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The Impact of Antimicrobial Substances on the Methanogenic Community during Methane Fermentation of Sewage Sludge and Cattle Slurry

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Abstract: This study showed the effect of amoxicillin (AMO), and oxytetracycline (OXY) at a concentration of 512 μg mL^{-1}, and sulfamethoxazole (SMX), and metronidazole (MET) at a concentration of 1024 μg mL^{-1} on the efficiency of anaerobic digestion (AD) of sewage sludge (SS) and cattle slurry (CS). The production of biogas and methane (CH_4) content, and the concentration of volatile fatty acids (VFAs) was analyzed in this study. Other determinations included the concentration of the *mcr*A gene, which catalyzes the methanogenesis, and analysis of *MSC* and *MST* gene concentration, characteristic of the families *Methanosarcinaceae* and *Methanosaetaceae* (*Archaea*). Both substrates differed in the composition of microbial communities, and in the sensitivity of these microorganisms to particular antimicrobial substances. Metronidazole inhibited SS fermentation to the greatest extent (sixfold decrease in biogas production and over 50% decrease in the content of CH_4). The lowest concentrations of the *mcr*A gene (10^6 $g_D{}^{-1}$) were observed in CS and SS digestates with MET. A decline in the number of copies of the *MSC* and *MST* genes was noted in most of the digestate samples with antimicrobials supplementation. Due to selective pressure, antimicrobials led to a considerably lowered efficiency of the AD process and induced changes in the structure of methanogenic biodiversity.

Keywords: methane fermentation efficiency; biogas; methanogens; sewage sludge; cattle slurry; antimicrobials; qPCR

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1. Introduction

Activities taken for the sake of environmental protection and energy security are the main motivation of research on renewable energy sources [1]. One of the alternatives to fossil fuels is methane (CH_4) obtained from methane fermentation in anaerobic conditions, i.e., anaerobic digestion (AD). Methane fermentation can be implemented in technologies designed for the safe disposal and immobilization of a variety of organic biomass, used as a substrate [2]. Anaerobic digestion is a process often applied to sewage sludge and cattle slurry, which are substrates that enable the acquisition of high CH_4 content in biogas. Methane obtained via biological transformations is an eco-friendly, cheap and renewable fuel [3]. In order to harvest the highest possible amount of biogas and CH_4 contained in it, strict monitoring of the AD process and composition of the involved microbial community, an extremely complex and specialized microbiome, is in order [4,5].

The four-stage AD process engages different groups of microorganisms, responsible for each of the stages and mutually dependent on one another. The following degradation stages can be distinguished (according to the subsequent organic matter conversions): hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which is the final, key stage of the process driven by the activity of methanogenic microorganisms [6]. Substrates subjected

to AD, and especially sewage sludge and cattle slurry, may contain pharmaceuticals (and products of their transformation), used in treatment of humans and animals, which have not been completely metabolized in their bodies [7,8]. There are numerous reports demonstrating that drugs administered in human and veterinary medicine have been detected in digestate from biogas plants fed sewage sludge or cattle slurry [9–11]. The presence of medications in substrates induces permanent selective pressure on microorganisms active in the first three stages of AD, and on methanogens directly responsible for generation of CH_4 [12,13].

Both sewage sludge and cattle slurry, often used as substrates in AD, can be loaded with antimicrobial substances present in different concentrations. The drugs most often detected in sewage sludge are quinolones, sulfonamides and MLS pharmaceuticals (macrolides-lincosamides-streptogramins) [14], whereas cattle slurry is most often found to contain tetracyclines, amoxicillin, and sulfamethoxazole [15]. Substrates undergoing AD are also frequently determined to be contaminated with metronidazole (MET), which effectively inhibits the growth and development of anaerobic microorganisms and, by being a fat-soluble substance, it is difficult to remove [16]. Despite more stringent drug monitoring procedures, it is still worrying to observe a constant growth in the global consumption of pharmaceuticals [17]. In 2013, it was estimated that the consumption of antibiotics in China alone reached about 8.4×10^7 kg, half of which was administered in veterinary medicine, and the overall amount greatly surpassed the annual consumption of antibiotics in the USA, which was 1.48×10^7 kg. Antimicrobial preparations used in veterinary treatment corresponded to 52% of the total consumption of antibiotics in China and 80% in the USA. At the same time, the consumption of antibiotics on animal farms in the EU countries averaged around 7.98×10^6 kg. The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) informs that in Poland alone in 2014, the total amount of consumed antibiotics was 5.8×10^5 kg [18]. Research by Gao et al. [19] and Wei et al. [20] showed that a large part of the residue, e.g., tetracycline, was discharged unchanged and/or active to domestic or animal wastewater at concentrations ranging from ng L^{-1} to mg L^{-1}. Similar concentrations of antibiotics were observed by Zhao et al. [21] in the analyzed samples of cattle manure, which were approx. 59 mg kg^{-1} for oxytetracycline, while Martinez-Carballo et al. [22], analyzing the same kind of samples, noted the concentration 90 mg kg^{-1} for sulfadiazine. Antimicrobial substances can affect methanogens and bacteria in various ways. Their direct effects may include the inhibition of DNA replication, RNA transcription or ATP generation. They can also retard cell division, protein translation or synthesis of the cell wall and nucleotides [23,24]. Antibiotics can also affect microorganisms indirectly by influencing the unbalance between microbial groups activities in four steps including hydrolysis, acidogenesis, acetogenesis and methanogenesis. For the carrying out the first three stages of methane fermentation, various groups of bacteria are responsible, which supply the substrates for biogas production to methanogens. Acetic bacteria convert the products like propionate to acetate, glucose to acetate, and ethanol to acetate, which can then be used by methanogenic microorganisms as a substrate. However, methanogens belonging to the *Archaea* domain are necessary for the success of the AD process and the stable operation of bioreactors. Among the methanogens, due to their ability to convert the substrate, there are acetotrophic and hydrogenotrophic methanogens [25]. These microorganisms are responsible for catalyzing the final and most sensitive stage of the anaerobic process (methanogenesis). The acetotrophic methanogens play an extremely important role in the production of CH_4, since 70% of the methane produced as a product of the process comes from acetate [26]. Antibiotics in wastewater and cattle slurry used as substrates in the AD process can pose serious problems for anaerobic microorganisms. Drugs can reduce the activity of methanogenic microorganisms or alter the microbial populations, influencing pollutant removal and biogas production. The presence of antibiotics during anaerobic treatment may also disturb the balance of the process through the accumulation of metabolic intermediates such as volatile fatty acids (VFAs), which consequently may lead to a complete inhibition of the process [27]. The occurrence of

