energies

MDPI

Article

Metagenomic Analysis of the Long-Term Synergistic Effects of Antibiotics on the Anaerobic Digestion of Cattle Manure

Izabela Wolak [1], Małgorzata Czatzkowska [1], Monika Harnisz [1], Jan Paweł Jastrzębski [2], Łukasz Paukszto [3], Paulina Rusanowska [4], Ewa Felis [5] and Ewa Korzeniewska [1,*]

1 Department of Water Protection Engineering and Environmental Microbiology, Faculty of Geoengineering, University of Warmia and Mazury in Olsztyn, Prawocheńskiego 1, 10-720 Olsztyn, Poland; izabela.koniuszewska@uwm.edu.pl (I.W.); malgorzata.czatzkowska@uwm.edu.pl (M.C.); monika.harnisz@uwm.edu.pl (M.H.)
2 Department of Physiology, Genetics and Plant Biotechnology, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10-957 Olsztyn, Poland; jan.jastrzebski@uwm.edu.pl
3 Department of Botany and Nature Protection, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Plac Łódzki 1, 10-721 Olsztyn, Poland; pauk24@gmail.com
4 Department of Environmental Engineering, University of Warmia and Mazury in Olsztyn, Warszawska 117, 10-950 Olsztyn, Poland; paulina.jaranowska@uwm.edu.pl
5 Department of Environmental Biotechnology, Faculty of Energy and Environmental Engineering, Silesian University of Technology, Akademicka 2, 44-100 Gliwice, Poland; ewa.felis@polsl.pl
* Correspondence: ewa.korzeniewska@uwm.edu.pl; Tel.: +48-89-523-37-52

Abstract: The conversion of cattle manure into biogas in anaerobic digestion (AD) processes has been gaining attention in recent years. However, antibiotic consumption continues to increase worldwide, which is why antimicrobial concentrations can be expected to rise in cattle manure and in digestate. This study examined the long-term synergistic effects of antimicrobials on the anaerobic digestion of cattle manure. The prevalence of antibiotic resistance genes (ARGs) and changes in microbial biodiversity under exposure to the tested drugs was investigated using a metagenomic approach. Methane production was analyzed in lab-scale anaerobic bioreactors. *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* were the most abundant bacteria in the samples. The domain *Archaea* was represented mainly by methanogenic genera *Methanothrix* and *Methanosarcina* and the order *Methanomassiliicoccales*. Exposure to antibiotics inhibited the growth and development of methanogenic microorganisms in the substrate. Antibiotics also influenced the abundance and prevalence of ARGs in samples. Seventeen types of ARGs were identified and classified. Genes encoding resistance to tetracyclines, macrolide–lincosamide–streptogramin antibiotics, and aminoglycosides, as well as multi-drug resistance genes, were most abundant. Antibiotics affected homoacetogenic bacteria and methanogens, and decreased the production of CH_4. However, the antibiotic-induced decrease in CH_4 production was minimized in the presence of highly drug-resistant microorganisms in AD bioreactors.

Keywords: anaerobic digestion; antibiotics; biodiversity; cattle manure; synergistic effect

Citation: Wolak, I.; Czatzkowska, M.; Harnisz, M.; Jastrzębski, J.P.; Paukszto, Ł.; Rusanowska, P.; Felis, E.; Korzeniewska, E. Metagenomic Analysis of the Long-Term Synergistic Effects of Antibiotics on the Anaerobic Digestion of Cattle Manure. *Energies* **2022**, *15*, 1920. https://doi.org/10.3390/en15051920

Academic Editors: Timo Kikas, Abrar Inayat and Lisandra Rocha Meneses

Received: 16 February 2022
Accepted: 4 March 2022
Published: 6 March 2022

1. Introduction

Methane fermentation (anaerobic digestion, AD) is a promising technology for stabilizing organic matter, including cattle manure from livestock production [1]. Anaerobic digestion leads to the production of environmentally friendly biogas containing methane (CH_4) as well as digestate ($_D$) [2]. Digestate is used mainly as organic fertilizer in agriculture [3]. However, antibiotics are widely used in livestock farms to promote animal growth, prevent disease and treat bacterial infections [4]. The use of antibiotics as growth promoters has been banned worldwide, excluding in China and Brasil. In the European Union, the use of bacteriostatic agents as growth promoters or feed additives in livestock production was banned in 2003 (Regulation (EC) No. 1831/2003 of 22 September 2003) [5]. Despite

the above, antimicrobials are still overused in agriculture, in particular in the livestock industry [6]. Broad-spectrum bacteriostatic drugs are most frequently used in agriculture and veterinary medicine [7]. The most popular classes of antibiotics include beta-lactams (such as amoxicillin, AMO) [8], fluoroquinolones (such as enrofloxacin, ENRO) [9], and nitroimidazole derivatives (such as metronidazole, MET) [10]. These antimicrobials inhibit the AD process, induce changes in microbial communities, promote the spread of drug resistance (DR), and influence the efficiency of biogas and CH_4 production [11]. Research has demonstrated that fluoroquinolones are highly persistent in the environment, and that the efficiency of fluoroquinolone removal during AD is close to zero [12]. In the livestock industry, MET is used mainly to treat infections caused by anaerobic bacteria [13]. This antimicrobial can be accumulated for up to 42 days in the host organism [10]. The main metabolic pathway of MET involves the oxidation of two side chains of the imidazole ring and glucuronidation. Metronidazole can be introduced to AD bioreactors with feces, and it can spread to the environment when soil is fertilized with digestate [14]. Amoxicillin is not highly persistent in the environment due to its specific chemical structure and the presence of a ring that is easily degraded by H^+, OH^-, Ca^{2+}, Mg^{2+} and Fe^{2+} ions [15]. However, even trace amounts of drug transformation products (TPs) in the environment can promote the spread of antibiotic resistance [16]. The presence and spread of broad-spectrum beta-lactamases in the environment pose a particularly serious threat to public health around the world [17].

Most antimicrobial substances detected in cattle manure are composed of several compounds. The above can be attributed to the fact that pharmaceuticals are widely used in livestock production and are excreted with urine and feces [18]. The overuse of antibiotics in the livestock industry has contributed to DR and the transmission of antibiotic resistance genes (ARGs) in the environment. Drug resistance also promotes the spread of ARGs in AD bioreactors where cattle manure containing drug residues is fermented [19]. The long-term synergistic effects of antibiotics and other parameters of the AD process on the fate of ARGs have not been fully elucidated because AD involves complex microbial communities that degrade chemical compounds and produce biogas and CH_4. Antibiotics indirectly influence methanogenic bacteria [20], but the relationships between methanogenic *Archaea*, bacteria, ARGs, and CH_4 production remain insufficiently investigated. Anaerobic digestion can pose a serious threat to public health, and it can contribute to the transmission of ARGs in the environment and the spread of DR, which is why the safety of the AD process should be thoroughly analyzed. Antibiotic resistance genes have been classified as persistent organic pollutants that are more harmful to the environment than antibiotics, which also gives serious cause for concern [21]. According to [22], human activities, expressed by the size of livestock populations in industrial farms, significantly influence the ARG profile in the environment, including in drinking water. Water contaminated with ARGs is administered to livestock, cattle manure is fermented in bioreactors, and the resulting digestate is used as agricultural fertilizer, which completes the cycle of acquisition and spread of ARGs in the environment. Research has demonstrated that the copy numbers of various ARG groups can increase in response to specific parameters of the AD process [23]. The associated risks require in-depth analysis, especially since the long-term synergistic effects of several antibiotics on the AD process have been poorly researched in the literature. The literature from the last decade in which the synergistic effect of drugs on the mesophilic AD process was investigated is summarized in Table 1.

The aim of the present study was to determine the long-term synergistic effects of a combination of AMO, ENRO and MET on mesophilic AD of cattle manure, including the efficiency of CH_4 production and the accumulation of volatile fatty acids (VFAs). Changes in the structure of microbial communities that participate in AD, and the spread and fate of ARGs were also examined. These parameters were analyzed in response to different combinations of antibiotics, different antibiotic concentrations, and different periods of antibiotic exposure. The combined effect of organic pollutants such as antibiotics may pose ecological risks and influence ARG profile in the environment. The study will provide

valuable insights into changes in the succession of methanogenic communities in fermented biomass during long-term exposure to antibiotics. The results will be used to identify the main problem areas and propose effective solutions for improving the efficiency of ARGs removal from digestate. The environmental threats associated with the spread of ARGs from digested cattle manure were also evaluated. The study will contribute valuable information to the existing knowledge base, which can be useful in reducing environmental pollution with ARGs. This study evaluates the anaerobic digestion environmental impacts of antibiotics to make the integration process more environmentally sustainable and identifies challenges and future prospects. The study relied on metagenomic techniques, which are the most effective tools for characterizing microbial communities and analyzing changes in microbial biodiversity.

Table 1. The summary of the literature from the last decade, about synergistic effect of drugs on the mesophilic AD process.

Kind of Substrate	Antibiotics Used in Investigate	Antibiotics Combinations Concentration	Effect of Antibiotics	References
Seed sludge	Short-term effect of sulfamethoxazole and tetracycline, erythromycin and sulfamethoxazole, erythromycin and tetracycline	0, 2, 20, 50, 100, 250, and 500 mg/L	Inhibition of CH_4 production in reactors fed with erythromycin-sulfamethoxazole and sulfamethoxazole-tetracycline and weak inhibition of CH_4 production in reactors fed with the mixture of erythromycin-tetracycline	[24]
Synthetic wastewater	Short-term effect of three antibiotics with four combinations; sulfamethoxazole-tetracycline; erythromycin-sulfamethoxazole; erythromycin-tetracycline and erythromycin-tetracycline-sulfamethoxazole	0, 1, 10, 25, 50, 100 and 250 mg/L	Inhibition of biogas production; microorganisms' development of resistance to antibiotics	[25]
Synthetic wastewater	Short-term effect of erythromycin and sulfamethoxazole mixture	In 10 stages, respectively (mg/L): 0.1 and 0.5; 0.2 and 5; 0.5 and 5; 0.5 and 10; 1 and 10, 1 and 15; 1.5 and 15; 1.5 and 20; 2 and 20; 2.5 and 25;	Inhibition of biogas production; microorganisms' development of resistance to antibiotics	[26]
Synthetic wastewater	Long-term effect of erythromycin-tetracycline-sulfamethoxazole and sulfamethoxazole-tetracycline	0.5; 5; 10; 15; 20; 25; 40 mg/L for SMX; 0.1; 0.2; 0.5; 1; 1.5; 2; 2.5; 3 mg/L for erythromycin-tetracycline	Increasing antibiotic concentrations has a negative impact on the microbial community structure and function in anaerobic wastewater treatment; increase of AR	[27]
Sewage sludge from a municipal WWTP	Amoxicillin, metronidazole, and ciprofloxacin	Static conditions—In first variant, respectively [mg/kg]; 2, 16 and 1024, in second variant: 1, 8, and 512; and last variant: 0.25, 2, and 512, semi-continuous conditions, respectively: 16, 8, and 2 mg/kg	Synergistic effect of antibiotics causes inhibition of CH_4 production and accumulation of VFAs	[28]

2. Materials and Methods

2.1. Experimental Setup and Sampling

Cattle manure was subjected to AD in 2 L digesters operating under dynamic, semi-continuous conditions. The process digester was fed with substrate containing a mixture of AMO, ENRO and MET. The antibiotics were chosen based on the results of our previous research [23]. In the process bioreactor, biomass was subjected to AD in the presence of AMO, ENRO and MET. In the control bioreactor, biomass was fermented without antibiotics. In the process bioreactor, AD was conducted in seven experimental series (I–VII) that differed in the concentrations of the antibiotics added to biomass Table 2. Antibiotic concentrations were gradually increased in successive experimental series.

