48 and its close derivative OXA-181/232, and GOB and VIM were identified, which may support previous findings of carbapenem resistance in AD processes, such as meropenem-resistant *Bacillus oleronius* from a reactor processing food waste (Sun et al., 2020) and imipenem-resistant *Clostridium perfringens* from biogas plants treating pig manure (Derongs et al., 2020). This clearly shows that, in addition to the common presence of BL genes in fecal waste, OXA variants and metallo-BLs can also be found in plant materials. This study is the first to identify these genes in crop-based AD substrates. The BLs identified as carbapenemases are most commonly associated with Gram-negative pathogenic bacteria, while the presence of OXA-48 derivatives may result from a pathogenic Gram-negative *Shewanella* species that is widely distributed in freshwater environments and has been shown to harbor various OXA variants (Potron et al., 2011). OXA-2 and OXA-10, but also OXA-48, are frequently found in Enterobacteriaceae and *Pseudomonas aeruginosa* (Brandt et al., 2017), pathogens widespread in soil and water. Similarly, the metallo-BLs, VIM, and GOB have been detected in different species, including *P. aeruginosa* (Meletis, 2016; Palzkill, 2013). However, such Gram-negative bacteria were not detected by the DNA array (Table 3), or by bacterial cultivation (Fig. 1). In rare cases, such BLs have been found in Gram-positive *Bacillus* and close relatives. For example, a carbapenemase gene, bla_{KPC-2}, has been found to be carried by *Paenibacillus* spp. isolated from a wastewater treatment plant (Yang et al., 2016). Moreover, a strain of *Bacillus oleronius* isolated from digestate derived from food waste has been found to display resistance to meropenem, an agent of carbapenems, suggesting presence of BL (Sun et al., 2020). Thus the possibility that some BL genes were carried by Gram-positive *Bacillus* cannot be completely ruled out, although the variants identified in the present study have not been reported to be associated with *Bacillus*. However, it seems more likely that the BLs were associated with Gram-negative bacteria, even though the isolation procedure showed mainly Gram-positive species. This discrepancy in the results could have been caused by culture-dependent method limitations. It has been pointed out that the diversity of ARB in natural environments is often underestimated, as some bacteria are viable but non-culturable in the laboratory conditions (Del Mar Lleò et al., 2003; Zandri et al., 2012).

Other resistance determinants for e.g., fluoroquinolones and aminoglycosides were also found in both types of substrates. In addition, an integrase gene *intI3*, which is associated with class 3 integron, was detected in the corn-cob mix substrate from BPB. Integrons of class 1, 2, and 3 (*intI1*, *intI2*, and *intI3*) were the first integrons to be identified as associated with MGEs

Table 2
Resistance pattern of bacterial species isolated from different sources in three biogas plants, according to antimicrobial susceptibility test (AST) using the agar diffusing method for: ceftazidime (CAZ), meropenem (MEM), vancomycin (VAN), colistin (CST), ciprofloxacin (CIP), tetracycline (TET), and gentamicin (GEN).

Sample no.	Source[a]	Isolate no.	Species	CAZ	MEM	VAN	CST	CIP	TET	GEN
1	SA-G	1-1	*Bacillus cereus*	R[b]	S[c]	S	R	S	S	S
		1-2	*Bacillus licheniformis*	R	S	S	R	S	S	S
2	SA-C	2-1	*Lysinibacillus fusiformis*	S	S	S	S	S	S	S
		2-2	*Bacillus licheniformis*	R	S	S	R	S	S	R
3	DA-F1	3-1	*Bacillus licheniformis*	R	R	S	R	S	S	S
		3-2	*Bacillus licheniformis*	R	R	S	S	R	S	R
		3-3	*Bacillus pumilus*	R	S	S	R	S	S	S
4	DA-F2	4-1	*Bacillus licheniformis*	R	S	S	R	S	S	R
		4-2	*Streptococcus equinus*	R	S	S	S	S	S	R
5	DA-S	5-1	*Paenibacillus lactis*	R	S	S	R	S	R	S
		5-2	*Paenibacillus polymyxa*	S	S	S	R	S	S	S
		5-3	*Bacillus subtilis*	S	S	S	R	S	S	S
6	SB-C	6-1	*Bacillus licheniformis*	R	S	S	R	S	S	S
		6-2	*Lysinibacillus fusiformis*	S	S	S	S	S	R	S
7	SB-M	7-1	*Pantoea agglomerans*	S	S	R	S	S	S	S
8	SB-G	8-1	*Paenibacillus amylolyticus*	R	S	S	R	S	S	S
		8-2	*Bacillus cereus*	R	S	S	R	S	S	S
		8-3	*Lysinibacillus fusiformis*	R	S	S	R	S	R	R
		8-4	*Bacillus licheniformis*	R	S	S	R	R	S	S
9	DB-H	9-1	*Bacillus subtilis*	S	R	S	R	S	S	S
		9-2	*Enterococcus faecium*	R	R	S	R	S	S	R
		9-3	*Bacillus oleronius*	R	S	S	R	S	R	R
		9-4	*Bacillus subtilis*	R	S	S	R	S	S	R
10	DB-F	10-1	*Bacillus licheniformis*	S	S	S	R	S	S	S
		10-2	*Bacillus clausii*	R	S	S	R	S	S	S
		10-3	*Bacillus licheniformis*	S	S	S	R	S	S	S
		10-4	*Bacillus licheniformis*	S	S	S	R	S	S	S
11	DB-FB	11-1	*Bacillus pumilus*	R	S	S	S	S	S	S
		11-2	*Bacillus pumilus*	R	S	S	S	S	S	S
		11-3	*Bacillus licheniformis*	S	S	S	R	S	S	S
		11-4	*Bacillus licheniformis*	R	S	S	R	S	S	S
		11-5	*Bacillus subtilis*	S	S	S	R	S	S	S
12	SC-C	12-1	*Lysinibacillus fusiformis*	S	S	S	S	S	S	S
		12-2	*Bacillus circulans*	R	S	S	S	S	S	S
		12-3	*Bacillus licheniformis*	R	S	S	R	S	S	S
		12-4	*Bacillus oleronius*	R	S	S	S	S	S	S
13	SC-P	13-1	*Staphylococcus lentus*	R	S	S	S	R	R	R
14	DC-F	14-1	*Bacillus licheniformis*	R	S	S	R	S	S	S
		14-2	*Bacillus altitudinis*	R	S	S	S	S	S	S
		14-3	*Bacillus oleronius*	S	S	S	R	S	S	S
		14-4	*Bacillus subtilis*	S	S	S	R	S	S	S
		14-5	*Bacillus subtilis*	R	S	S	S	S	R	S
		14-6	*Bacillus subtilis*	S	S	S	R	S	S	S
15	DC-S	15-1	*Paenibacillus lactis*	R	S	S	R	S	R	S
		15-2	*Bacillus licheniformis*	R	S	S	R	S	S	S
		15-3	*Lysinibacillus massiliensis*	R	S	S	S	S	S	S