drugs in AD-processed substrates has a direct influence on metabolic functions of both methanogens and bacteria active in the first three stages of the process, which are responsible for supplying methanogenic *Archaea* with substrates for CH_4 production [28]. Many authors have confirmed the negative impact of several antimicrobial substances on the efficiency of AD, with such manifestations as a decreased yield of biogas or its lower CH_4 content [29,30]. As evidenced in a study by Aydin et al. [25], the presence of different combinations of antibiotics in a substrate undergoing AD first and foremost inhibits the activity methanogens and acetogenic bacteria. This may be a consequence of changes in the structure of microorganisms in bioreactors due to their exposure to antibacterial compounds, which has a direct effect on biogas production [30].

To the best of our knowledge, no studies have been conducted so far that would analyze the effect of the most common antimicrobial substances used in veterinary and human medicine on the AD process, inclusive of the processing parameters and the microbiological aspect of AD and using a model of substrates of different origin. It is worth noting that the relevant scientific references [31,32] do not provide data concerning the effect of metronidazole (characterized by the direct influence on methanogens) on the efficiency of biogas production. The purpose of this study has been to determine the effect of amoxicillin (AMO), oxytetracycline (OXY), sulfamethoxazole (SMX) and metronidazole (MET) on the efficiency of the AD process of sewage sludge (SS) and cattle slurry (CS). The effect of the drugs on the production of biogas and its content of CH_4 was determined, while simultaneously analyzing concentrations of VFAs. Furthermore, the influence of the mentioned antimicrobial substances on changes in the structure and activity of the methanogenic community was studied, using the concentrations of the gene of methyl-coenzyme M reductase (*mcr*A), catalyzing the last stage of AD—methanogenesis—and the concentration of 16S rRNA genes characteristic of the methanogenic *Archaea* families, i.e., *Methanosarcinaceae* (named in the text of this manuscript as MSC) and *Methanosaetaceae* (named in the text of this manuscript as MST). The research results will enrich the global database with information about the influence of such inhibitors as antimicrobial substances on the AD process.

2. Materials and Methods

2.1. Substrates and Inoculum

The tested substrates were sewage sludge (SS) from the Wastewater Treatment Plant (WWTP) in Olsztyn (Poland) and cattle slurry (CS), which was obtained from a farm located in Bałdy (Poland). Anaerobic sludge from the WWTP was used as the inoculum in AD of both SS and CS substrates. The characteristics of the substrates and inoculum are described in Table 1.

Table 1. The characteristics of substrates used in the anaerobic digestion (AD) process.

	TS [a] g_D^{-1} [b] (mg)	VS [c] g_D^{-1} (mg)	pH	TP [d] g_{TS}^{-1} (mg)	TN [e] g_{TS}^{-1} (mg)
SS	55.7 ± 1.5	42.8 ± 2.3	8.01 ± 0.4	0.6 ± 0.2	2.1 ± 0.4
CS	150.0 ± 10.8	123.1 ± 14.5	8.2 ± 0.5	1.6 ± 2.4	5.3 ± 2.8
Inoculum	38.8 ± 5.2	25.2 ± 3.8	8.1 ± 0.5	0.9 ± 0.4	5.5 ± 1.9

[a] TS—total solids, [b] g_D^{-1}—value of parameter per one gram of digestate samples, [c] VS—volatile solids, [d] TP—total phosphorus, [e] TN—total nitrogen, SS—sewage sludge, CS—cattle slurry.

2.2. Methane Fermentation

The choice of the antimicrobial substances tested in the AD process was dictated by the information about the most popular drugs in human and veterinary medicine [18]. The impact of antimicrobials such as metronidazole (MET), amoxicillin (AMO), and oxytetracycline (OXY) at a concentration of 512 μg mL^{-1}, and sulfamethoxazole (SMX), and metronidazole (MET) at a concentration of 1024 μg mL^{-1} was determined with use the Automatic Methane Potential Test System II (Bioprocess Control, Lund, Sweden), by testing

the production of biogas and its CH_4 content (Table 2; Supplementary Materials Figure S1). In the experiment, high doses of antibiotics were intentionally injected into bioreactors in order to induce a reaction of microorganisms. Details about the experiment were described in our previous study [33]. The antimicrobial substances were added to the substrate in the tested bioreactors, later referred to as the process bioreactors. These bioreactors are abbreviated in the graphics according to the type of antibiotic and substrate (for example, "MET SS" means a bioreactor with sewage sludge exposed to MET). Mesophilic anaerobic digestion (37 °C) with an organic loading of 5 g VS L^{-1} was carried out in bioreactors with a volume of 250 mL Anaerobic digestion was carried out in two replications, for 40 days. Anaerobic conditions were achieved by continuous flushing of pure nitrogen through the sludge/slurry. Control samples were prepared in the same way as the tested samples, but without antimicrobials supplementation. Control samples were labeled as SSC (sewage sludge) and CSC (cattle slurry).