Table 2. Antibiotic concentrations (μg/mL $_D$) and ID numbers of the samples collected from the experimental reactor (experimental series I–VII) and the control reactor (CS) without antibiotics.

Experimental Series	Antibiotic Concentrations (μg/mL $_D$)			Collected Samples	Sample ID
	AMO	ENRO	MET		
Series I	1	0.25	0.25	Sample 1	S1 I
				Sample 2	S2 I
				Sample 3	S3 I
				Control sample	CS I
Series II	2	0.5	0.5	Sample 1	S1 II
				Sample 2	S2 II
				Sample 3	S3 II
Series III	2.5	0.75	0.75	Sample 1	S1 III
				Sample 2	S2 III
Series IV	5	1.5	1.5	Sample 1	S1 IV
				Sample 2	S2 IV
				Control sample	CS IV
Series V	10	3	3	Sample 1	S1 V
				Sample 2	S2 V
Series VI	16	4	4	Sample 1	S1 VI
				Sample 2	S2 VI
				Sample 3	S3 VI
Series VII	32	8	8	Sample 1	S1 VII
				Sample 2	S2 VII
				Sample 3	S3 VII
				Control sample	CS VII

Cattle manure was collected in the field at the Agricultural Experiment Station in Bałdy, Poland, operated by the University of Warmia and Mazury in Olsztyn. The collected manure was stored at a temperature of 5 °C until analysis. The analyzed substrate had the following characteristics: total solids (TS)—107.5 ± 29.0 mg TS/g; volatile solids (VS)—84.2 ± 22.2 mg VS/g; pH—7.75 ± 0.4; total phosphorus (TP)—1.0 ± 0.3 mg TP/g of TS; total nitrogen (TN)—4.1 ± 1.6 mg TN/g of TS. The inoculum (anaerobic sludge) was obtained from a laboratory methane fermentation reactor fed with cattle manure and *Sida hermaphrodita* silage. Anaerobic sludge had the following characteristics: 35.2 ± 4.8 mg TS/g; 23.2 ± 2.1 mg VS/g; pH of 7.9 ± 0.9; 0.8 ± 0.6 mg TP/g of TS; 5.0 ± 0.9 mg TN/g of TS. The digester was operated at a daily organic loading rate of 2.8 kg × m^{-3} × d^{-1}.

Hydraulic retention time (HRT) was 28 days. The digesters were equipped with mechanical stirrers, feeding and discharge systems, and they were coupled with the Automatic Methane Potential Test System (AMPTS II, Bioprocess control, Lund, Sweden) which measured CH_4 production. Gas was normalized for standard temperature (273.2 K) and pressure (1.01325 bar). The AD process was conducted at mesophilic temperature (37 °C), and the substrate was stirred for 30 s at 100 rpm at 10min intervals. The experiment lasted 417 days, with two replicates per bioreactor. To monitor the digestion process, the substrates were sampled twice a week to determine their pH, FOS/TAC ratio (the FOS

value denotes VFA content, and the TAC value denotes the estimated buffer capacity of the sample), and the content of TS, VS, TN and TP.

The content of VFAs was determined with the use of a gas chromatograph (Brüker, 450-GC) with a flame ionization detector (FID) based on a previously described method [29]. FOS/TAC ratio was determined with the TitraLab AT1000 Series Titrator (Hatch). The content of TS and vs. in biomass samples was determined according to APHA [30]. The content of TN and TP in mineralized samples was determined in HachLange cuvette tests. All measurements were performed in duplicate.

Microbial diversity was determined by metagenomic analysis at the beginning, in the middle and at the end of the AD process in seven experimental series. For this purpose, digestate samples were collected in the first week of AD from each experimental series (sample No. 1), in the middle of each experimental series (sample No. 2) and in the last week of each experimental series (sample No. 3). At least two digestate samples were collected in duplicate in each experimental series. The samples were numbered (1 to 3) and labeled with the number of the corresponding experimental series (I to VII) based on increasing concentrations of the tested antibiotics Table 1. The concentrations of the antibiotics added to the experimental digester increased after doubling the digester's hydraulic volume. The collected samples and the adopted nomenclature are described in Table 1. The substrate fed to the control digester (two replicates) was not supplemented with antibiotics (control samples, CS); Table 1. To compare changes in the structure of microbial communities during the AD process, control samples were collected from experimental series I at the beginning of AD, from series IV in the middle of AD, and from series VII at the end of AD.

2.2. Genomic DNA Isolation

Total DNA was isolated in triplicate from digestate samples of 1 g collected from each reactor using the MP Biomedicals™ FastDNA™ SPIN Kit for Soil (MP Biomedicals™, Solon, OH, USA). DNA was isolated according to the manufacturer's instructions. The quality and yield of the extracted DNA were verified with the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. DNA Sequencing

The DNA from each sample was used to construct a shotgun library with the TruSeq Nano DNA Library Kit for Illumina (Macrogen, Amsterdam, The Netherlands) with paired-end 2 × 150 bp sequence reads and 350 bp insert. Binary base call (BCL) files were converted to FASTQ with the bcl2fastq Illumina package. The prepared libraries were sequenced on the NovaSeq6000 Illumina platform. Twenty-one DNA samples generated approximately 3.3 Gbp of metagenomic data, and the entire dataset had a size of 144.1 Gbp. Metagenomics datasets were deposited in the European Nucleotide Archive (ENA) database under accession numbers PRJEB48924. The quality of paired-end metagenomic sequences were evaluated in the Kneaddata v. 0.7.6 software (https://github.com/biobakery/kneaddata, accessed on 12 November 2021). In the first step, low-quality and rich adapter sequences were filtered with the Trimmomatic tool [31] in the Kneaddata v. 0.7.6 software (Huttenhower Lab, Harvard Chan Center for the Microbiome in Public Health, Boston, MA, USA) pipeline based on the following parameters '2:30:10:8:TRUE SLIDINGWINDOW:4:20 MINLEN:75'. In the second step, the reads were aligned to bovine contaminants with a known reference genome (NCBI GenBank accession No. GCA_000003055.5, accessed on 12 November 2021). Unmapped sequences were classified for metagenomic analyses. To determine bacterial distribution the remaining reads were mapped to bacterial reference sequences using bowtie2 software v. 2.4.5, (accessed on November 2021) [32]. Bacterial diversity was estimated by Metaphlan v. 2.0 (Huttenhower Lab) [33] and visualized by circlize [34] packages in R v. 4.0.2 (R Core Team, 2017, Vienna, Austria). The Kneaddata and Metaphlan software packages were used as components of the Biobakery v.0.15.1 pipeline (https://github.com/biobakery/biobakery_workflows, accessed on 12 November 2021).

The abundance of ARGs was determined based on the number of trimmed paired-end reads in ARG-OAP v.2.0 software (Environmental Microbiome Engineering and Biotechnology Laboratory, The University of Hong Kong) [35]. The number of ARG-like sequences was normalized to the number of metagenome and ARG sequences in each sample to compare ARG levels across digestate samples. ARG levels were expressed in terms of "total metagenome sequences" (reads per million reads, ppm) [36] or "total ARG-like sequences" (%).

2.4. Statistical Analysis

Statistical analyses were performed in the Statistica program (ver. 13.1., StatSoft, Kraków, Poland, accessed on 10 December 2021). Data were visualized with the use of GraphPad Prism software (GraphPad Software, CA, USA). The correlations between CH_4 production, microorganisms, ARG types, and VFA production were determined by calculating Spearman's rank correlation coefficients at a significance level of $p \leq 0.05$.

3. Results and Discussion

3.1. Long-Term Synergistic Effects of AMO, ENRO and MET on CH_4 Production and VFA Concentrations

Anerobic digestion is a process composed of four mutually dependent stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis [37]. Bacteria and methanogenic microorganisms are responsible for the efficiency of CH_4 production during AD. The first three stages of AD involve bacteria, whereas methanogens are responsible for the last stage of the process and CH_4 production [11]. Bacteria and methanogens enter into syntrophic interactions to produce the required substrates for growth and development. These interactions are essential to maximize CH_4 yields [26]. The efficiency of CH_4 production is determined by numerous factors, including the type of added antibiotics, antibiotic concentrations, temperature inside AD bioreactors, and duration of AD [38,39].

The effects of long-term exposure to increasing concentrations of a mixture of AMO, ENRO and MET on the inhibition of CH_4 production in manure digestates were analyzed in seven experimental series; see Section 2.1 and Figure 1. This approach was adopted to determine the long-term influence antibiotics on the efficiency of CH_4 production. Methane production was expressed in liters per kilogram of volatile solids (L/kg VS).

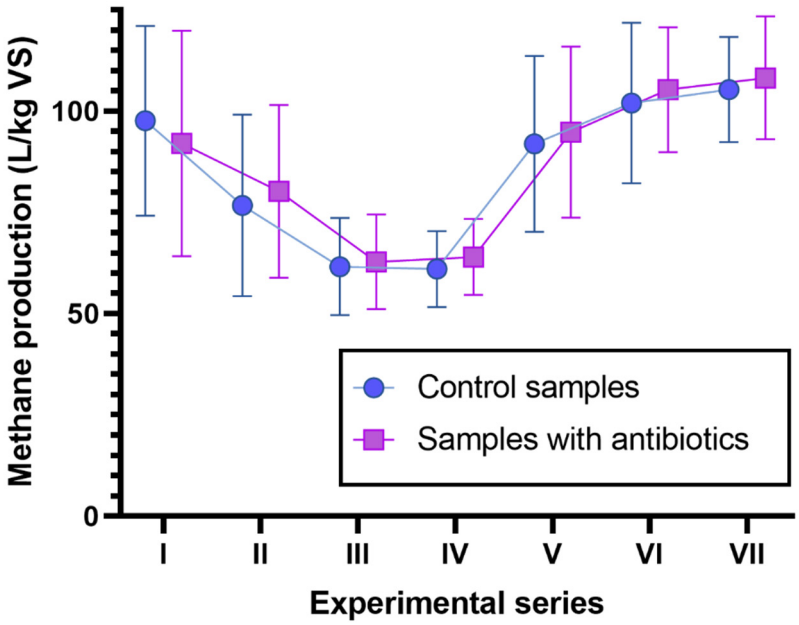

Figure 1. Methane production in seven experimental series (L/kg VS). The presented data are the average values in each experimental series with triplicate measurements and two replicates. Error bars represent standard deviation (SD).