[a] SA, SB, and SC represent substrate of biogas plant A, plant B, and plant C, respectively. DA, DB, and DC represent digestate from plant A, plant B, and plant C, respectively. G represents grass silage, C corn silage, M corn-cob mix, and P poultry manure. F, F1, F2, and FB represent primary fermenter, primary fermenter1, primary fermenter2, and fixed bed, respectively. H and S represent hydrolysis and secondary fermenter, respectively.
[b] Resistant.
[c] Sensitive.

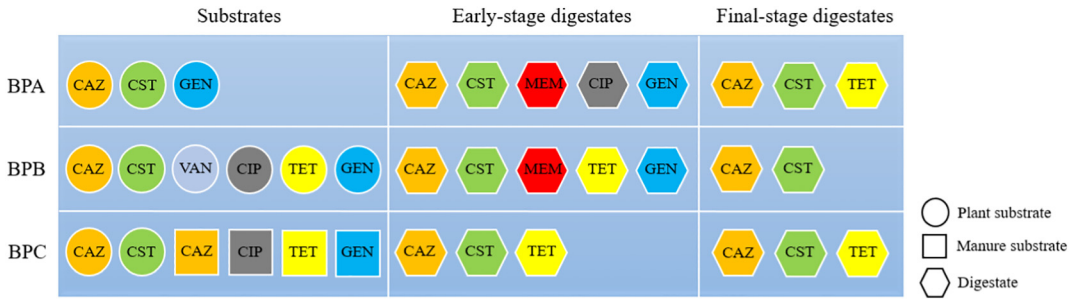


Fig. 3. Phenotypic resistance to ceftazidime (CAZ), meropenem (MEM), vancomycin (VAN), colistin (CST), ciprofloxacin (CIP), tetracycline (TET), and gentamicin (GEN) in antibiotic-resistant bacteria (ARB) isolated from substrates and digestates in three biogas plants (BPA, BPB, and BPC). Early-stage digestate is from primary fermenters, hydrolysis + fermenter, and primary fermenter in BPA, BPB, and BPC, respectively. Final-stage digestate is from secondary fermenter, fixed bed, and secondary fermenter in BPA, BPB, and BPC, respectively. Colors indicate resistance to different antibiotic agents, while shapes indicate the sampling source.

Table 3
Identification using ArrayMate of antibiotic resistance genes and Gram-negative bacterial genera/species from substrates and digestates of three biogas plants.

No.	Source[a]	β-Lactamases			Fluoroquinolones	Aminoglycosides	Macrolides	Trimethoprim	Sulfonamide	Virulence factors	Bacterial genus/species
		Narrow	ESBL	Carbapenemase							
1	SA-G	[b]									
2	SA-C	OXA-2	OXA-2, OXA-18	OXA-48	Positive	Positive		Positive			
3	DA-F1	TEM	TEM		Positive			Positive			
4	DA-F2	OXA-10	OXA-10		Positive					tnpISEcp1	
5	DA-S	OXA-10	OXA-10	OXA-48, TMB			Positive			tnpISEcp1	
6	SB-C										
7	SB-M	OXA-10	OXA-10, CME	GOB						intI3	
8	SB-G			VIM, OXA-181/232							
9	DB-H	OXA-10, OXA-1	OXA-10, OXA-1, OXA-18, CME	OXA-48		Positive					
10	DB-F										
11	DB-FB										
12	SC-C										
13	SC-P	OXA-10, TEM	OXA-10, TEM, OXA-18	OXA-48		Positive	Positive	Positive	Positive		
14	DC-F	TEM	TEM		Positive			Positive			
15	DC-S										

[a] SA, SB, and SC represent substrate of biogas plant A, plant B, and plant C, respectively. DA, DB, and DC represent digestate from plant A, plant B, and plant C, respectively. G represents grass silage, C corn silage, M corn-cob mix, and P poultry manure. F, F1, F2, and FB represent primary fermenter, primary fermenter1, primary fermenter2, and fixed bed, respectively. H and S represent hydrolysis and secondary fermenter, respectively.
[b] Blank cells indicate nothing was detected.

(Deng et al., 2015). They play an important role in ARG dissemination. The Class 3 integron shows a similar function to the class 1 integron, which is most frequently found in Gram-negative bacteria (Deng et al., 2015). Class 1 integrons have been found in many AD processes operating with WWTP sludge and animal manure substrates (Miller et al., 2016; Wolters et al., 2016b; Zou et al., 2020), as has as *intI2*, but less commonly (Wolters et al., 2016b). However, to our knowledge, *intI3* has not yet been found in biogas processes. Thus, this study may represent the first time finding of *intI3* in an AD process.