The AD process settings were described in our previous study [34]. Anaerobic conditions were achieved by continuous flushing of pure nitrogen through sludge. The following were determined in the samples of digestate before and after AD: the content of volatile fatty acids (VFAs), pH, the FOS/TAC ratio (the TAC value is an estimation of the buffer capacity of the sample, and the FOS value indicates the volatile fatty acids content), and the TS (total solids), and VS (volatile solids), and the TN (total nitrogen), and TP (total phosphorus) content.

2.3. Genomic DNA Isolation from Digestate Samples

Digestate samples in amounts of 2 g each were transferred to 2 mL Eppendorf centrifuge tubes. The samples were then centrifuged for 10 min at 8000 rpm. In the next step, the supernatant was removed from centrifuged digestate samples. Next, DNA was isolated from the pellet in duplicate, using a Fast DNA Spin Kit for Soil® (MP Biomedicals™, Carlsbad, CA, USA) according to the manufacturer's instructions. Multiskan Sky (Thermo Scientific™, Waltham, MA, USA) was used for determination of the concentration and quality of extracted genetic material. gDNA from digestate samples was stored in a freezer (−20 °C) for quantitative analysis by qPCR.

2.4. Analysis of the Genes mcrA, MSC and MSC by Quantitative Real-Time Polymerase Chain Reactions (qPCR)

The analysis of the presence and activity of methanogenic microorganisms was based on Real-Time PCR (qPCR). With the help of this method, determination of the concentrations of genes characteristic of two families which belong to the order *Methanosarcinales*: *Methanosarcinaceae* (*MSC*) and *Methanosaetaceae* (*MST*) was planned, both responsible for CH_4 production, according to Yu et al. [35] and Denman et al. [36]. Preliminary investigations enabled us to exclude any significant share in the samples of other genera from the domain *Archaea*, such as *Methanococcales*, *Methanobacteriales*, or *Methanomicrobiales*. Evaluation of the activity of methanogens was based on an analysis of the concentration of the gene of methyl-coenzyme M reductase (*mcr*A), catalyzing the last step of AD—methanogensis. A LightCycler® instrument (Roche Diagnostics GmbH, Mannheim, Germany) with LightCycler® software (version 1.5.0) was used to identify the abundance of genes specific for methanogenic *Archaea* (*mcr*A, *MSC*, *MST*) during AD of sewage sludge and AD of cattle slurry. The abundance of the genes was calculated as a copy number in 1 g digestate (D). All qPCR reactions were carried out with three replications. Each reaction was performed using a mixture of 15 µL in capacity, containing 0.8 µL gDNA, 0.75 µL of each forward and reverse oligonucleotide starters (10 µM in concentration), 7.5 µL SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and sterile water. During the amplification of genes in a qPCR reaction, both negative control, without DNA matrix, and positive control, characterized by a known number of copies of a given gene, were employed. Sequences of primers and parameters used for qPCR analyses are shown in Table S1 (Supplementary Materials).

Table 2. The results of methane fermentation in the process reactors with antimicrobial addition and control samples.

Antibiotic Concentration ($\mu g\ mL^{-1}$)	Substrate	CH_4 * Production ($L\ kg^{-1}$ VS)	CH_4 Content in Biogas (%)	VFAs Concentration ($g\ L^{-1}$)								
				Acetic Acid	Propionic Acid	Iso-Butyric Acid	Butyric Acid	Iso-Valeric Acid	Valeric Acid	Iso-Caproic Acid	Caproic Acid	Heptanoic Acid
MET	SS	44.3 * ± 3.5	12.8 * ± 4.0	17.52 * ± 1.85	4.96 * ± 0.99	5.04 * ± 1.20	3.69 ± 0.79	5.89 * ± 1.02	2.73 * ± 0.84	4.36 * ± 0.78	0.67 * ± 0.11	0.01 ± 0.01
(512)	CS	143.4 * ± 44.0	70.7 ± 4.7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AMO	SS	198.1 * ± 17.3	43.9 ± 2.7	0.83 ± 0.17	0.38 ± 0.13	0.44 ± 0.13	253.00 * ± 15.89	0.00	0.00	0.49 ± 0.09	0.00	0.00
(1024)	CS	51.2 * ± 27.0	61.5 * ± 11.4	16.11 * ± 2.09	5.96 * ± 0.99	6.46 * ± 1.12	4.56 * ± 0.89	7.75 * ± 1.45	2.93 * ± 0.43	4.34 * ± 0.85	0.89 ± 0.29	0.23 ± 0.11
OXY	SS	181.1 * ± 14.3	69.8 ± 0.5	0.95 ± 0.1	0.14 ± 0.09	0.12 ± 0.05	0.07 ± 0.04	0.53 ± 0.21	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
(1024)	CS	91.5 * ± 33.1	68.2 ± 5.4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SMX	SS	209.4 ± 3.2	70.2 ± 1.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(512)	CS	183.1 * ± 54.1	71.4 ± 3.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CONTROL	SSC	272.8 ± 21.1	65.5 ± 2.3	0.26 ± 0.10	0.03 ± 0.03	0.03 ± 0.03	0.00	0.04 ± 0.03	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.02
	CSC	201.2 ± 9.7	70.8 ± 3.5	0.26 ± 0.10	0.03 ± 0.03	0.03 ± 0.03	0.00	0.04 ± 0.03	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.02

(VFAs) volatile fatty acids, (MET) metronidazole, (AMO) amoxicillin, (OXY) oxytetracycline, (SMX) sulfamethoxazole, (SS) sewage sludge, (CS) cattle slurry, (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry. (*) indicates statistically significant differences regarding the proper control.