Methane production was somewhat higher (3 L/kg vs. per sample) in the experimental samples containing a mixture of AMO, ENRO and MET than in control samples (ANOVA, Kruskal–Wallis, $p > 0.05$); Figure 1. In the first few weeks of the AD process, CH_4 yields were high at around 90 L/kg vs. in the control sample from series I (CS I) and in the experimental samples from series I and II (Supplementary Materials, Table S1). The above trend was maintained until the end of experimental series II, during which AMO, ENRO and MET were added to the bioreactor at concentrations of 2, 0.5 and 0.5 μg/mL $_D$, respectively; Table 2.

In experimental series III, Figure 1, a sudden decrease (ANOVA, Kruskal–Wallis, $p > 0.05$) in CH_4 production was observed in experimental samples S1 III and S2 III Table S1. Methane production was approximately 40% lower in comparison with series I (approximately 90 L/kg VS) and series VII when CH_4 production peaked (108.2 L/kg VS). This trend was maintained until the end of series IV. The above experimental series were conducted in December, January, February, March and the first half of April; Table S2. In these months, livestock diets are often modified due to the lower availability of nutrients from fresh fodder [40,41]. In winter, the prevalence of infections is also higher, and antimicrobials are more frequently used in livestock farming [42]. Therefore, the presence of chemical compounds such as ammonia in biomass could have inhibited the growth and development of bacteria and methanogens in the analyzed digestates [11]. Specific bacterial groups supply substrates that are used by methanogenic microorganisms in the production of CH_4 [43]. Therefore, the sudden drop in CH_4 yields in series III and IV, Figure 1, and the absence of significant differences (ANOVA, Kruskal–Wallis, $p > 0.05$) in CH_4 production could be attributed to periodic disruptions in AD and the low supply of substrates that are essential for microbial growth and development in fermented biomass. In conclusion, these unfavorable process conditions temporarily inhibited the growth and development of methanogens and, consequently, decreased CH_4 production in both control and experimental samples.

Methane production peaked in experimental series VII when digestates were supplemented with the highest concentrations of the tested antibiotics (AMO—32 μg/mL $_D$, ENRO—8 μg/mL $_D$, MET—8 μg/mL $_D$) Table 1. In series VII, CH_4 yields were somewhat higher in the experimental sample (108.2 L/kg VS; SD = 15.2) than in the control sample (105.3 L/kg VS; SD = 13).

The CH_4 production curves for the experimental samples and the control samples were similar throughout the entire AD process; Figure 1. These results indicate that antimicrobials exerted a temporary effect on CH_4 production. Probably, biomass microorganisms could have probably easily adapted to the applied drug concentrations due to the high supply of antibiotics in livestock farming [44]. Acquired antibiotic resistance decreased the tested drugs' inhibitory effect on the growth of microbial groups involved in CH_4 production and, consequently, on the efficiency of biogas production. According to some authors, antibiotic-resistant bacteria (ARB) and ARGs are naturally present in cattle gut microbiota; they are excreted with feces and transferred to digestates during AD [45,46]. In fermented substrates, DR can spread via horizontal gene transfer (HGT) [47], and it can ultimately decrease antibiotics' inhibitory effect on CH_4 production. This hypothesis was confirmed by the results of the metagenomic analysis which revealed high abundance and diversity of the examined ARGs Section 3.3 in fermented biomass in different stages of the experiment. In our previous study, the presence of antibiotics in cattle manure clearly influenced CH_4 production, but antimicrobials were administered to bioreactors at much higher doses than in the present experiment. In the cited study, AMO and oxytetracycline applied at a concentration of 512 μg/mL $_D$ and MET and sulfamethoxazole applied at a concentration of 1024 μg/mL $_D$ clearly inhibited CH_4 production [48]. According to other researchers, antimicrobials significantly affect AD, but only after a certain threshold concentration of a drug has been achieved [49,50]. In the work of Wen et al. [51], sulfamethoxazole administered at 50 μg/mL TS exerted a minor influence on CH_4 production. Yin et al. [52] found that biogas production began to decrease when oxytetracycline and chlortetracycline

concentrations exceeded 40 and 60 mg/kg TS, respectively. Antibiotics had no significant effect on biogas production when digestates were exposed to oxytetracycline concentrations below 40 mg/kg TS and chlortetracycline concentrations below 60 mg/kg TS [52]. Other published findings are consistent with the results of this study, where the highest concentrations of the tested antimicrobials were 32 µg/mL $_D$ for AMO and 8 µg/mL $_D$ for ENRO and MET. In the present study, antibiotics induced a minor and temporary decrease in CH_4 yields in experimental series III and IV, which could be attributed to changes in methanogen biodiversity. These changes could have resulted from the limited availability of substrates for CH_4 production, the presence of toxic substances in the bioreactor, high microbial loads, and temporary disruptions in the physicochemical parameters of the AD process [53].

3.2. Concentrations of Volatile Fatty Acids

The products of protein and carbohydrate hydrolysis, such as VFAs, including acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids and heptane, play an important role in AD [54]. In the current study, VFA concentrations were measured in each experimental series throughout the entire 417-day experiment. A minor increase (ANOVA, Kruskal–Wallis, $p > 0.05$) in acetate production was observed at the beginning of the AD process in experimental series I (approximately 6 g/L $_D$); Figure 2.

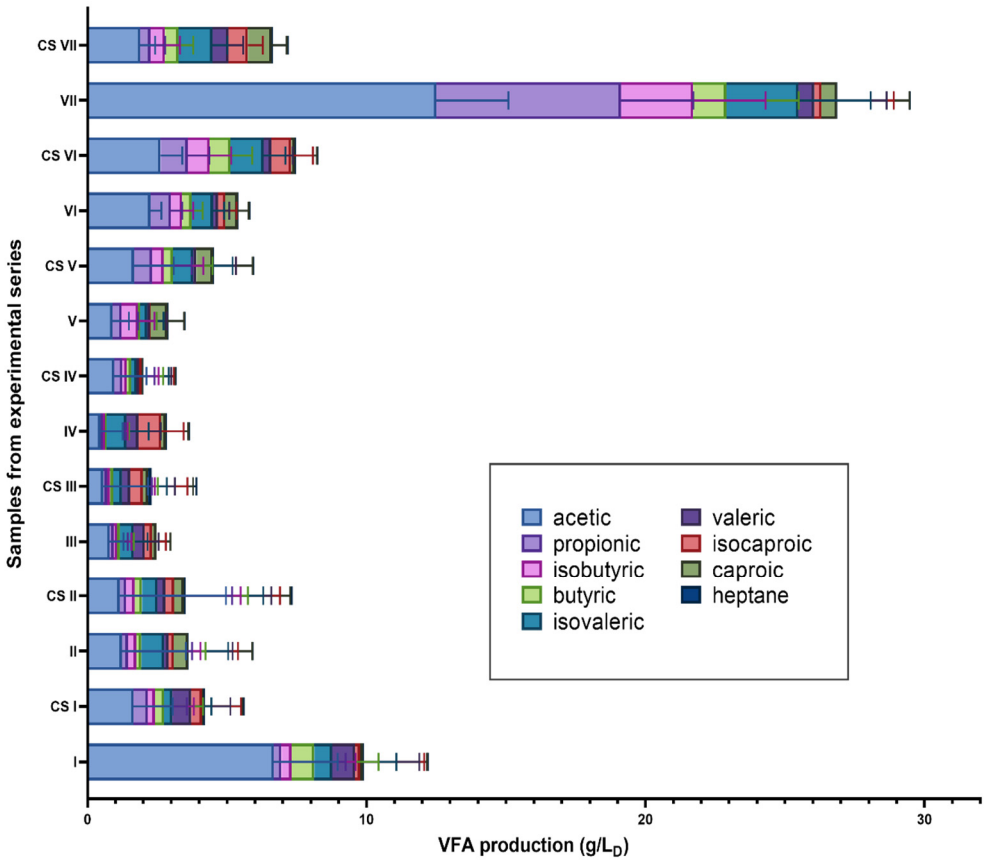

Figure 2. Average concentrations of VFAs (g/L $_D$ ± SD) in the experimental samples collected from series I–VII. Control samples without AMO, ENRO and MET are marked as CS and are labeled with the number of the corresponding experimental series (I–VII).

A minor increase (ANOVA, Kruskal–Wallis, $p > 0.05$) in the concentrations of acetic acid (approximately 13 g/L $_D$) and propionic acid (approximately 6 g/L $_D$) was noted in experimental series VII; Figure 2. The content of isovaleric and isobutyric acids also increased (from 3 g/L $_D$ to nearly 5 g/L $_D$) in the samples collected during experimental series VII when digestates were exposed to the highest antibiotic concentrations; Figure 2

(ANOVA, Kruskal–Wallis, $p > 0.05$). The concentrations of most of the remaining VFAs did not exceed 3 g/L $_D$. The increase in VFA content could have resulted from high antibiotic concentrations in series VII, i.e., 32 µg/mL $_D$ for AMO and 8 µg/mL $_D$ for ENRO and MET. However, the observed increase in the content of acetate, which is utilized by microorganisms of the genera *Methanosarcina* and *Methanothrix* to produce CH_4 in different stages of AD, appears to be a natural phenomenon [55,56]. Methane yields were high in the last experimental series, which suggests that microorganisms relied on increased VFA concentrations to produce CH_4 during methanogenesis [57].

3.3. Long-Term Synergistic Effects of AMO, ENR and MET on the Microbial Biodiversity of Digestates

3.3.1. Bacteria

A total of 2 × 1,049,430,401 high-quality paired-end sequences were obtained from 21 biomass samples exposed to increasing concentrations of AMO, ENRO and MET over a long period of time; Table 2. The number of sequences per sample ranged from 47,053,977 to 51,020,960. The sequences were grouped between 23 and 54 OTUs at 97% similarity level, respectively, for the least and most abundant samples. It was assumed that the structure of microbial communities in digestates would be similar to the cattle gut microbiome, which is significantly influenced by, among other things, the presence of pharmaceuticals in feed. The duration of the experiment was yet another factor that induced changes in the composition of microbial consortia during AD. As a result, bacterial OTUs in digestates varied considerably subject to the antibiotic dose and the duration of the AD process. Eight bacterial phyla were identified in digestates during the AD process and at the end of the 417-day experiment. These were: *Bacteroidetes* (*Bacteroidia* class), *Firmicutes* (*Bacilli*, *Clostridia*, *Mollicutes*, and *Erysipelotrichia* classes), *Proteobacteria* (*Alphaprotebocateria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* classes), *Acidobacteria* (*Blastocatellia* class), *Spirochaetes*, *Synergistetes* (*Synergistia* class) *Actinobacteria* and *Tenericutes*; Figure 3. *Firmicutes* and *Bacteroidetes* are the dominant phyla during AD [58]. *Bacteroidetes* are well-known proteolytic bacteria [59], whereas *Proteobacteria* are among the key consumers oxidizing long-chain VFAs during AD [60]. Soil-dwelling *Actinobacteria* degrade various organic compounds and xenobiotics, whereas *Acidobacteria* are obligate anaerobes that ferment aromatic compounds and acetates [61]. *Synergistetes* and *Spirochaetes* convert products such as propionate, butyrate, isobutyrate, valeric acid, isovaleric acid and ethanol to acetate, H_2 and CO_2. The resulting products are used by methanogens in CH_4 production [62].