Comparing the presence of ARGs in the different processing steps of the biogas plants revealed lower levels in the intermediate and final digestate compared with the substrates, except for BPA (Table 3 and Fig. S1). This difference between the biogas plants may be explained by the higher free ammonia (NH_3) level in BPB and BPC (both ~1000 mg L^{-1}) than in BPA (<200 mg L^{-1}) (Table 1). It has been shown that an NH_3 concentration of about 600 mg L^{-1} can effectively reduce pathogen levels in the AD process (Ottoson et al., 2008; Park and Diez-Gonzalez, 2003). Moreover, high concentrations of NH_3 have been shown to decrease microbial diversity in the AD process (Müller et al., 2016; Peng et al., 2018), and greater reduction in ARGs have been observed at low microbial diversity (Ma et al., 2011; Sun et al., 2016). Alternatively, the higher process temperature in BPB and BPC than in BPA might have contributed to the removal of ARGs, as it has been shown that the removal rate of ARGs increases with increasing temperature (Sun et al., 2016). In general, this finding of reduced ARGs levels during the AD process is in line with results in previous studies. For example, ARGs encoding for resistance to sulfamethoxazole, ciprofloxacin, and enrofloxacin have been found to decline by up to 80% in a full-scale biogas plant processing cattle manure (Visca et al., 2021), and five sulfonamide- and tetracycline- resistance genes have been found to decrease significantly under mesophilic and thermophilic operations in batch AD processes treating pig manure (Zou et al., 2020).

3.4. Detection of plasmids

In total, the presence of 18 different Gram-negative plasmid groups based on their replicons (Inc grouping) was detected in both the substrate and digestate samples. Presence of plasmids has been detected previously in pig manure and the corresponding biogas facilities, and broad-host range plasmid groups (IncP-1, IncN, IncW, and IncQ) have been found in digestates (Wolters et al., 2016b). To our knowledge, the present work is the first to identify Gram-negative plasmid groups in plant-based substrates and the following AD process. In this study, the Inc groups FIB (n = 6), followed by W (n = 3) and K and B/O (each n = 2), were most frequently identified in the plant-based substrates. The Inc groups FIB, K, and B/O were also identified in the poultry manure substrate (Fig. 4). All these are particularly widespread in Gram-negative pathogenic bacteria, including genera such as *Escherichia*, *Salmonella*, *Shigella*, *Pseudomonas*, and *Providencia* (Fernández-López et al., 2006; Khajanchi et al., 2017; Rozwandowicz et al., 2018, 2017). Therefore, the identified plasmid groups, in addition to the identified ARGs, suggest presence of Gram-negative bacteria in both categories of substrate samples. IncFIB plasmids can encode both virulence factors and ARGs, e.g., *str*A, *sul*2, and *tet* (A) encode resistance to streptomycin, sulfonamide, and tetracycline, respectively (Han et al., 2012; Johnson et al., 2006), and have been shown to contribute to the virulence of extra-intestinal pathogenic *E. coli* (Johnson et al., 2006). IncW plasmids have a wide spectrum of antibiotic resistances with a broad host range (Fernández-López et al., 2006). Successful transfer and stable inheritance of IncW plasmids have been reported in many bacterial genera, most belonging to the Proteobacteria (Fernández-López et al., 2006). Inc groups K and B/O are highly related and both belong to the I complex based on morphological and serological similarities of their pili (Bradley, 1984). IncK plasmids are mainly associated with the spread of the most prevalent ESBL variants, bla_{CMY-2} and $bla_{CTX-M-14}$, in Europe (especially in Spain and the UK) and are frequently found in *E. coli* from animal sources (Rozwandowicz et al., 2017). IncB/O plasmids are less prevalent, but carry a greater variety of resistance genes, such as $bla_{CTX-M-1}$, bla_{CMY-2}, bla_{TEM-1}, *sul*1, *sul*1, etc. (Rozwandowicz et al., 2018). Additionally, members of the IncF group (IncFIA, IncFIB, and IncFIIS) were detected in some substrates, e.g., corn silage, at all three biogas plants. This group has been shown to be associated with ESBLs, such as highly prevalent $bla_{CTX-M-15}$ (Coque et al., 2008), but also carbapenemase KPC (Fu et al., 2019), and with the spread of plasmid-mediated genes, such as bla_{CMY} and bla_{DHA} (Villa et al., 2010), and quinolone and aminoglycoside resistance genes, such as *qnr* (Lascols et al., 2008) and *arm*A (Galimand et al., 2005). Therefore, the plasmids identified in the plant-based substrate, but also poultry manure, represent potential for transfer of resistance via HGT, including resistance to beta-lactams, sulfonamides, tetracyclines, quinolones, and aminoglycosides. The DNA-based ARG array showed fluoroquinolone and aminoglycoside resistance in the corn silage substrate at BPA and poultry manure at BPC, which could have been associated with