2.5. Data Analysis

Statistica 13.1 software was used for data analysis. Differences in the number of copies of *Archaea* specific genes were determined by the Kruskal–Wallis test (KW) (for values deviating from normal distribution). Results were regarded as statistically significant at $p < 0.05$. Principal component analysis (PCA) was used to visualize the correlations between the concentration of genes, the accumulation of VFAs and the production of biogas in individual processes and control bioreactors with SS and CS. The distributions of genes in analysed substrates were visualized using a heatmap and clustering method implemented in the software R Studio. Ward's method was used for hierarchical clustering of methane production values and VFAs concentration in SS and CS from process and control bioreactors [37].

3. Results and Discussion

3.1. The Effect of Antimicrobials on Biogas Yield

The methane fermentation of SS led to a statistically significant decrease in the efficiency of anaerobic processing after the substrate had been added AMO, OXY and MET. Compared to the control, MET most strongly inhibited the AD process, which resulted in a nearly six-fold decline in the biogas yield and a decrease in its content of CH_4 by over 50%. The concentration of seven out of nine analyzed VFAs was significantly higher in a sample of the digestate from SS exposed to MET than in the control (Table 2; Supplementary Materials Figure S1). The highest concentration was recorded for acetic acid. The SS digestate sample exposed to the presence of AMO was shown to have accumulated a considerable amount of butyric acid. Another study [38] analyzing the effect of antimicrobial substances on sewage sludge AD proved that MET, AMO, OXY and SMX in low doses (8, 16, 8, and 8 mg kg^{-1}, respectively) did not have a significant effect on the efficiency of this process. The research results clearly indicate that the efficiency of the AD processing of sewage sludge is significantly decreased in the presence of high concentrations of antimicrobial substances. Drugs which enter WWTPs in high quantities, having been, for example, excreted by people [39], can induce such consequences as a lower efficiency or complete inhibition of SS methane fermentation.

Antimicrobial substances are also present in by-products from animal farms, most probably due to their administration in treatment of animals. The presence of antibiotics can lower the rate or retard the conversion of such substrates by microorganisms engaged in the AD process [40]. The conducted research showed statistically significant decreased biogas production in the presence of all the four microbial substances when CS was used as a substrate. The most severe inhibition was due to the exposure to amoxicillin (AMO) and oxytetracycline (OXY). The presence of these microbial substances in CS resulted in a nearly four-fold and over two-fold decrease in biogas production due to the presence of AMO and OXY, respectively (Table 2; Supplementary Materials Figure S1). Moreover, the introduction of AMO to CS caused a statistically significant decrease in the biogas content of CH_4, which declined by nearly 10%. Additionally, exposure to AMO caused significant accumulation of seven out of nine VFAs, indicating the inhibition of AD, and this was unobserved in any other bioreactor (Table 2). Volatile fatty acids are the major products of protein and carbohydrate synthesis in the AD process [41,42], and their accumulation can implicate the inhibition of the activity of acetogenic microorganisms [43,44]. The current results are convergent with the ones reported by Sun et al. [45], who noted that higher AMO concentrations in sewage from a piggery submitted to AD, particularly when combined with the presence of other antibiotics, caused a decrease in the rate of CH_4 production. Other researchers [46] noticed that AMO and OXY only slightly decreased the efficiency of the AD processing of swine slurry, although the concentrations of antimicrobial substances used in the cited experiment were several-fold lower than in the study reported in this paper (60–120 and 125–250 mg L^{-1}, respectively). Thus, our results demonstrate that higher concentrations of an antibiotic in a substrate of animal origin can substantially alter the efficiency of anaerobic processing, which is an extremely important finding in light

of the currently widespread application of antimicrobial compounds in both human and veterinary medicine. Concentrations of pharmaceuticals in a substrate in fermentation tanks have a direct influence on the efficiency of CH_4 production.

As demonstrated by Hashemi et al. [47], yield of biogas is negatively correlated with an increase in the concentration of an antibiotic in a substrate undergoing AD processing. Our experiment proves that anaerobic processing of SS and CS becomes less efficient when exposed to high concentrations of antimicrobial substances [47]. Importantly, both substrates are characterized by the distinctly different composition of their microbiota [48], which means that they differ in the drug sensitivity of particular groups of microorganisms engaged in the AD process. This is manifested by the different extent to which drugs affect the course and efficiency of AD. When SS served as a substrate, MET most strongly affected the efficiency of methane fermentation, causing a decrease in both biogas production and methane content in biogas, as well as the accumulation of most of VFAs. Exposure of CS to MET resulted in a 25% decrease in biogas production, without affecting the biogas content of CH_4 or accumulation of VFAs (Table 2). In the case of CS, the broadest spectrum of the process inhibition, similar to that induced by MET in SS, was detected when AMO had been added to the substrate. Exposure of SS to AMO resulted in the inhibition of biogas production and accumulation of only one of VFAs (from ten analysed in total), which was butyric acid. Values of methane production and VFAs concentration in SS and CS from process and control bioreactors were grouped in hierarchical cluster analysis with the use of Ward's method (Figure 1). Three main clusters were identified. The first cluster (I) included both control bioreactors (SSC, CSC) and SS exposed to OXY (OXY SS). The analysis of the results concerning the production of VFA in individual bioreactors (Table 2) shows that these values for OXY SS were closest to the control bioreactors with SS and CS, which resulted in grouping these three bioreactors in one cluster. MET SS and AMO CS formed the second cluster (II). As a result of exposure of the SS to MET and the CS to AMO, the highest differences compared to the control bioreactors were noted, manifested by the greatest changes in methane production and VFAs accumulation. The distinction of these bioreactors in a separate cluster also reflects the results concerning the parameters of methane fermentation (Table 2). The third cluster (III) consisted of other process bioreactors. The results point to the mentioned differences in the composition and drug-sensitivity of the microbiota that are characteristic of these substrates, with respect to particular antimicrobial substances.