The microbial community was represented mainly by *Bacteroidetes* species, which accounted for 89% of the bacteria in experimental series I (samples S1 I, S2 I, S3 I) and more than 70% of the bacteria in experimental series II (S1 II, S2 II, S3 II); Figure 3. In comparison with the experimental samples from series I and II, the proportion of *Bacteroidetes* decreased to 50% in series III (S1 III, S2 III). *Bacteroidetes* also accounted for 50% of the bacteria in the control sample from series I. The proportion of *Bacteroidetes* was also determined to be 50% in samples S2 VII and S3 VII collected during experimental series VII. The abundance of *Bacteroidetes* in samples S2 IV, S1 VI, S2 VI and S3 VI from experimental series IV and VI did not exceed 30%. In the remaining experimental samples and in control samples, the abundance of *Bacteroidetes* was estimated at 20%. Interestingly, the proportion of *Bacteroidetes* in most experimental samples ranged from 30% to 50%, but it was determined to be nearly 20% in control samples. This observation suggests that *Bacteroidetes* species were highly resistant to the tested antimicrobials [63]. *Bacteroidetes* widely colonize the gastrointestinal tract of cattle, and they are ubiquitous in manure [64].

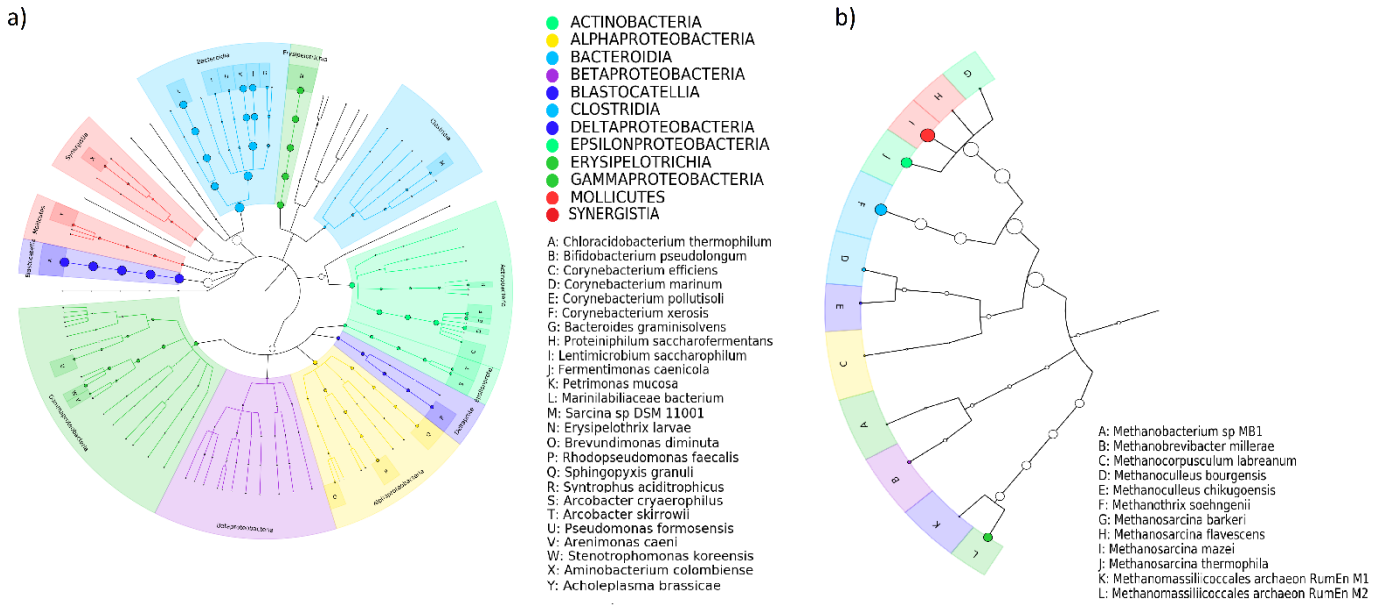

Figure 3. Circos presenting the (**a**) most abundant bacterial consortia in digestate samples and (**b**) most abundant *Archaea* consortia in digestate samples. The OTU values of the presented species exceeded 1 ppm.

Acidobacteria were also highly prevalent in the microbial community colonizing cattle manure digestates; Figure 3. In experimental series I, *Acidobacteria* accounted for 1% to 3% of the bacteria identified in the experimental samples (S1 I, S2 I, S3 I), whereas their share in the control sample (CS I) exceeded 11%. The proportion of *Acidobacteria* species in fermented biomass continued to increase in successive weeks of the experiment, and it exceeded 50% in sample S2 II in series II. The share of *Acidobacteria* reached up to 60% in samples S3 II, S1 III, S2 III and S1 IV. In the control sample from series IV (CS IV), *Acidobacteria* also represented around 60% of the microbial community. In series V, the proportions of *Acidobacteria* in sample S2 V decreased rapidly to around 20%, and this trend was maintained in the experimental samples containing a mixture of AMO, ENRO and MET until the end of AD. In the control sample collected in the last stage of the AD process in series VII, *Acidobacteria* accounted for 64% of the microbial community. These results suggest *Acidobacteria* were resistant only to lower concentrations of the tested drugs. Prolonged exposure to antimicrobials increases the risk of selection pressure and the spread of ARGs among microorganisms [65]. Therefore, the use of digestates as agricultural fertilizers can contribute to the transfer and dissemination of ARGs in the soil environment [15,66]. Other authors [67] have reported growing levels of DR in endophytes colonizing lettuce roots, leaves and the phyllosphere in fields fertilized with manure, and the observed changes in the plant resistome were correlated with bacterial taxa such as *Acidobacteria*, *Proteobacteria* and *Firmicutes*, which were also identified in the present study.

Bacteria of the phylum *Firmicutes* accounted for 4% to 8% of the microorganisms in samples S1 I, S2 I and S3 I from experimental series I; Figure 3. In the control sample collected from series I, more than 17% of the bacterial community was represented by *Firmicutes* species. In series II and III (samples S1 II, S2 II, S3 II, S1 III and S2 III), the proportion of *Firmicutes* ranged from 9% to 12%. The share of *Firmicutes* was estimated at 12% to around 20% in the samples collected in series IV, V and VI. Interestingly, in experimental series VII, *Firmicutes* accounted for 23% of the microorganisms in the experimental samples, but for only 8% in the control sample. *Firmicutes* can easily degrade complex polysaccharide and protein substrates under anerobic conditions, and synthesize acetate from amino acids that are produced during protein hydrolysis [11,68]. This bacterial phylum is predominant in the gastrointestinal tracts of humans and animals, and it is also ubiquitous in untreated wastewater, which suggests that *Firmicutes* species are highly resistant to antimicrobials [63].

Huerta et al. [69] concluded that *Firmicutes* probably harbor and transmit ARGs in the environment, including in aquatic ecosystems. According to Shi et al. [70], *Firmicutes* are the main ARG hosts in agricultural soils. Other authors have shown in previous studies that bacteria can acquire resistance to selected antimicrobial agents (tetracycline, streptomycin or sulfonamide) without any contact with these drugs [71]. It is also a problem that resistant bacteria and ARGs can persist for years, even with short-term administration of antibiotics [72]. In addition, it is noteworthy that ARGs can be horizontally transferred between microbes through MGEs. The HGT of ARGs can occur between nonpathogens, pathogens, and even distantly related organisms [73,74]. Recent studies have demonstrated specific instances of the HGT of ARGs in human and animal guts, soil, sediments, and water [75,76].

3.3.2. Archaea

The abundance of the *Euryarchaeota* species in digestate samples differed considerably across experimental series in the AD process; Figure 3. In most samples, the predominant methanogens were bacteria of the genera *Methanothrix* and *Methanosarcina* and order *Methanomassiliicoccales*. The genus *Methanothrix* is composed of acetoclastic methanogens [54] that are sensitive to unfavorable environmental conditions, whereas *Methanosarcina* spp. use various substrates, including acetate, H_2 and methyl compounds [77].

Methanothrix soehngenii, *Methanosarcina mazei* and *Methanomassiliicoccales archaeon-RumEnM2* were the dominant species in both experimental and control digestate samples. *Methanobrevibacter millerae* was additionally identified in five experimental samples containing a mixture of AMO, ENRO and MET (S3 I, S2 II, S3 II, S1 III and S3 VII), and in two control samples (CS IV and CS VII). *Methanosarcina thermophila* was detected in the experimental samples collected in series I, II and III. The growth of *Methanosarcina thermophila* was probably inhibited due to unfavorable conditions during prolonged AD as well as the bacterium's sensitivity to the applied combination of drugs [78]. *Methanosarcina thermophila* was not identified in successive experimental series in which antibiotic concentrations were higher, which suggests that prolonged exposure to antimicrobials can decrease the diversity of methanogenic communities in biomass. *Methanosarcina mazei* was also abundant in the experimental samples collected in series I–III, but its prevalence decreased rapidly in response to higher antibiotic concentrations in series IV, and it remained low until the end of the AD process.

3.4. The Effects of Antibiotics on the Total Abundance of ARGs and Resistant Types

A total of 401 ARG subtypes belonging to 17 ARG types that encode resistance to bleomycin, kasugamycin, trimethoprim, polymyxin, fosfomycin, quinolone, rifamycin, fosmidomycin, chloramphenicol, beta-lactam, sulfonamide, vancomycin, bacitracin, aminoglycoside, macrolide–lincosamide–streptogramin (MLS) antibiotics, and tetracycline, as well as multidrug resistance, were identified in manure digestates; Figure 4. Genes conferring resistance to MLS antibiotics (32.6%), tetracycline (22.7%), and aminoglycosides (19.5%), as well as genes encoding resistance to multiple drugs (10.3%), were most abundant in digestate samples; Figure 4. The abundance of the remaining ARG types in digestate samples did not exceed 6%.

The most prevalent ARG subtypes encoding resistance to MLS drugs were *erm*F, *lnu*B, *lsa*, *mac*A, *mac*B, and *mef* A; Figure 5. At the end of the 417-day experiment, a minor increase was observed in the abundance of the *ermF* gene, from 23 ppm in series I to 25 ppm in series VII. The *ermF* gene encodes 23S *r*RNA adenine-specific N-6-methyltransferases that methylate bacterial 23S *r*RNA. Methylation prevents MLS drugs from binding to bacterial ribosomes and, consequently, encodes resistance to this group of antibiotics [79,80]. The *erm*F gene is one of the most prevalent ARGs conferring resistance to MLS antibiotics, and it is also one of the major acquired resistance genes in bacteria [81]. An increase in *erm*F abundance in digestates can contribute to the spread of other ARGs in the environment via

HGT because in addition to the *erm*F gene, *tet*(X1) and *tet*(X2) genes were also identified on conjugative transposon CTnDOT [82].