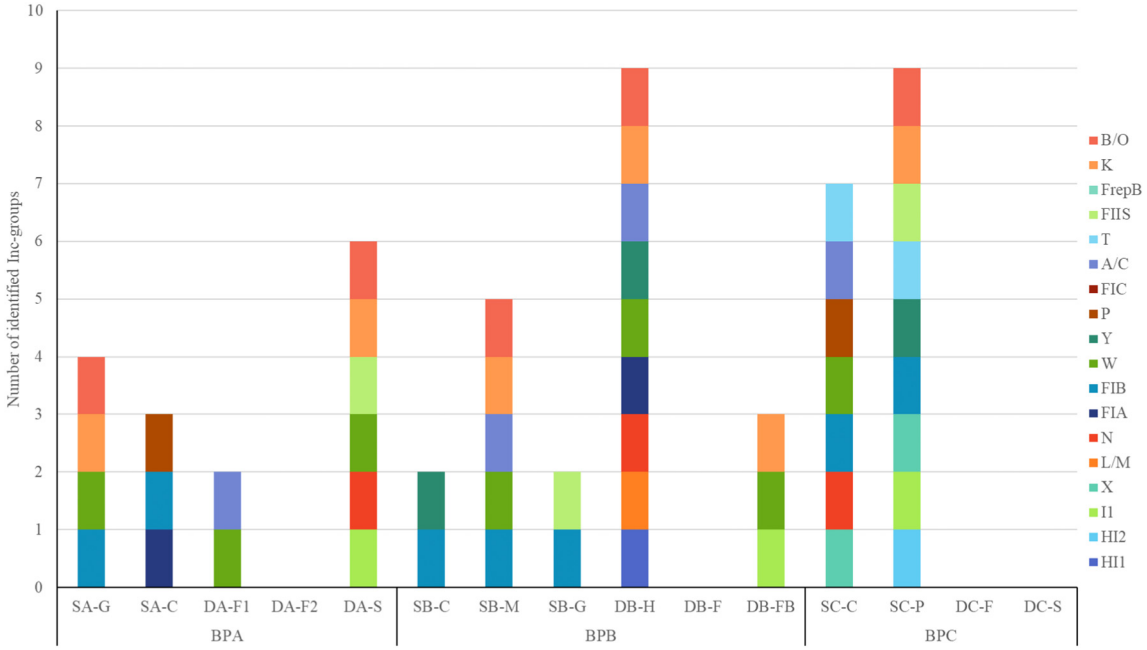


Fig. 4. Presence of identified plasmids and their incompatibility groups in substrate and digestate samples of three biogas plants (BPA, BPB, and BPC). SA, SB, and SC represent substrate of biogas plant A, plant B, and plant C, respectively. DA, DB, and DC represent digestate of plant A, plant B, and plant C, respectively. G represents grass silage, C corn silage, M corn-cob mix, and P poultry manure. F, F1, F2, and FB represent primary fermenter, primary fermenter1, primary fermenter2, and fixed bed, respectively. H and S represent hydrolysis and secondary fermenter, respectively.

the IncF group plasmids. However, the plasmid-associated BLs and carbapenemases reported in the studies cited above were not found in the present study. This could possibly have been caused by insufficient DNA concentrations in the plant-based substrates, resulting in underestimation of ARG variances. Alternatively, the identified BLs and carbapenemases could still be associated with the plasmid groups identified in the present study. A previous study on a manure-based biogas system showed that transferable antibiotic resistance plasmids in digestate often belong to the IncP-1ε subgroup (Wolters et al., 2014). In the present study, IncP plasmids were detected in the corn silage substrates at both BPA and BPC, and thus this plasmid group, combined with other highly mobile groups such as IncF and IncI, could undoubtedly contribute to HGT throughout the process. However, it is not clear whether these Gram-negative specific plasmid-mediated resistances can influence the ARB community isolated in this study (mainly Gram-positive *Bacillus*).

The number of Inc groups identified in the substrates varied, with lower numbers in plant substrates (n = 2–7; Fig. 4) compared with the manure-containing substrate (n = 9). This indicates a lower load of human-pathogenic Gram-negative species to the AD process via the plant substrates. Comparing the presence of identified Inc groups in the different processing steps of the biogas plants, plasmid removal was indicated in BPB and BPC throughout the process. In BPB, only Inc groups I1, W, and K were retained in the final digestate, and no Inc group was detected in BPC digestate. This was similar to the removal pattern of ARGs, and might be explained by a high level of NH_3 and/or temperature effectively reducing the level of Gram-negative pathogens. The plasmid removal results were in line with those in a previous study investigating eight full-scale biogas reactors using pig manure, which revealed presence of IncP-1 and low GC-content plasmids in samples from different steps of the biogas process, with a trend for lower levels in the fermenters compared with the manure (Wolters et al., 2016a). Among the digestate samples in the present study, the Inc groups W (n = 4) and K (n = 3) were most frequently detected. Both plasmid groups originated from the substrates and persisted throughout the process in BPA and BPB. This may indicate difficulty in removal of such plasmids during the AD process.

4. Conclusions

Plant-based substrates were found to be associated with AMR contamination, including culturable Gram-positive ARB (mainly Gram-positive *Bacillus* spp.), and Gram-negative pathogenic bacteria-associated ARGs and plasmids. The observed discrepancy for Gram-negative bacteria by culture-independent and culture-dependent methods might have been caused by culture-dependent method limitations. Alternatively, it could have been caused by Gram-positive bacteria harboring ARGs and plasmids typically detected in Gram-negative bacteria, which has been found in rare cases but seems unlikely for all the ARGs and plasmids identified in the present study. Conclusively, AMR factors in plant substrates should be considered in agricultural biogas processing, although lower levels of cultured ARB, ARGs, and plasmids were found in digestate compared with the raw substrates.

Funding

This work was supported by funding by the China Scholarship Council (CSC) [Grant No. 201606350190] and Swedish University of Agricultural Sciences.

CRediT authorship contribution statement

He Sun: Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Visualization; Writing original draft; Funding acquisition. **Anna Schnürer**: Project administration; Supervision; Validation; Formal analysis; Writing- review & editing; Funding acquisition. **Bettina Müller**: Conceptualization; Project administration; Supervision; Validation; Formal analysis; Writing-review & editing. **Bettina Mößnang**: Resources; Validation; Writing - review & editing. **Michael Lebuhn**: Resources; Validation; Writing - review & editing. **Olivia Makarewicz**: Conceptualization; Methodology; Supervision; Formal analysis; Validation; Visualization; Writing- review & editing.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154556.

References

Al Seadi, T., Drosg, B., Fuchs, W., Rutz, D., Janssen, R., 2013. Biogas digestate quality and utilization. The Biogas Handbook. Elsevier, pp. 267–301 https://doi.org/10.1533/9780857097415.2.267.