3.2. Quantification of mcrA Gene

Monitoring of AD parameters, especially the yield of CH_4, makes it possible to assess the efficiency of anaerobic decomposition of organic matter. However, to achieve a complete picture of the activity of methanogens, which are the key group of microorganisms involved in this process, it is necessary to evaluate the quantities of CH_4 yield and to analyze quantitative changes in methanogenic populations on a molecular level. Back in the last years of the 20th century, it was suggested to use the *mcr*A gene as a phylogenetic tool to analyze the activity of *Archaea* methanogenic populations [48,49]. This gene, encoding the methyl-coenzyme M reductase, is characteristic of the metabolism of methanogens [25], and was therefore considered to be a molecular marker for this group of microorganisms [50]. Analysis of the occurrence of the *mcr*A gene became a potential tool to evaluate the presence of methanogens in samples originating from different environments [50]. A great advantage of the *mcr*A gene lies in the fact that only one or two copies of this gene have been found in sequenced methanogen genomes, which makes it a more precise instrument than the 16S *r*RNA gene for evaluation of the number of these groups of microorganisms, as the latter can reach, on average, from four to over a dozen copies per genome [51].

The statistical analysis showed significant differences in the concentration of *the mcr*A gene relative to the control in all digestate samples exposed to antibiotics. The lowest *mcr*A concentrations, in both CS and SS digestate samples, were determined in the presence of MET. In the case of SS, the presence of *mcr*A gene copies in 1 g of digestate from the

process and control bioreactors remained on a high level of 10^6–10^7. A decrease in the number of *mcr*A gene copies by one order relative to the control (10^7 of copies in 1 g of digestate) was recorded only in a sample of the digestate with MET (number of copies in the order of 10^6 w 1 $g_D{}^{-1}$) (Figure 2A). Although the presence of AMO and OXY led to a significant decrease in the yield of CH_4, lower by nearly 75 and 92 L kg^{-1} VS, respectively (Table 2), the concentration of the *mcr*A gene in digestate samples including these antibiotics remained on the level in the same order of magnitude as in the control sample. In this case, the concentrations of the *mcr*A gene, which is a functional gene of methanogenic microorganisms, do not reflect the actual production of methane in bioreactors.

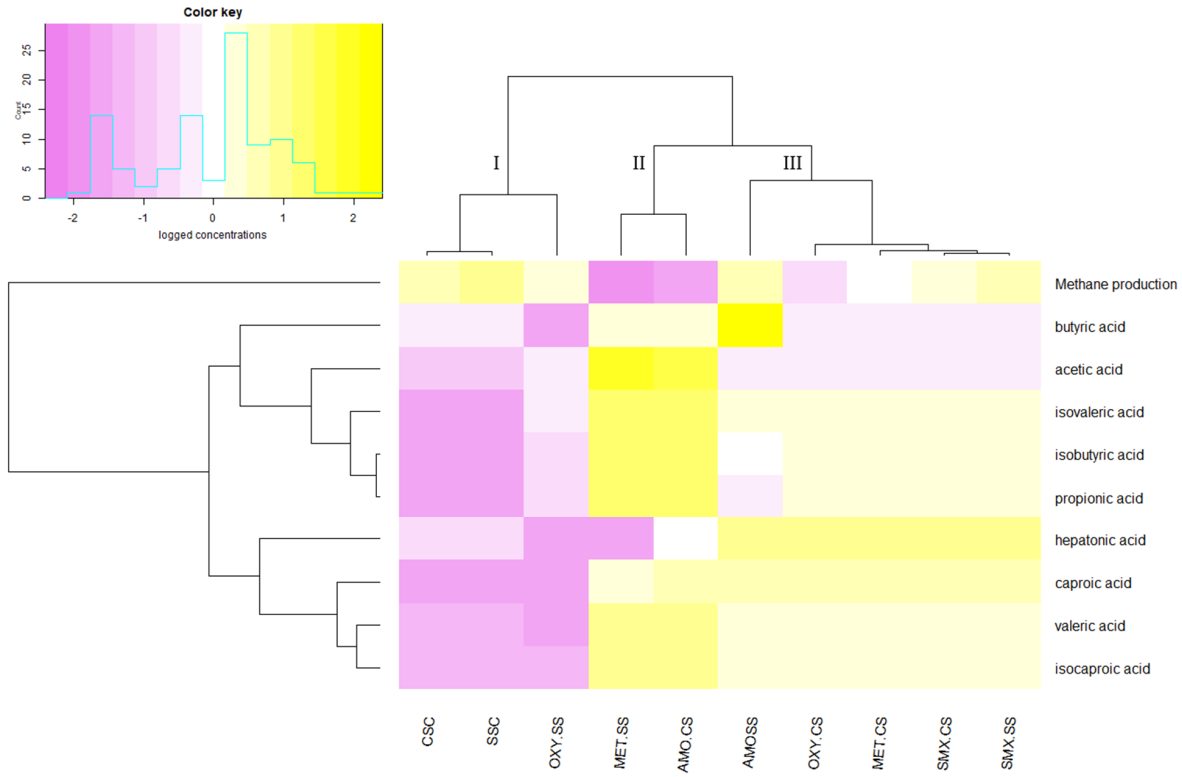

Figure 1. Heatmap with the logged values of methane production (L kg^{-1} VS) and VFAs concentrations (g L^{-1}) in process and control bioreactors with sewage sludge (SS) and cattle slurry (CS). (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry.