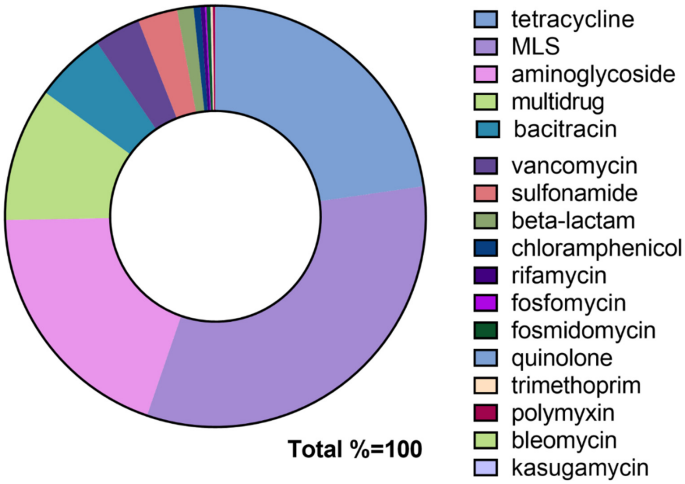

Figure 4. Percentage (%) distribution of different ARG types in digestate samples. MLS—macrolide–lincosamide–streptogramin antibiotics.

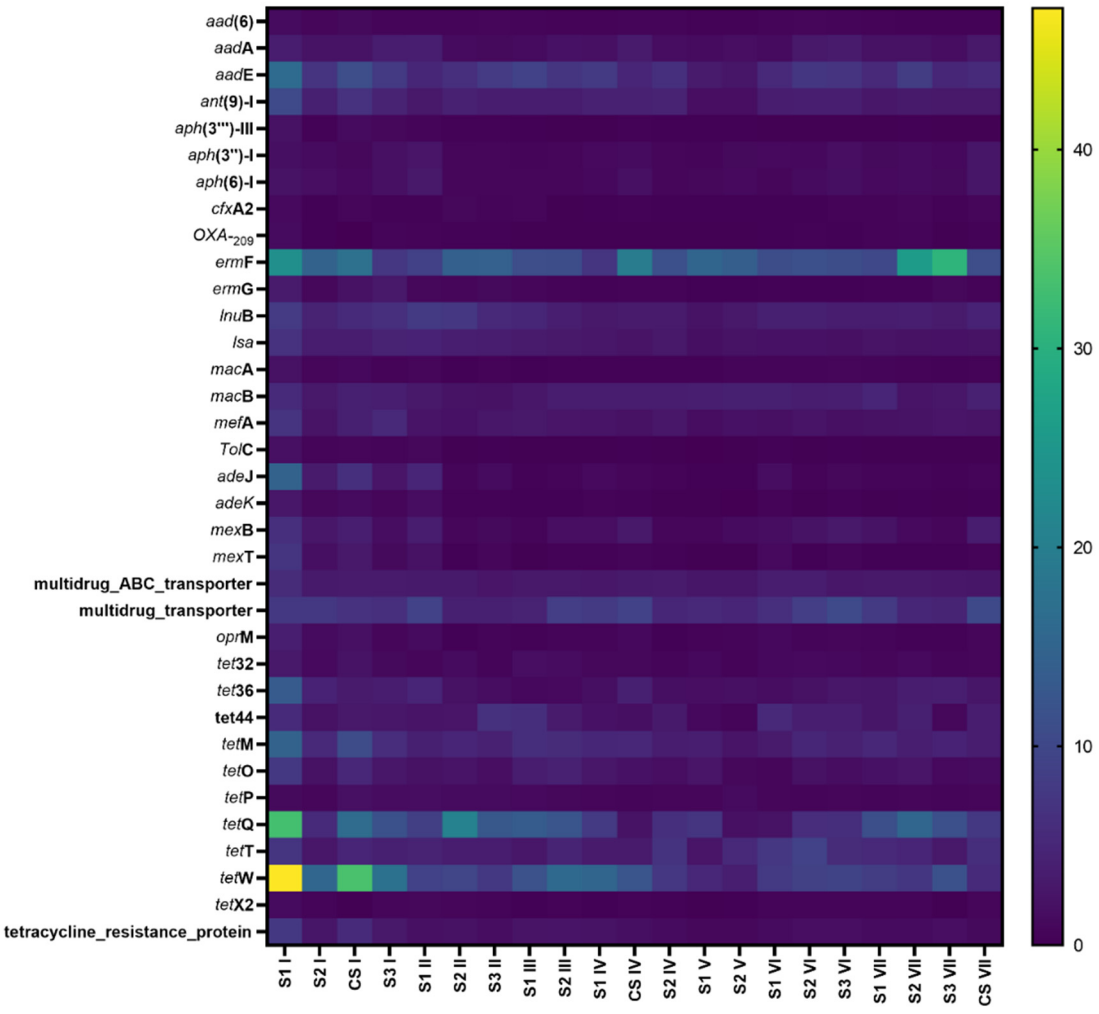

Figure 5. Heatmap presenting the abundance (ppm) of ARG subtypes in the experimental samples (S1, S2, S3) collected from seven experimental series (I–VII) and in control samples (CS).

The dominant tetracycline resistance genes were *tet*32, *tet*36, *tet*44, *tet*M, *tet*O, *tet*P, *tet*Q, *tet*T, *tet*W, *tet*X2 and *tetracycline_resistance_protein*. These ARG subtypes encode two mechanisms of specific tetracycline resistance. *tet*32, *tet*36, *tet*44, *tet*M, *tet*O, *tet*P, *tet*Q, *tet*T and *tet*W genes are linked with ribosomal protection proteins (RPP), whereas *tet*X2 encodes a tetracycline-degrading enzyme [83]. The RPP mechanism is more ubiquitous in the environment, which explains the presence of the identified tetracycline resistance genes in this study. In the work of Santamaria et al. [84], genes encoding RPP were also most abundant in cattle manure. Numerous researchers have reported that cattle manure is a reservoir of ARGs encoding RPP. Therefore, the AD of cattle manure contributes to the spread of *tet* genes in bioreactors and crop fields. This observation was confirmed by Chen et al. [85] and Wu et al. [86], who identified numerous tetracycline resistance genes in topsoil samples collected from arable fields.

The most prevalent aminoglycoside resistance genes were *aad*(6), *aad*A, *aad*E, *ant(9)-I*, *aph(3″)-III*, *aph(3′)-I* and *aph(6)-I*. These putative genes encode *O*-nucleotidyltransferases, i.e., aminoglycoside-modifying enzymes. This enzymatic modification process is one of the most common DR mechanisms in the environment [87]. Progressive DR and the spread of ARGs reduce the efficacy of aminoglycosides. In several medical reports, the identified bacteria were totally resistant to aminoglycosides, rendering these drugs completely useless in pharmacotherapy [88].

The most abundant multidrug resistance genes were *Tol*C, *ade*J, *ade*K, *mex*B, *mex*T, *multidrug_ABC_transporter*, *multidrug_transporter* and *opr*M. The *ade*J gene is responsible for drug recognition and proton motive force generation that provides the cellular energy required for substrate transport [89]. *Tol*C belongs to the resistance–nodulation–division (RND) family of transporters, and it confers resistance to chloramphenicol, aminoglycoside, macrolide, acriflavine, doxorubicin, erythromycin, puromycin and beta-lactams [90]. Two of the detected genes, *mex*B and *mex*T, are most often identified in clinical isolates, which gives serious cause for concern [91]. The presence of these genes in cattle manure digestates indicates that *mex*B and *mex*T are being spread from the hospital setting to the natural environment and agriculture [8].

Interestingly, despite the fact that AMO was one of the tested antibiotics, *cfx*A and OXA_{-209} were the only beta-lactamase resistance genes in the digestate samples. In experimental series I, the abundance of *cfx*A and OXA_{-209} exceeded 1 ppm, but in successive series, their abundance decreased rapidly to 0.26 ppm and 0.02 ppm. Beta-lactams have an unstable ring and they are not highly persistent in the environment, which could explain the low abundance of genes encoding resistance to this class of antibiotics [92].

The total number of sequences characteristic of the identified ARGs was higher in the experimental samples containing a mixture of antibiotics, but numerous ARG sequences were also identified in control samples. The abundance of beta-lactam resistance genes was reduced by 50%. However, the prevalence of genes encoding resistance to tetracyclines, aminoglycosides and MLS drugs increased considerably in digestate samples. The abundance of ARGs in the experimental samples (with the addition of AMO, ENRO and MET) and control samples is worrying because it indicates that the spread of ARGs in cattle manure is a process that had begun before AD. However, experimental and control samples of cattle manure digestates differed significantly in their ARG profiles, which indicates that ARGs are transferred via various pathways in the presence of antibiotics. The analyzed samples differed in the prevalence of ARG subtypes. Both the abundance and diversity of ARGs were much higher in the experimental samples with the addition of antibiotics than in the control samples. In a study evaluating the influence of drugs on AD, Wen et al. [51] also reported an increase in the abundance of individual ARGs during AD, and concluded that microbial communities and mobile genetic elements played the key role in the transmission of ARGs and an increase in their abundance. The fact that ARGs can reach other pathogenic microorganisms via HGT [63] is particularly worrying. It can endanger the efficacy of the available antibiotics, thus increasing the prevalence of infections caused by pathogens that do not respond to pharmacotherapy [93].

3.5. Results of Statistical Analyses

The correlations between microbial abundance and ARG abundance were determined by calculating Spearman's rank correlation coefficient (R > 0.7; $p < 0.01$); Figure 6, Table S3.