Beneragama, N., Iwasaki, M., Lateef, S.A., Yamashiro, T., Ihara, I., Umetsu, K., 2013. The survival of multidrug-resistant bacteria in thermophilic and mesophilic anaerobic co-digestion of dairy manure and waste milk. Anim. Sci. J. 84, 426–433. https://doi.org/10.1111/asj.12017.

Bradley, D.E., 1984. Characteristics and function of thick and thin conjugative pili determined by transfer-derepressed plasmids of incompatibility groups I1, I2, I5, B, K and Z. J. Gen. Microbiol. 130, 1489–1502. https://doi.org/10.1099/00221287-130-6-1489.

Brandt, C., Braun, S.D., Stein, C., Slickers, P., Ehricht, R., Makarewicz, O., 2017. In silico serine β-lactamases analysis reveals a huge potential resistome in environmental and pathogenic species. Sci. Rep. 7. https://doi.org/10.1038/srep43232.

Braun, S.D., Monecke, S., Thürmer, A., Ruppelt, A., Makarewicz, O., Ehricht, R., 2014. Rapid identification of carbapenemase genes in gram-negative bacteria with an oligonucleotide microarray-based assay. PLoS One 9, e102232. https://doi.org/10.1371/journal.pone.0102232.

<collab>Bundesministerium für Gesundheit Bundesministerium für Bildung und Forschung</collab>, Bundesministerium für Ernährung und Landwirtschaft, 2020. DART 2020. Fighting Antibiotic Resistance for the Good of Both Humans and Animals.

Calli, B., Mertoglu, B., Inanc, B., Yenigun, O., 2005. Effects of high free ammonia concentrations on the performances of anaerobic bioreactors. Process Biochem. 40, 1285–1292. https://doi.org/10.1016/j.procbio.2004.05.008.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods 63, 219–228. https://doi.org/10.1016/j.mimet.2005.03.018.

Coque, T.M., Novais, Â., Carattoli, A., Poirel, L., Pitout, J., Nordmann, P., 2008. Dissemination of clonally related Escherichia coli strains expressing extended-spectrum β-lactamase CTX-M-15. Emerg. Infect. Dis. 14, 195–200. https://doi.org/10.3201/eid1402.070350.

Corden, C., Bougas, K., Cunningham, E., Tyrer, D., Kreißig, J., Crookes, M., 2019. Digestate and compost as fertilisers: risk assessment and risk management options. Wood Environment & Infrastructure Solutions UK Limited. https://ec.europa.eu/environment/chemicals/reach/pdf/40039_Digestate_and_Compost_RMOA-Final_report_i2_20190208.pdf.

Cox, G., Wright, G.D., 2013. Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. Int. J. Med. Microbiol. https://doi.org/10.1016/j.ijmm.2013.02.009.

Cruz, A.T., Cazacu, A.C., Allen, C.H., 2007. Pantoea agglomerans, a plant pathogen causing human disease. J. Clin. Microbiol. 45, 1989–1992. https://doi.org/10.1128/JCM.00632-07.

Daniel-Gromke, J., Rensberg, N., Denysenko, V., Stinner, W., Schmalfuß, T., Liebetrau, J., 2018. Current developments in production and utilization of biogas and biomethane in Germany. Chemie-Ingenieur-Technik https://doi.org/10.1002/cite.201700077.

Del Mar Lleò, M., Bonato, B., Signoretto, C., Canepari, P., 2003. Vancomycin resistance is maintained in enterococci in the viable but nonculturable state and after division is resumed. Antimicrob. Agents Chemother. 47, 1154–1156. https://doi.org/10.1128/AAC.47.3.1154-1156.2003.

Deng, Y., Bao, X., Ji, L., Chen, L., Liu, J., Yu, G., 2015. Resistance integrons: class 1, 2 and 3 integrons. Ann. Clin. Microbiol. Antimicrob. https://doi.org/10.1186/s12941-015-0100-6.

Derongs, L., Druilhe, C., Ziebal, C., Le Maréchal, C., Pourcher, A.M., 2020. Characterization of clostridium perfringens isolates collected from three agricultural biogas plants over a one-year period. Int. J. Environ. Res. Public Health 17, 1–12. https://doi.org/10.3390/ijerph17155450.

Driehuis, F., Wilkinson, J.M., Jiang, Y., Ogunade, I., Adesogan, A.T., 2018. Silage review: animal and human health risks from silage. J. Dairy Sci. https://doi.org/10.3168/jds.2017-13836.

Dutkiewicz, J., Mackiewicz, B., Lemieszek, M.K., Golec, M., Milanowski, J., 2016. Pantoea agglomerans: a mysterious bacterium of evil and good. Part III. Deleterious effects: infections of humans, animals and plants. Ann. Agric. Environ. Med. https://doi.org/10.5604/12321966.1203878.

EFSA panel on additives and products or substances used in animal Feed (FEEDAP), 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10, 1–10. https://doi.org/10.2903/j.efsa.2012.2740.

Enne, V.I., Delsol, A.A., Roe, J.M., Bennett, P.M., 2006. Evidence of antibiotic resistance gene silencing in Escherichia coli. Antimicrob. Agents Chemother. 50, 3003–3010. https://doi.org/10.1128/AAC.00137-06.

EUCAST, 2019. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 9.0.

Ewers, C., Bethe, A., Semmler, T., Guenther, S., Wieler, L.H., 2012. Extended-spectrum β-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. Clin. Microbiol. Infect. https://doi.org/10.1111/j.1469-0691.2012.03850.x.

Fernández-López, R., Pilar Garcillán-Barcia, M., Revilla, C., Lázaro, M., Vielva, L., De La Cruz, F., 2006. Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. FEMS Microbiol. Rev. https://doi.org/10.1111/j.1574-6976.2006.00042.x.