With respect to CS as a substrate, the number of copies of the *mcr*A gene in 1 g of digestate of the control sample and of experimental samples exposed to OXY or SMX oscillated on a high level, in the order of 10^8. Importantly, the presence of OXY in CS resulted in an over twofold decline in biogas yield relative to the control. Paradoxically, the analyzed samples of digestate from both SS and CS exposed to the presence of OXY were observed to generate a significantly lower CH_4 yield with the simultaneous increase in the *mcr*A concentration in comparison with the control sample. In turn, exposure of CS to MET and AMO resulted in a decreased concentration of the *mcr*A gene by two orders of magnitude (10^6 copies in 1 g of digestate) (Figure 2B).

The research results therefore implicate that a decrease in the frequency of presence of *mcr*A gene copies was associated with the efficiency of biogas production, content of CH_4 in biogas and concentration of VFAs only when samples were exposed to MET in the case of the SS substrate and AMO when CS was the substrate. These relationships were illustrated in a PCA diagram (Figure 3), which shows a positive correlation between the exposure of SS and CS to metronidazole (MET SS) and amoxicillin (AMO CS), respectively, and the VFAs accumulation. Moreover, a negative correlation with the production of CH_4 and concentration of the *mcr*A gene is noticeable.

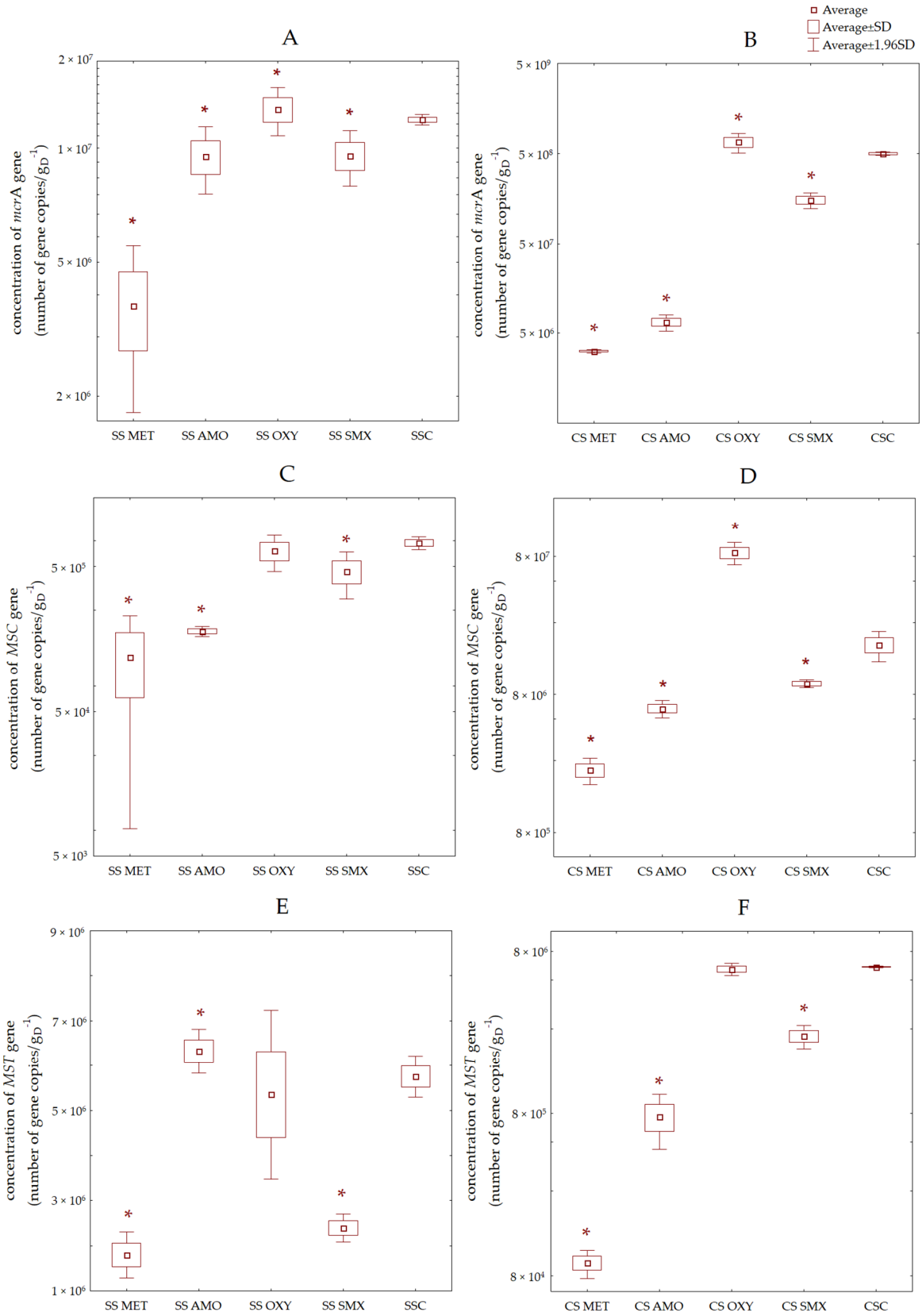

Figure 2. Average concentrations of *mcr*A, *MSC* and *MST* genes in 1 g_D^{-1} of sewage sludge samples (**A**,**C**,**E**, respectively) from the process and control bioreactors and cattle slurry samples (**B**,**D**,**F**, respectively) from the process and control bioreactors. (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry, (*) indicates statistically significant differences compared to the proper control ($p < 0.05$).