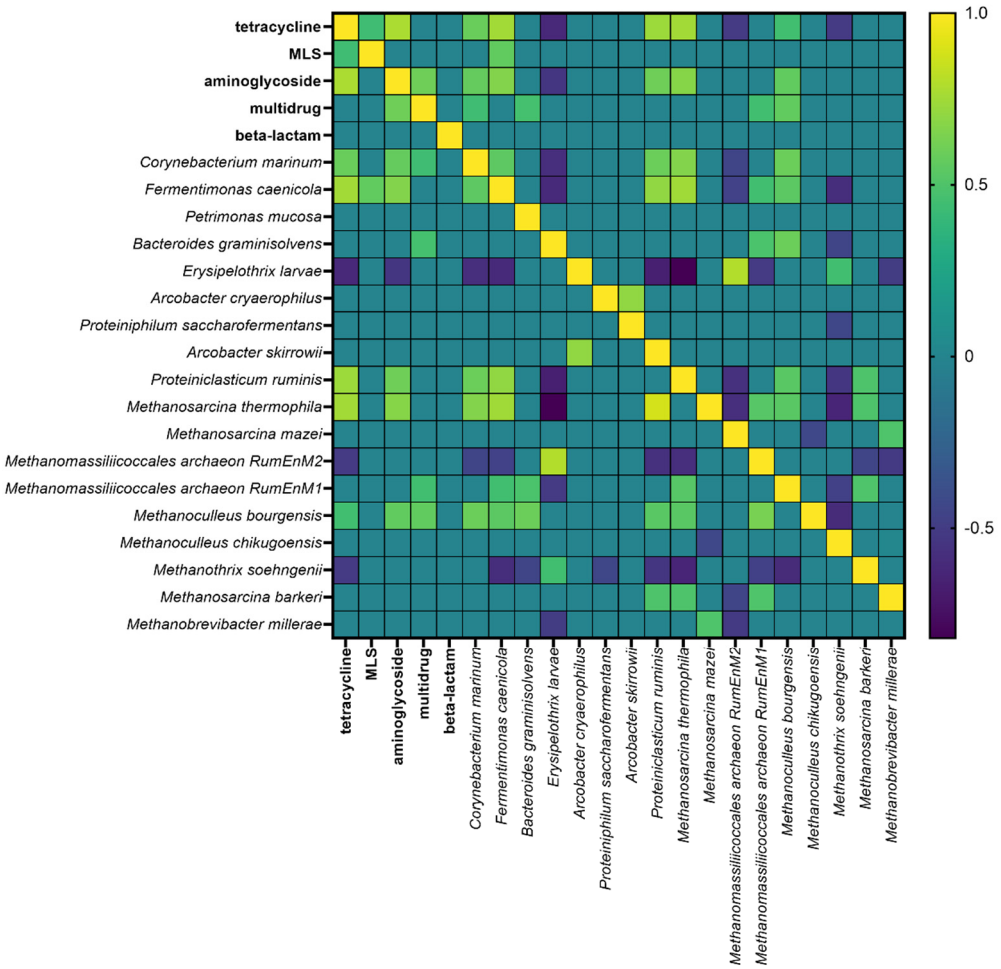

Figure 6. Spearman's rank order correlation coefficients for the relationships between microbial abundance and the abundance of different ARG types.

A strong and significant correlation ($p < 0.05$, ANOVA) was noted between genes encoding resistance to tetracyclines and aminoglycosides (R = 0.77). Tetracycline resistance genes were significantly correlated with the abundance of *Fermentiomonas caenicola* (R = 0.75); *Proteiniclasticum ruminis* (R = 0.73) and *Methanosarcina thermophila* (R = 0.75). These correlations suggest that the above microorganisms could be potential carriers of tetracycline resistance genes. A strong and significant correlation was found between the abundance of *Proteiniclasticum ruminis* and *Methanosarcina thermophila* (R = 0.87). Strong mutual correlations were also observed between the abundance of *Erysipelothrix larvae* and *Methanomassiliicoccales archaeon RumEnM2* (R = 0.78), and *Methanothrix soehngenii* and *Syntrophus aciditrophicus* (R = 0.71). The presence of mutual correlations between microorganisms indicates that they cooperate and enter into syntrophic interactions during the AD process [94], because hydrolysis, acidogenesis, and methanogenesis are the key phases of the AD process, which involves a diverse group of microbial communities [95].

4. Conclusions

This study demonstrated that biomass supplementation with increasing concentrations of AMO, ENRO and MET exerted a transient effect on CH_4 production. The metagenomic analysis revealed that prolonged mesophilic AD of cattle manure in the presence of all three

tested antibiotics can modify the resistome profile and increase the relative abundance of individual ARGs in digestates. However, there is also a risk of spreading ARGs in an environment where genes can be transferred from the digestate to farmland, to surface waters, to the soil, plant parts, and further down the food chain to livestock and humans. The abundance of genes conferring resistance to tetracyclines, aminoglycosides and MLS drugs increased significantly in digestate samples. The present findings indicate that at the current level of technological development, ARGs are not removed during the AD process, which can compromise the functions of soil-dwelling microorganisms in fields fertilized with digestate. The above poses a direct health threat for humans as well as livestock that consume fodder crops grown in digestate-fertilized fields. Therefore, effective strategies for managing the AD process and the resulting digestates are needed to reduce the abundance of ARGs in fermented biomass, and limit their further spread in the environment.

Supplementary Materials: The following Supplementary Materials can be downloaded at https://www.mdpi.com/article/10.3390/en15051920/s1: Table S1: Methane (CH_4) production in samples from experimental bioreactors (I–VII) containing a mixture of amoxicillin (AMO), enrofloxacin (ENRO) and metronidazole (MET), and in samples from control bioreactors without antibiotics (CS); Table S2: Dates and duration of seven experimental anaerobic digestion (AD) series; Table S3: Correlations between microbial counts[a] and concentrations of antibiotic resistance genes (ARGs).

Author Contributions: Conceptualization, I.W., M.C., M.H. and E.K.; methodology, M.H., E.F. and P.R.; software, Ł.P. and J.P.J.; validation, I.W. and M.H.; investigation, I.W.; resources, M.H.; data curation, Ł.P. and J.P.J.; writing—original draft preparation, I.W. and M.C.; writing—review and editing, I.W., E.K. and M.H.; visualization, I.W. and Ł.P.; supervision, E.K.; project administration, M.H.; funding acquisition, M.H. and I.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the NATIONAL SCIENCE CENTER (Poland), grant number No. 2016/23/B/NZ9/03669 and 2020/37/N/NZ9/00431. Moreover, the research was funded by the Minister of Science and Higher Education under the "Regional Initiative of Excellence" program for the years 2019–2022 (Project No. 010/RID2018/19, funding 12.000.000 PLN).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: A total of 21 metagenomic samples were submitted to the European Nucleotide Archive (ENA) under accession No. PRJEB48924. The submitted data can be accessed at https://www.ebi.ac.uk/ena/browser/view/PRJEB48924 (accessed on 3 March 2022).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Reyes-Contreras, C.; Vidal, G. Methanogenic toxicity evaluation of chlortetracycline hydrochloride. *Electron. J. Biotechnol.* **2015**, *18*, 445–450. [CrossRef]
2. Moulod, M.; Jalali, A.; Asmatulu, R. Biogas derived from municipal solid waste to generate electrical power through solid oxide fuel cells. *Int. J. Energy Res.* **2016**, *45*, 2091–2104. [CrossRef]
3. Thompson, T.M.; Young, B.R.; Baroutian, S. Advances in the pretreatment of brown macroalgae for biogas production. *Fuel Process. Technol.* **2019**, *195*, 106151. [CrossRef]
4. Wu, N.; Qiao, M.; Zhang, B.; Cheng, W.D.; Zhu, Y.G. Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environ. Sci. Technol.* **2010**, *44*, 6933–6939. [CrossRef] [PubMed]
5. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (Text with EEA relevance). Available online: https://eur-lex.europa.eu/eli/reg/2003/1829/oj (accessed on 3 March 2022).
6. Aus der Beek, T.; Weber, F.A.; Bergmann, A.; Hickmann, S.; Ebert, I.; Hein, A.; Küster, A. Pharmaceuticals in the environment-Global occurrences and perspectives. *Environ. Toxicol. Chem.* **2016**, *35*, 823–835. [CrossRef]
7. Guo, J.; Li, J.; Chen, H.; Bond, P.L.; Yuan, Z. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Res.* **2017**, *123*, 468–478. [CrossRef] [PubMed]

8. KIES, F.K.; Boutchebak, S.; Bendaida, N. Soil Contamination by Pharmaceutical Pollutants: Adsorption of an Antibiotic (Amoxicillin) on an Agricultural Land. *Proceedings* **2020**, *30*, 60. [CrossRef]
9. Álvarez-Esmorís, C.; Conde-Cid, M.; Ferreira-Coelho, G.; Fernández-Sanjurjo, M.J.; Núñez-Delgado, A.; Álvarez-Rodríguez, E.; Arias-Estévez, M. Adsorption/desorption of sulfamethoxypyridazine and enrofloxacin in agricultural soils. *Sci. Total Environ.* **2020**, *706*, 136015. [CrossRef]
10. Pan, Y.; Yi, J.; Zhou, B.; Xie, S.; Chen, D.; Tao, Y.; Qu, W.; Liu, Z.; Huang, L.; Yuan, Z. Disposition and Residue Depletion of Metronidazole in Pigs and Broilers. *Sci. Rep.* **2017**, *7*, 7203. [CrossRef]
11. Czatzkowska, M.; Harnisz, M.; Korzeniewska, E.; Koniuszewska, I. Inhibitors of the methane fermentation process with particular emphasis on the microbiological aspect: A review. *Energy Sci. Eng.* **2020**, *8*, 1880–1897. [CrossRef]
12. Mohammed, S.J.; M-Ridha, M.J.; Abed, K.M.; Elgharbawy, A.A.M. Removal of levofloxacin and ciprofloxacin from aqueous solutions and an economic evaluation using the electrocoagulation process. *Int. J. Environ. Anal. Chem.* **2021**, 1–19. [CrossRef]
13. Eitel, Z.; Sóki, J.; Urbán, E.; Nagy, E. The prevalence of antibiotic resistance genes in Bacteroides fragilis group strains isolated in different European countries. *Anaerobe* **2013**, *21*, 43–49. [CrossRef] [PubMed]
14. Ingerslev, F.; Halling-Sørensen, B. Biodegradability of metronidazole, olaquindox, and tylosin and formation of tylosin degradation products in aerobic soil-manure slurries. *Ecotoxicol. Environ. Saf.* **2001**, *48*, 311–320. [CrossRef] [PubMed]
15. Buta, M.; Korzeniewska, E.; Harnisz, M.; Hubeny, J.; Zieliński, W.; Rolbiecki, D.; Bajkacz, S.; Felis, E.; Kokoszka, K. Microbial and chemical pollutants on the manure-crops pathway in the perspective of "One Health" holistic approach. *Sci. Total Environ.* **2021**, *785*, 147411. [CrossRef]
16. Korzeniewska, E.; Harnisz, M. Sources, Occurrence, and Environmental Risk Assessment of Antibiotics and Antimicrobial-Resistant Bacteria in Aquatic Environments of Poland. In *Handbook of Environmental Chemistry*; Springer: Berlin/Heidelberg, Germany, 2020; Volume 87, pp. 179–193.
17. Amador, P.P.; Fernandes, R.M.; Prudêncio, M.C.; Barreto, M.P.; Duarte, I.M. Antibiotic resistance in wastewater: Occurrence and fate of Enterobacteriaceae producers of Class A and Class C β-lactamases. *J. Environ. Sci. Health—Part A Toxic/Hazardous Subst. Environ. Eng.* **2015**, *50*, 26–39. [CrossRef]
18. Flores-Orozco, D.; Patidar, R.; Levin, D.B.; Sparling, R.; Kumar, A.; Çiçek, N. Effect of ceftiofur on mesophilic anaerobic digestion of dairy manure and the reduction of the cephalosporin-resistance gene cmy-2. *Bioresour. Technol.* **2020**, *301*, 122729. [CrossRef]
19. Gurmessa, B.; Pedretti, E.F.; Cocco, S.; Cardelli, V.; Corti, G. Manure anaerobic digestion effects and the role of pre- and post-treatments on veterinary antibiotics and antibiotic resistance genes removal efficiency. *Sci. Total Environ.* **2020**, *721*, 137532. [CrossRef]
20. Huang, X.; Zheng, J.; Tian, S.; Liu, C.; Liu, L.; Wei, L.; Fan, H.; Zhang, T.; Wang, L.; Zhu, G.; et al. Higher temperatures do not always achieve better antibiotic resistance gene removal in anaerobic digestion of swine manure. *Appl. Environ. Microbiol.* **2019**, *85*, e02878-18. [CrossRef]
21. Larrañaga, O.; Brown-Jaque, M.; Quirós, P.; Gómez-Gómez, C.; Blanch, A.R.; Rodríguez-Rubio, L.; Muniesa, M. Phage particles harboring antibiotic resistance genes in fresh-cut vegetables and agricultural soil. *Environ. Int.* **2018**, *115*, 133–141. [CrossRef]
22. Bai, Y.; Ruan, X.; Xie, X.; Yan, Z. Antibiotic resistome profile based on metagenomics in raw surface drinking water source and the influence of environmental factor: A case study in Huaihe River Basin, China. *Environ. Pollut.* **2019**, *248*, 438–447. [CrossRef]
23. Koniuszewska, I.; Harnisz, M.; Korzeniewska, E.; Czatzkowska, M.; Jastrzębski, J.P.; Paukszto, Ł.; Bajkacz, S.; Felis, E.; Rusanowska, P. The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure. *Energies* **2021**, *14*, 1125. [CrossRef]
24. Ozbayram, E.G.; Arikan, O.; Ince, B.; Cetecioglu, Z.; Aydin, S.; Ince, O. Acute effects of various antibiotic combinations on acetoclastic methanogenic activity. *Environ. Sci. Pollut. Res.* **2015**, *22*, 6230–6235. [CrossRef] [PubMed]
25. Aydin, S.; Cetecioglu, Z.; Arikan, O.; Ince, B.; Ozbayram, E.G.; Ince, O. Inhibitory effects of antibiotic combinations on syntrophic bacteria, homoacetogens and methanogens. *Chemosphere* **2015**, *120*, 515–520. [CrossRef] [PubMed]
26. Aydin, S.; Ince, B.; Ince, O. Application of real-time PCR to determination of combined effect of antibiotics on Bacteria, Methanogenic Archaea, Archaea in anaerobic sequencing batch reactors. *Water Res.* **2015**, *76*, 88–98. [CrossRef]
27. Aydin, S.; Ince, B.; Cetecioglu, Z.; Ozbayram, E.G.; Shahi, A.; Okay, O.; Arikan, O.; Ince, O. Performance of anaerobic sequencing batch reactor in the treatment of pharmaceutical wastewater containing erythromycin and sulfamethoxazole mixture. *Water Sci. Technol.* **2014**, *70*, 1625–1632. [CrossRef]
28. Rusanowska, P.; Harnisz, M.; Zieliński, M.; Dębowski, M.; Korzeniewska, E.; Kisielewska, M.; Amenda, E. Individual and Synergistic Effects of Metronidazole, Amoxicillin, and Ciprofloxacin on Methane Fermentation with Sewage Sludge. *Clean—Soil Air Water* **2020**, *48*, 1900281. [CrossRef]
29. Kisielewska, M.; Dębowski, M.; Zieliński, M. Improvement of biohydrogen production using a reduced pressure fermentation. *Bioprocess Biosyst. Eng.* **2015**, *38*, 1925–1933. [CrossRef]
30. Federation, W.E. APHA, AWWA, WEF. "Standard Methods for examination of water and wastewater". *An. Hidrol. Médica* **2012**, *5*, 185–186. [CrossRef]
31. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef]
32. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357–359. [CrossRef]