Fontana, R., Ligozzi, M., Pedrotti, C., Padovani, E.M., Cornaglia, G., 1997. Vancomycin-resistant Bacillus circulans carrying the vanA gene responsible for vancomycin resistance in enterococci [1]. Eur. J. Clin. Microbiol. Infect. Dis. https://doi.org/10.1007/BF02471915.

Fu, P., Tang, Y., Li, G., Yu, L., Wang, Y., Jiang, X., 2019. Pandemic spread of blaKPC-2 among Klebsiella pneumoniae ST11 in China is associated with horizontal transfer mediated by IncFII-like plasmids. Int. J. Antimicrob. Agents 54, 117–124. https://doi.org/10.1016/j.ijantimicag.2019.03.014.

Galimand, M., Sabtcheva, S., Courvalin, P., Lambert, T., 2005. Worldwide disseminated armA aminoglycoside resistance methylase gene is borne by composite transposon Tn1548. Antimicrob. Agents Chemother. 49, 2949–2953. https://doi.org/10.1128/AAC.49.7.2949-2953.2005.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Stokes, H.W., 2008. The evolution of class 1 integrons and the rise of antibiotic resistance. J. Bacteriol. 190, 5095–5100. https://doi.org/10.1128/JB.00152-08.

Glaeser, S.P., Sowinsky, O., Brunner, J.S., Dott, W., Kämpfer, P., 2016. Cultivation of vancomycin-resistant enterococci and methicillin-resistant staphylococci from input and output samples of German biogas plants. FEMS Microbiol. Ecol. 92, 10. https://doi.org/10.1093/femsec/fiw010.

Government Offices of Sweden, 2016. Swedish Strategy to Combat Antibiotic Resistance, pp. 1–24.

Grossman, T.H., 2016. Tetracycline antibiotics and resistance. Cold Spring Harb. Perspect. Med. 6, a025387. https://doi.org/10.1101/cshperspect.a025387.

Gurmessa, B., Pedretti, E.F., Cocco, S., Cardelli, V., Corti, G., 2020. Manure anaerobic digestion effects and the role of pre- and post-treatments on veterinary antibiotics and antibiotic resistance genes removal efficiency. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2020.137532.

Han, J., Lynne, A.M., David, D.E., Tang, H., Xu, J., Foley, S.L., 2012. DNA sequence analysis of plasmids from multidrug resistant Salmonella enterica serotype Heidelberg isolates. PLoS One 7, e51160. https://doi.org/10.1371/journal.pone.0051160.

Hay, C.Y., Sherris, D.A., 2020. Staphylococcus lentus sinusitis: a new sinonasal pathogen. Ear Nose Throat J. 99, NP62–NP63. https://doi.org/10.1177/0145561319848990.

Huber, H., Ziegler, D., Pflüger, V., Vogel, G., Zweifel, C., Stephan, R., 2011. Prevalence and characteristics of methicillin-resistant coagulase-negative staphylococci from livestock, chicken carcasses, bulk tank milk, minced meat, and contact persons. BMC Vet. Res. 7, 1–7. https://doi.org/10.1186/1746-6148-7-6.

Johnson, T.J., Siek, K.E., Johnson, S.J., Nolan, L.K., 2006. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian Escherichia coli strains. J. Bacteriol. 188, 745–758. https://doi.org/10.1128/JB.188.2.745-758.2006.

Kanger, K., Guilford, N.G.H., Lee, H.W., Nesbø, C.L., Truu, J., Edwards, E.A., 2020. Antibiotic resistome and microbial community structure during anaerobic co-digestion of food waste, paper and cardboard. FEMS Microbiol. Ecol. 96, 6. https://doi.org/10.1093/femsec/fiaa006.

Khajanchi, B.K., Hasan, N.A., Choi, S.Y., Han, J., Zhao, S., Foley, S.L., 2017. Comparative genomic analysis and characterization of incompatibility group FIB plasmid encoded virulence factors of Salmonella enterica isolated from food sources. BMC Genomics 18, 1–14. https://doi.org/10.1186/s12864-017-3954-5.

Kotiranta, A., Lounatmaa, K., Haapasalo, M., 2000. Epidemiology and pathogenesis of Bacillus cereus infections. Microbes Infect. 2, 189–198. https://doi.org/10.1016/S1286-4579(00)00269-0.

Kougias, P.G., Angelidaki, I., 2018. Biogas and its opportunities—a review. Front. Environ. Sci. Eng. https://doi.org/10.1007/s11783-018-1037-8.

Lascols, C., Podglajen, I., Verdet, C., Gautier, V., Gutmann, L., Cambau, E., 2008. A plasmid-borne Shewanella algae gene, qnrA3, and its possible transfer in vivo between Kluyvera ascorbata and Klebsiella pneumoniae. J. Bacteriol. 190, 5217–5223. https://doi.org/10.1128/JB.00243-08.

Lebuhn, M., Munk, B., Effenberger, M., 2014. Agricultural biogas production in Germany - from practice to microbiology basics. Energy. Sustain. Soc. https://doi.org/10.1186/2192-0567-4-10.

Liu, Q.H., Shao, T., Zhang, J.G., 2013. Determination of aerobic deterioration of corn stalk silage caused by aerobic bacteria. Anim. Feed Sci. Technol. 183, 124–131. https://doi.org/10.1016/j.anifeedsci.2013.05.012.

Lu, Y., Li, J., Meng, J., Zhang, J., Zhuang, H., Shan, S., 2021. Long-term biogas slurry application increased antibiotics accumulation and antibiotic resistance genes (ARGs) spread in agricultural soils with different properties. Sci. Total Environ. 759. https://doi.org/10.1016/j.scitotenv.2020.143473.