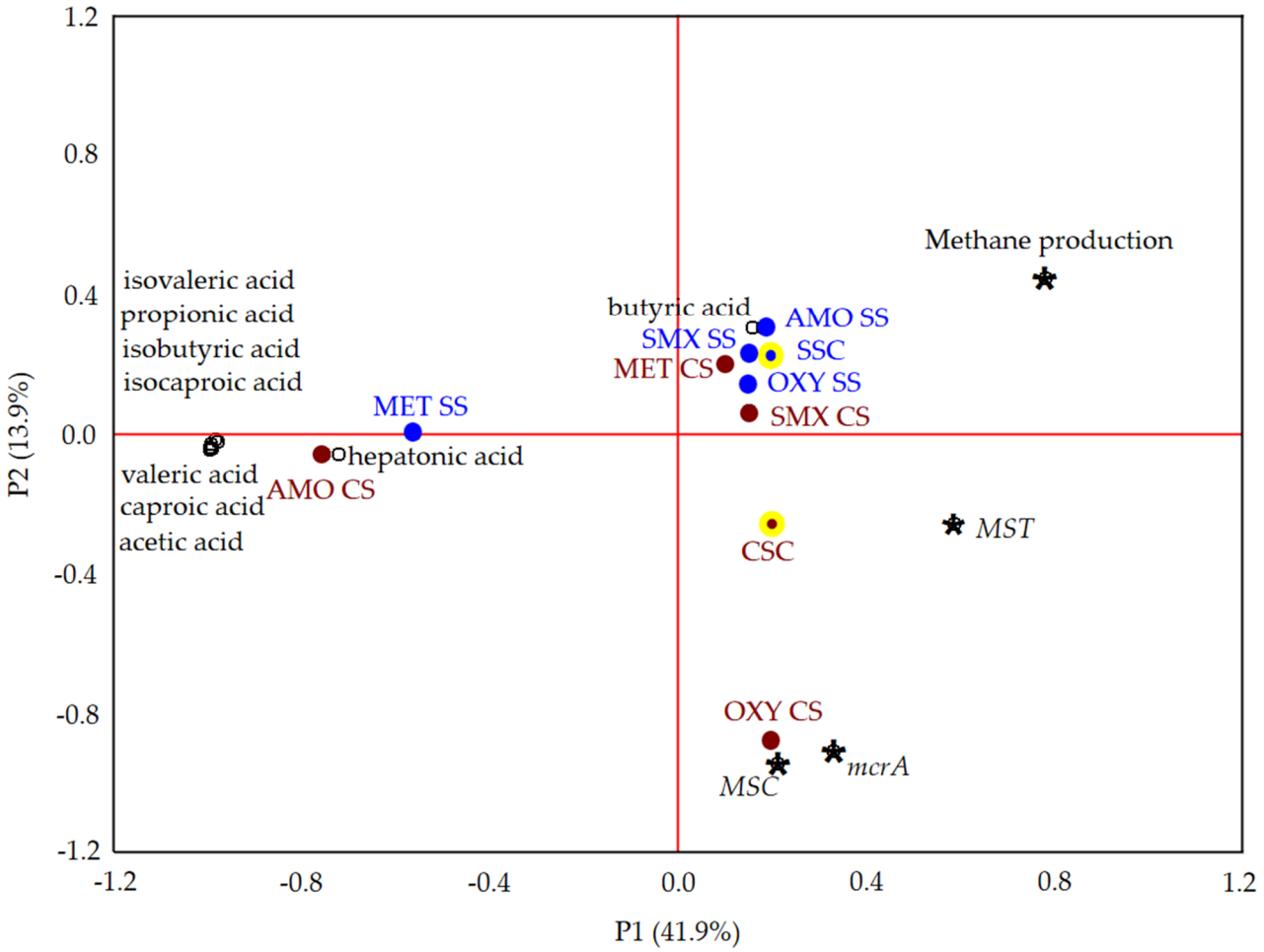

Figure 3. Principal component analysis (PCA) based on the distribution of the analyzed genes characteristic of methanogens and parameters of methane fermentation in bioreactors with sewage sludge (SS) and cattle slurry (CS). (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry.

Furthermore, an analysis was made of the presence of statistically significant differences in concentrations of the *mcr*A gene between appropriate samples of SS and CS digestates (exposed to the same antimicrobial substance) and control samples SSC and CSC. Statistically significant differences ($p < 0.05$) were noted in each case (Figure 3). Importantly, the number of the *mcr*A gene copies in the CSC control sample ranged around 5.0×10^8 in 1 g of digestate (Figure 2B), whereas, in the SSC sample, this concentration was just 1.34×10^7 copies per gram of analyzed sample (Figure 2A), which was 37-fold lower. With these data, one might expect that the concentration and activity of methanogens in the CSC samples would be higher, which should result in a higher efficiency of the process. However, the results of an analysis of AD parameters (Table 2) demonstrated that the CH_4 yield was higher by 72 L kg^{-1} VS in the SSC samples, not exposed to the presence of antimicrobial substances.

Ma et al. [52] demonstrated in their study that the number of *mcr*A gene copies in substrates undergoing AD may significantly differ from the concentration of transcripts of this gene. In their investigation of the soil from rice fields, these authors showed that the number of *mcr*A gene transcripts changed in different environmental conditions while the number of copies of this gene remained virtually unchanged [52]. These results suggest that only some of the methanogenic *Archaea* were metabolically active and responsible for CH_4 production. It can be suspected that the underlying reason is that the AD reaction environment presented conditions unfavorable for methanogens, causing the inhibition of the *mcr*A gene transcription processes. In the experiment reported in this article, first

and foremost such unfavorable conditions meant exposure to high doses of antimicrobial substances, in addition to the resulting accumulation of VFAs in the selected process bioreactor chambers. Our experiment proved that an evident decrease in the production of CH_4, caused by the presence of each of the tested drugs, may not correlate with the abundance of the *mcr*A gene, characteristic of methanogens [53].