33. Segata, N.; Waldron, L.; Ballarini, A.; Narasimhan, V.; Jousson, O.; Huttenhower, C. Metagenomic microbial community profiling using unique clade-specific marker genes. *Nat. Methods* **2012**, *9*, 811–814. [CrossRef] [PubMed]
34. Gu, Z.; Gu, L.; Eils, R.; Schlesner, M.; Brors, B. Circlize implements and enhances circular visualization in R. *Bioinformatics* **2014**, *30*, 2811–2812. [CrossRef] [PubMed]
35. Yin, X.; Jiang, X.T.; Chai, B.; Li, L.; Yang, Y.; Cole, J.R.; Tiedje, J.M.; Zhang, T. ARGs-OAP v2.0 with an expanded SARG database and Hidden Markov Models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. *Bioinformatics* **2018**, *34*, 2263–2270. [CrossRef] [PubMed]
36. Chambers, L.; Yang, Y.; Littier, H.; Ray, P.; Zhang, T.; Pruden, A.; Strickland, M.; Knowlton, K. Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. *PLoS ONE* **2015**, *10*, 1–18. [CrossRef]
37. Xiao, L.; Wang, Y.; Lichtfouse, E.; Li, Z.; Kumar, P.S.; Liu, J.; Feng, D.; Yang, Q.; Liu, F. Effect of Antibiotics on the Microbial Efficiency of Anaerobic Digestion of Wastewater: A Review. *Front. Microbiol.* **2021**, *11*, 1–11. [CrossRef]
38. Sun, C.; Li, W.; Chen, Z.; Qin, W.; Wen, X. Responses of antibiotics, antibiotic resistance genes, and mobile genetic elements in sewage sludge to thermal hydrolysis pre-treatment and various anaerobic digestion conditions. *Environ. Int.* **2019**, *133*, 105156. [CrossRef]
39. Koniuszewska, I.; Korzeniewska, E.; Harnisz, M.; Czatzkowska, M. Intensification of biogas production using various technologies: A review. *Int. J. Energy Res.* **2020**, *44*, 6240–6258. [CrossRef]
40. Schulz, F.; Wagner, K.; Brinkmann, J.; March, S.; Hinterstoißer, P.; Schüler, M.; Warnecke, S.; Paulsen, H.M. Welfare of dairy cattle in summer and winter—A comparison of organic and conventional herds in a farm network in Germany. *Landbauforschung* **2020**, *70*, 83–96. [CrossRef]
41. Tufan, T.; Arslan, C. Beef Cattle Feeding Principles of Kars Province Enterprises During the Winter Period. *Bahri Dağdaş Hayvancılık Araştırma Derg.* **2021**, *10*, 1–9.
42. Pan, X.; Qiang, Z.; Ben, W.; Chen, M. Residual veterinary antibiotics in swine manure from concentrated animal feeding operations in Shandong Province, China. *Chemosphere* **2011**, *84*, 695–700. [CrossRef]
43. Nascimento, A.L.; Souza, A.J.; Andrade, P.A.M.; Andreote, F.D.; Coscione, A.R.; Oliveira, F.C.; Regitano, J.B. Sewage sludge microbial structures and relations to their sources, treatments, and chemical attributes. *Front. Microbiol.* **2018**, *9*, 1–11. [CrossRef] [PubMed]
44. Tian, M.; He, X.; Feng, Y.; Wang, W.; Chen, H.; Gong, M.; Liu, D.; Clarke, J.L.; Van Eerde, A. Pollution by antibiotics and antimicrobial resistance in live stock and poultry manure in china, and countermeasures. *Antibiotics* **2021**, *10*, 539. [CrossRef] [PubMed]
45. Hu, Y.; Yang, X.; Qin, J.; Lu, N.; Cheng, G.; Wu, N.; Pan, Y.; Li, J.; Zhu, L.; Wang, X.; et al. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat. Commun.* **2013**, *4*, 2151. [CrossRef] [PubMed]
46. Wang, Y.; Hu, Y.; Cao, J.; Bi, Y.; Lv, N.; Liu, F.; Liang, S.; Shi, Y.; Jiao, X.; Gao, G.F.; et al. Antibiotic resistance gene reservoir in live poultry markets. *J. Infect.* **2019**, *78*, 445–453. [CrossRef]
47. Smillie, C.S.; Smith, M.B.; Friedman, J.; Cordero, O.X.; David, L.A.; Alm, E.J. Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* **2011**, *480*, 241–244. [CrossRef]
48. Koniuszewska, I.; Czatzkowska, M.; Harnisz, M.; Korzeniewska, E. The impact of antimicrobial substances on the methanogenic community during methane fermentation of sewage sludge and cattle slurry. *Appl. Sci.* **2021**, *11*, 369. [CrossRef]
49. Cheng, D.; Feng, Y.; Liu, Y.; Xue, J.; Li, Z. Dynamics of oxytetracycline, sulfamerazine, and cipro fl oxacin and related antibiotic resistance genes during swine manure composting. *J. Environ. Manage.* **2019**, *230*, 102–109. [CrossRef]
50. Nurk, L.; Knörzer, S.; Jacobi, H.F.; Spielmeyer, A. Elimination of sulfonamides and tetracyclines during anaerobic fermentation—A " Cheshire Cat " phenomenon. *Sustain. Chem. Pharm.* **2019**, *13*, 100157. [CrossRef]
51. Wen, Q.; Yang, S.; Chen, Z. Mesophilic and thermophilic anaerobic digestion of swine manure with sulfamethoxazole and norfloxacin: Dynamics of microbial communities and evolution of resistance genes. *Front. Environ. Sci. Eng.* **2021**, *15*, 94. [CrossRef]
52. Yin, F.; Dong, H.; Ji, C.; Tao, X.; Chen, Y. Effects of anaerobic digestion on chlortetracycline and oxytetracycline degradation efficiency for swine manure. *Waste Manag.* **2016**, *56*, 540–546. [CrossRef]
53. García-Ruíz, M.J.; Castellano-Hinojosa, A.; Armato, C.; González-Martínez, A.; González-López, J.; Osorio, F. Biogas production and microbial community structure in a stable-stage of a two-stage anaerobic digester. *AIChE J.* **2020**, *66*, e16807. [CrossRef]
54. Wang, S.; Du, K.; Yuan, R.; Chen, H.; Wang, F.; Zhou, B. Effects of sulfonamide antibiotics on digestion performance and microbial community during swine manure anaerobic digestion. *Environ. Eng. Res.* **2020**, *26*, 190471. [CrossRef]
55. Zhao, Z.; Li, Y.; Quan, X.; Zhang, Y. Towards engineering application: Potential mechanism for enhancing anaerobic digestion of complex organic waste with different types of conductive materials. *Water Res.* **2017**, *115*, 266–277. [CrossRef] [PubMed]
56. Park, M.J.; Jo, J.H.; Park, D.; Lee, D.S.; Park, J.M. Comprehensive study on a two-stage anaerobic digestion process for the sequential production of hydrogen and methane from cost-effective molasses. *Renew. Energy* **2010**, *35*, 6194–6202. [CrossRef]
57. Mitchell, S.M.; Ullman, J.L.; Teel, A.L.; Watts, R.J.; Frear, C. Bioresource Technology The effects of the antibiotics ampicillin, florfenicol, sulfamethazine, and tylosin on biogas production and their degradation efficiency during anaerobic digestion. *Bioresour. Technol.* **2013**, *149*, 244–252. [CrossRef]