Luo, G., Li, B., Li, L.G., Zhang, T., Angelidaki, I., 2017. Antibiotic resistance genes and correlations with microbial community and metal resistance genes in full-scale biogas reactors as revealed by metagenomic analysis. Environ. Sci. Technol. 51, 4069–4080. https://doi.org/10.1021/acs.est.6b05100.

Ma, Y., Wilson, C.A., Novak, J.T., Riffat, R., Aynur, S., Pruden, A., 2011. Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class i integrons. Environ. Sci. Technol. 45, 7855–7861. https://doi.org/10.1021/es200827t.

MacFaddin, J.F., 1985. Media for Isolation-cultivation-identification-maintenance of Medical Bacteria. vol. 1. Williams & Wilkins, Baltimore, MD.

Meletis, G., 2016. Carbapenem resistance: overview of the problem and future perspectives. Ther. Adv. Infect. Dis. 3, 15–21. https://doi.org/10.1177/2049936115621709.
Mell, J.C., Redfield, R.J., 2014. Natural competence and the evolution of DNA uptake specificity. J. Bacteriol. https://doi.org/10.1128/JB.01293-13.
Miller, J.H., Novak, J.T., Knocke, W.R., Pruden, A., 2016. Survival of antibiotic resistant bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic digesters. Front. Microbiol. 7, 263. https://doi.org/10.3389/fmicb.2016.00263.
Mitchell, S.M., Ullman, J.L., Teel, A.L., Watts, R.J., Frear, C., 2013. The effects of the antibiotics ampicillin, florfenicol, sulfamethazine, and tylosin on biogas production and their degradation efficiency during anaerobic digestion. Bioresour. Technol. 149, 244–252. https://doi.org/10.1016/j.biortech.2013.09.048.
Müller, B., Sun, L., Westerholm, M., Schnürer, A., 2016. Bacterial community composition and fhs profiles of low- and high-ammonia biogas digesters reveal novel syntrophic acetate-oxidising bacteria. Biotechnol. Biofuels 9, 1–18. https://doi.org/10.1186/s13068-016-0454-9.
Naghmouchi, K., Hammami, R., Fliss, I., Teather, R., Baah, J., Drider, D., 2012. Colistin a and colistin B among inhibitory substances of Paenibacillus polymyxa JB05-01-1. Arch. Microbiol. 194, 363–370. https://doi.org/10.1007/s00203-011-0764-z.
Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274. https://doi.org/10.1093/molbev/msu300.
Nieminen, T., Rintaluoma, N., Andersson, M., Taimisto, A.M., Ali-Vehmas, T., Salkinoja-Salonen, M., 2007. Toxinogenic Bacillus pumilus and bacillus licheniformis from mastitic milk. Vet. Microbiol. 124, 329–339. https://doi.org/10.1016/j.vetmic.2007.05.015.
Ottoson, J.R., Schnürer, A., Vinnerås, B., 2008. In situ ammonia production as a sanitation agent during anaerobic digestion at mesophilic temperature. Lett. Appl. Microbiol. 46, 325–330. https://doi.org/10.1111/j.1472-765X.2007.02317.x.
Palzkill, T., 2013. Metallo-β-lactamase structure and function. Ann. N. Y. Acad. Sci. 1277, 91–104. https://doi.org/10.1111/j.1749-6632.2012.06796.x.
Park, G.W., Diez-Gonzalez, F., 2003. Utilization of carbonate and ammonia-based treatments to eliminate Escherichia coli O157:H7 and Salmonella typhimurium DT104 from cattle manure. J. Appl. Microbiol. 94, 675–685. https://doi.org/10.1046/j.1365-2672.2003.01899.x.
Peng, X., Zhang, S.Y., Li, L., Zhao, X., Ma, Y., Shi, D., 2018. Long-term high-solids anaerobic digestion of food waste: effects of ammonia on process performance and microbial community. Bioresour. Technol. 262, 148–158. https://doi.org/10.1016/j.biortech.2018.04.076.
Potron, A., Poirel, L., Nordmann, P., 2011. Origin of OXA-181, an emerging carbapenem-hydrolyzing oxacillinase, as a chromosomal gene in Shewanella xiamenensis. Antimicrob. Agents Chemother. 55, 4405–4407. https://doi.org/10.1128/AAC.00681-11.
Pulami, D., Schauss, T., Eisenberg, T., Wilharm, G., Blom, J., Glaeser, S.P., 2020. Acinetobacter baumannii in manure and anaerobic digestates of German biogas plants. FEMS Microbiol. Ecol. 96, 176. https://doi.org/10.1093/femsec/fiaa176.
Resende, J.A., Silva, V.L., de Oliveira, T.L.R., de Oliveira Fortunato, S., da Costa Carneiro, J., Diniz, C.G., 2014. Prevalence and persistence of potentially pathogenic and antibiotic resistant bacteria during anaerobic digestion treatment of cattle manure. Bioresour. Technol. 153, 284–291. https://doi.org/10.1016/j.biortech.2013.12.007.
Risberg, K., Cederlund, H., Pell, M., Arthurson, V., Schnürer, A., 2017. Comparative characterization of digestate versus pig slurry and cow manure – chemical composition and effects on soil microbial activity. Waste Manag. 61, 529–538. https://doi.org/10.1016/j.wasman.2016.12.016.
Roberts, M.C., 2011. Mechanisms of bacterial antibiotic resistance and lessons learned from environmental tetracycline-resistant bacteria. Antimicrobial Resistance in the Environment. John Wiley & Sons, Ltd, pp. 93–121 https://doi.org/10.1002/9781118156247.ch7.
Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488.
Rozwandowicz, M., Brouwer, M.S.M., Zomer, A.L., Bossers, A., Harders, F., Hordijk, J., 2017. Plasmids of distinct IncK lineages show compatible phenotypes. Antimicrob. Agents Chemother. 61. https://doi.org/10.1128/AAC.01954-16.