58. Chen, S.; Cheng, H.; Wyckoff, K.N.; He, Q. Linkages of Firmicutes and Bacteroidetes populations to methanogenic process performance. *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 771–781. [CrossRef]
59. Xin, L.; Guo, Z.; Xiao, X.; Peng, C.; Zeng, P.; Feng, W.; Xu, W. Feasibility of anaerobic digestion on the release of biogas and heavy metals from rice straw pretreated with sodium hydroxide. *Environ. Sci. Pollut. Res.* **2019**, *26*, 19434–19444. [CrossRef]
60. She, Y.; Hong, J.; Zhang, Q.; Chen, B.Y.; Wei, W.; Xin, X. Revealing microbial mechanism associated with volatile fatty acids production in anaerobic acidogenesis of waste activated sludge enhanced by freezing/thawing pretreatment. *Bioresour. Technol.* **2020**, *302*, 122869. [CrossRef]
61. Krakat, N.; Schmidt, S.; Scherer, P. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. *Bioresour. Technol.* **2011**, *102*, 5692–5701. [CrossRef]
62. Zhang, X.; Gu, J.; Wang, X.; Zhang, K.; Yin, Y.; Zhang, R.; Zhang, S. Effects of tylosin, ciprofloxacin, and sulfadimidine on mcrA gene abundance and the methanogen community during anaerobic digestion of cattle manure. *Chemosphere* **2019**, *221*, 81–88. [CrossRef]
63. Niestępski, S.; Harnisz, M.; Ciesielski, S.; Korzeniewska, E.; Osińska, A. Environmental fate of Bacteroidetes, with particular emphasis on Bacteroides fragilis group bacteria and their specific antibiotic resistance genes, in activated sludge wastewater treatment plants. *J. Hazard. Mater.* **2020**, *394*, 122544. [CrossRef] [PubMed]
64. Gou, M.; Hu, H.W.; Zhang, Y.J.; Wang, J.T.; Hayden, H.; Tang, Y.Q.; He, J.Z. Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils. *Sci. Total Environ.* **2018**, *612*, 1300–1310. [CrossRef] [PubMed]
65. Zhang, R.; Gu, J.; Wang, X.; Li, Y.; Liu, J.; Lu, C.; Qiu, L. Response of antibiotic resistance genes abundance by graphene oxide during the anaerobic digestion of swine manure with copper pollution. *Sci. Total Environ.* **2019**, *654*, 292–299. [CrossRef]
66. Sarmah, A.K.; Meyer, M.T.; Boxall, A.B.A. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* **2006**, *65*, 725–759. [CrossRef] [PubMed]
67. Zhang, Q.; Wang, M.; Ma, X.; Gao, Q.; Wang, T.; Shi, X. High variations of methanogenic microorganisms drive full-scale anaerobic digestion process. *Environ. Int.* **2019**, *126*, 543–551. [CrossRef] [PubMed]
68. Kim, J.; Kim, W.; Lee, C. Absolute dominance of hydrogenotrophic methanogens in full-scale anaerobic sewage sludge digesters. *J. Environ. Sci.* **2013**, *25*, 2272–2280. [CrossRef]
69. Huerta, B.; Marti, E.; Gros, M.; López, P.; Pompêo, M.; Armengol, J.; Barceló, D.; Balcázar, J.L.; Rodríguez-Mozaz, S.; Marcé, R. Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs. *Sci. Total Environ.* **2013**, *456–457*, 161–170. [CrossRef] [PubMed]
70. Shi, W.; Liu, Y.; Li, J.; Zhang, H.; Shi, R.; Chen, J.; Li, H. Distribution pattern of antibiotic resistance genes and bacterial community in agricultural soil samples of Wuliangsuhai watershed. China. *Agric. Ecosyst. Environ.* **2020**, *295*, 106884. [CrossRef]
71. Gebreyes, W.A.; Thakur, S.; Morrow, W.E.M. Comparison of prevalence, antimicrobial resistance, and occurrence of multidrug-resistant Salmonella in antimicrobial-free and conventional pig production. *J. Food Prot.* **2006**, *69*, 743–748. [CrossRef]
72. Marano, R.B.M.; Gupta, C.L.; Cozer, T.; Jurkevitch, E.; Cytryn, E. Hidden Resistome: Enrichment Reveals the Presence of Clinically Relevant Antibiotic Resistance Determinants in Treated Wastewater-Irrigated Soils. *Environ. Sci. Technol.* **2021**, *55*, 6814–6827. [CrossRef]
73. Zhou, Z.; Yao, H. Effects of composting different types of organic fertilizer on the microbial community structure and antibiotic resistance genes. *Microorganisms* **2020**, *8*, 268. [CrossRef] [PubMed]
74. Cerqueira, F.; Matamoros, V.; Bayona, J.M.; Berendonk, T.U.; Elsinga, G.; Hornstra, L.M.; Piña, B. Antibiotic resistance gene distribution in agricultural fields and crops. A soil-to-food analysis. *Environ. Res.* **2019**, *177*, 18–26. [CrossRef] [PubMed]
75. Xie, S.; Wu, N.; Tian, J.; Liu, X.; Wu, S.; Mo, Q.; Lu, S. Review on the removal of antibiotic resistance genes from livestock manure by composting. *IOP Conf. Ser. Earth Environ. Sci.* **2019**, *237*, 052010. [CrossRef]
76. Zhu, Z.; Cao, M.; Wang, W.; Zhang, L.; Ma, T.; Liu, G.; Zhang, Y.; Shang, Z.; Chen, X.; Shi, Y.; et al. Exploring the Prevalence and Distribution Patterns of Antibiotic Resistance Genes in Bovine Gut Microbiota Using a Metagenomic Approach. *Microb. Drug Resist.* **2020**, *00*, 980–990. [CrossRef] [PubMed]
77. Lins, P.; Reitschuler, C.; Illmer, P. Methanosarcina spp., the key to relieve the start-up of a thermophilic anaerobic digestion suffering from high acetic acid loads. *Bioresour. Technol.* **2014**, *152*, 347–354. [CrossRef]
78. Smith, K.S.; Ingram-Smith, C. Methanosaeta, the forgotten methanogen? *Trends Microbiol.* **2007**, *15*, 150–155. [CrossRef] [PubMed]
79. Chen, J.; Yu, Z.; Michel, F.C.; Wittum, T.; Morrison, M. Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Appl. Environ. Microbiol.* **2007**, *73*, 4407–4416. [CrossRef]
80. Chen, J.; Fluharty, F.L.; Morrison, M.; Yu, Z. Technical note: Occurrence in fecal microbiota of genes conferring resistance to both macrolide-lincosamide-streptogramin B and tetracyclines concomitant with feeding of beef cattle with tylosin 1. *J. Anim. Sci.* **2004**, *86*, 2385–2391. [CrossRef]
81. Sóki, J. Extended role for insertion sequence elements in the antibiotic resistance of Bacteroides. *World J. Clin. Infect. Dis.* **2013**, *3*, 1–12. [CrossRef]
82. Whittle, G.; Hund, B.D.; Shoemaker, N.B.; Salyers, A.A. Characterization of the 13-Kilobase ermF Region of the Bacteroides Conjugative Transposon CTnDOT. *Appl. Environ. Microbiol.* **2001**, *67*, 3488–3495. [CrossRef]

83. Zhanel, G.G.; Cheung, D.; Adam, H.; Zelenitsky, S.; Golden, A.; Schweizer, F.; Gorityala, B.; Lagacé-Wiens, P.R.S.; Walkty, A.; Gin, A.S.; et al. Review of Eravacycline, a Novel Fluorocycline Antibacterial Agent. *Drugs* **2016**, *76*, 567–588. [CrossRef] [PubMed]
84. Santamaría, J.; López, L.; Soto, C.Y. Detection and diversity evaluation of tetracycline resistance genes in grassland-based production systems in Colombia, South America. *Front. Microbiol.* **2011**, *2*, 1–10. [CrossRef]
85. Chen, Q.; An, X.; Li, H.; Su, J.; Ma, Y.; Zhu, Y.G. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. *Environ. Int.* **2016**, *92–93*, 1–10. [CrossRef]
86. Wu, N.; Zhang, W.; Xie, S.; Zeng, M.; Liu, H.; Yang, J.; Liu, X.; Yang, F. Increasing prevalence of antibiotic resistance genes in manured agricultural soils in northern China. *Front. Environ. Sci. Eng.* **2020**, *14*, 1. [CrossRef]
87. Khurshid, M.; Rasool, M.H.; Ashfaq, U.A.; Aslam, B.; Waseem, M.; Ali, M.A.; Almatroudi, A.; Rasheed, F.; Saeed, M.; Guo, Q.; et al. Acinetobacter baumannii sequence types harboring genes encoding aminoglycoside modifying enzymes and 16srRNA methylase; a multicenter study from Pakistan. *Infect. Drug Resist.* **2020**, *13*, 2855–2862. [CrossRef]
88. Ramirez, M.S.; Tolmasky, M.E. Aminoglycoside modifying enzymes. *Drug Resist. Updat.* **2010**, *13*, 151–171. [CrossRef] [PubMed]
89. Gupta, S.K.; Shin, H.; Han, D.; Hur, H.G.; Unno, T. Metagenomic analysis reveals the prevalence and persistence of antibiotic- and heavy metal-resistance genes in wastewater treatment plant. *J. Microbiol.* **2018**, *56*, 408–415. [CrossRef]
90. He, P.; Wu, Y.; Huang, W.; Wu, X.; Lv, J.; Liu, P.; Bu, L.; Bai, Z.; Chen, S.; Feng, W.; et al. Characteristics of and variation in airborne ARGs among urban hospitals and adjacent urban and suburban communities: A metagenomic approach. *Environ. Int.* **2020**, *139*, 105625. [CrossRef] [PubMed]
91. Gao, Q.; Li, Y.; Qi, Z.; Yue, Y.; Min, M.; Peng, S.; Shi, Z.; Gao, Y. Diverse and abundant antibiotic resistance genes from mariculture sites of China's coastline. *Sci. Total Environ.* **2018**, *630*, 117–125. [CrossRef] [PubMed]
92. Hirte, K.; Seiwert, B.; Schüürmann, G.; Reemtsma, T. New hydrolysis products of the beta-lactam antibiotic amoxicillin, their pH-dependent formation and search in municipal wastewater. *Water Res.* **2016**, *88*, 880–888. [CrossRef]
93. Wallace, J.S.; Aga, D.S. Enhancing extraction and detection of veterinary antibiotics in solid and liquid fractions of manure. *J. Environ. Qual.* **2016**, *45*, 471–479. [CrossRef] [PubMed]
94. Fernández-Gómez, B.; Richter, M.; Schüler, M.; Pinhassi, J.; Acinas, S.G.; González, J.M.; Pedrós-Alió, C. Ecology of marine bacteroidetes: A comparative genomics approach. *ISME J.* **2013**, *7*, 1026–1037. [CrossRef] [PubMed]
95. Kumar, M.; Dutta, S.; You, S.; Luo, G.; Zhang, S. A critical review on biochar for enhancing biogas production from anaerobic digestion of food waste and sludge. *J. Clean. Prod.* **2021**, *305*, 127143. [CrossRef]