Schauss, T., Glaeser, S.P., Gütschow, A., Dott, W., Kämpfer, P., 2015. Improved detection of extended spectrum beta-lactamase (ESBL)-producing Escherichia coli in input and output samples of German biogas plants by a selective pre-enrichment procedure. PLoS One 10, 1–24. https://doi.org/10.1371/journal.pone.0119791.
Schauss, T., Wings, T.K., Brunner, J.S., Glaeser, S.P., Dott, W., Kämpfer, P., 2016. Bacterial diversity and antibiotic resistances of abundant aerobic culturable bacteria in input and output samples of 15 German biogas plants. J. Appl. Microbiol. 121, 1673–1684. https://doi.org/10.1111/jam.13277.
Schnürer, A., Jarvis, Å., 2018. Substrates. Microbiology of the Biogas Process. ISBN: 9789157695468, p. 41.
Stewardson, A.J., Allignol, A., Beyersmann, J., Graves, N., Schumacher, M., Falcone, C., 2016. The health and economic burden of bloodstream infections caused by antimicrobial-susceptible and non-susceptible Enterobacteriaceae and Staphylococcus aureus in European hospitals, 2010 and 2011: a multicentre retrospective cohort study. Eurosurveillance 21. https://doi.org/10.2807/1560-7917.ES.2016.21.33.30319.
Sun, H., Bjerketorp, J., Levenfors, J.J., Schnürer, A., 2020. Isolation of antibiotic-resistant bacteria in biogas digestate and their susceptibility to antibiotics. Environ. Pollut. 266, 115265. https://doi.org/10.1016/j.envpol.2020.115265.
Sun, W., Qian, X., Gu, J., Wang, X.J., Duan, M.L., 2016. Mechanism and effect of temperature on variations in antibiotic resistance genes during anaerobic digestion of dairy manure. Sci. Rep. 6, 1–9. https://doi.org/10.1038/srep30237.
Tao, Y., Ersahin, M.E., Ghasimi, D.S.M., Ozgun, H., Wang, H., van Lier, J.B., 2020. Biogas productivity of anaerobic digestion process is governed by a core bacterial microbiota. Chem. Eng. J. 380. https://doi.org/10.1016/j.cej.2019.122425.
Villa, L., García-Fernández, A., Fortini, D., Carattoli, A., 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. J. Antimicrob. Chemother. 65, 2518–2529. https://doi.org/10.1093/jac/dkq347.
Visca, A., Caracciolo, A.B., Grenni, P., Patrolecco, L., Rauseo, J., Spataro, F., 2021. Anaerobic digestion and removal of sulfamethoxazole, enrofloxacin, ciprofloxacin and their antibiotic resistance genes in a full-scale biogas plant. Antibiotics 10, 502. https://doi.org/10.3390/antibiotics10050502.
Wallace, J.S., Garner, E., Pruden, A., Aga, D.S., 2018. Occurrence and transformation of veterinary antibiotics and antibiotic resistance genes in dairy manure treated by advanced anaerobic digestion and conventional treatment methods. Environ. Pollut. 236, 764–772. https://doi.org/10.1016/j.envpol.2018.02.024.
Wishart, D.S., Knox, C., Guo, A.C., Cheng, D., Shrivastava, S., Hassanali, M., 2008. DrugBank: a knowledgebase for drugs, drug actions and drug targets. Nucleic Acids Res. 36, 901–906. https://doi.org/10.1093/nar/gkm958.
Wolters, B., Ding, G.C., Kreuzig, R., Smalla, K., 2016a. Full-scale mesophilic biogas plants using manure as C-source: bacterial community shifts along the process cause changes in the abundance of resistance genes and mobile genetic elements. FEMS Microbiol. Ecol. 92, 1–17. https://doi.org/10.1093/femsec/fiv163.
Wolters, B., Kyselková, M., Krögerrecklenfort, E., Kreuzig, R., Smalla, K., 2014. Transferable antibiotic resistance plasmids 1 from biogas plant digestates often belong to the IncP-1ε subgroup. Front. Microbiol. 5. https://doi.org/10.3389/fmicb.2014.00765.
Wolters, B., Widyasari-Mehta, A., Kreuzig, R., Smalla, K., 2016b. Contaminations of organic fertilizers with antibiotic residues, resistance genes, and mobile genetic elements mirroring antibiotic use in livestock? Appl. Microbiol. Biotechnol. 100, 9343–9353. https://doi.org/10.1007/s00253-016-7742-y.
Yang, F., Mao, D., Zhou, H., Luo, Y., 2016. Prevalence and fate of carbapenemase genes in a wastewater treatment plant in northern China. PLoS One 11, 156383. https://doi.org/10.1371/journal.pone.0156383.
Zandri, G., Pasquaroli, S., Vignaroli, C., Talevi, S., Manso, E., Biavasco, F., 2012. Detection of viable but non-culturable staphylococci in biofilms from central venous catheters negative on standard microbiological assays. Clin. Microbiol. Infect. 18. https://doi.org/10.1111/j.1469-0691.2012.03893.x.
Zhang, T., Yang, Y., Pruden, A., 2015. Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. Appl. Microbiol. Biotechnol. 99, 7771–7779. https://doi.org/10.1007/s00253-015-6688-9.
Zhang, Y.J., Hu, H.W., Chen, Q.L., Singh, B.K., Yan, H., He, J.Z., 2019. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. Environ. Int. 130, 104912. https://doi.org/10.1016/j.envint.2019.104912.
Zou, Y., Xiao, Y., Wang, H., Fang, T., Dong, P., 2020. New insight into fates of sulfonamide and tetracycline resistance genes and resistant bacteria during anaerobic digestion of manure at thermophilic and mesophilic temperatures. J. Hazard. Mater. 384. https://doi.org/10.1016/j.jhazmat.2019.121433.