Considering the above information, it can be concluded that the abundance of both the *mcr*A gene and its transcripts should be analyzed as part of an evaluation of the metabolic activity of methanogenic microorganisms. The experiment reported herein has shown that an assessment based solely on the presence of a functional gene of methanogens can lead to unreliable data. As the transcription of genes is more closely connected with the activity of microorganisms, determination of the number of *mcr*A gene transcripts seems to be a more adequate measure than just the number of copies of this gene [54–57].

3.3. The Effects of Antimicrobials on Changes in the Diversity of Methanogenic Microorganisms

Among the methanogenic *Archaea* involved in the AD processes, the ones most often mentioned in the literature are microorganisms from the order *Methanosarcinales*, with the prevalence of two families, *Methanosarcinaceae* and *Methanosaetaceae* [55]. In this experiment, the number of copies of the genes that are characteristic of two families, *Methanosarcinaceae* (*MSC* gene) and *Methanosaetaceae* (*MST* gene), were assayed, excluding in the preliminary study the presence in CS and SS digestate samples of other families from the domain *Archaea*. The families *Methanosaetaceae* and *Methanosarcinaceae* are acetoclastic microorganisms, sensitive to changes in environmental conditions and to the presence of such inhibitors as antimicrobial pharmaceuticals. *Methanosarcinaceae* continue stable growth at higher doses of acetate [36], unlike *Methanosaetaceae*, whose growth can be restrained in the presence of this compound [56].

In our research, regardless of the type of antibiotic added to a substrate, a decrease in the number of *MSC* and *MST* gene copies was observed in most of the digestate samples from SS and CS substrates (Figure 2C–F). It is significant to notice that the SS digestate samples were dominated by the *MST* genes (Figure 2E), while the CS digestate was observed to be dominated by *MSC* genes (Figure 2D), which proves that different groups of methanogens can be responsible for CH_4 production, regardless of the substrate applied in AD.

The number of copies of the *MSC* gene in SS samples ranged from 1.19×10^5 to 6.33×10^6, whereas the number of the *MST* gene copies was from 1.80×10^6 to 6.33×10^6 in 1 g of digestate (Figure 2C,E). In the control sample, the number of copies of the *MSC* gene was 7.26×10^5, whereas the number of the *MST* gene copies equaled 5.76×10^6 per gram of digestate. Compared to the control sample, a statistically significant decrease in the number of the *MST* gene copies was recorded, which may have been due to the selection pressure exerted by the drugs and to the concentrations of VFAs recorded in the AD reactors [56] (Table 2). The research results concerning the number of gene copies justify the claim that despite the presence of antibiotics in digestates from SS and a decline in the number of the *MST* gene copies, microorganisms from the family *Methanosetaceae* persisted in a large number until the AD process terminated. These research results are inconsistent with the data reported by other authors [57–59], who also demonstrated that of the two families analyzed in this experiment, it is *Methanosaetaceae* that dominates throughout the anaerobic conversion of sewage sludge biomass.

Relative to the control, the CS digestate samples exposed to MET, AMO or SMX demonstrated a considerable decrease in the number of *MST* and *MSC* genes. Interestingly, the presence of OXY in the CS samples resulted in a significant rise in the concentration of *MSC* genes compared with the control, coinciding with a significant increase in the number of the *mcr*A gene copies, which nevertheless did not translate into a higher efficiency of CH_4 production, which in fact was considerably decreased due to the exposure to this antibiotic (Figure 2B). The antimicrobial drugs MET and AMO had the strongest impact on the increase in the abundance of methanogens in substrates, which is evidenced by a decrease in the

number of the *MST* gene copies by as much as two orders of magnitude (10^4 copies in 1 g of digestate with MET) compared to the control (10^6 copies in 1 g of digestate) (Figure 2F). Out of the two analyzed families, *Methanosarcinaceae* occurred most numerously in the CS digestate, which is evidenced by the high number of the *MSC* gene copies (from 2.14×10^6 to 8.02×10^7 copies in 1 g of analyzed digestate). The number of the *MSC* gene copies in 1 g of CS digestate samples was lower by one order of magnitude (2.14×10^6) in comparison with the control sample. Despite the observed decrease in the number of copies of the *MST* and *MSC* genes, both gene copies in CS digestate samples remained constantly high (in the order of 10^7 in 1 g of digestate). The persistently high number of copies of the *MST* and *MSC* genes in CS digestate proves that *Methanosarcinaceae* and *Methanosaetaceae* have adapted to the antibiotics and products of their metabolism present in the digestate. The results of our study are convergent with the reports by other researchers, who applied molecular methods to monitor the composition of microbial communities in manure digestate, and also noted the most numerous presence of *Methanosarcinaceae* [60–62]. A possible underlying cause of the dominance of *Methanosarcinaceae* in CS digestate could be the degree of dilution of the substrate, characteristic of cattle slurry and beneficial for the microorganisms in question. The prevalence of *Methanosarcinaceae* could also result in the decreasing abundance of *Methanosaetaceae* in the substrate fed to a bioreactor, as these two families of microorganisms compete for such products as acetate [36].

The influence of antibiotics on changes in the methanogenic community in the samples is well illustrated by the Ward method based on gene concentrations determined in the qPCR assay (Figure 4). Three main clusters were identified. In the case of SS, the heatmap shows that the highest differences compared to the control (SSC) were observed in MET SS and AMO SS, which formed clusters IIIa and IIIb, respectively. Moreover, cluster II included MET SC and AMO SC. Exposure of CS and SS to MET and AMO caused the greatest changes in the concentrations of genes that are characteristic of the methanogenic community. In Figure 1 discussed above, among others the distinctness of MET SS and AMO CS is visible. However, the present analysis clearly distinguishes four process bioreactors, both SS and CS exposed to MET and AMO. This may indicate that both MET and AMO had the greatest impact on the disturbance of the AD efficiency in both substrates by its characteristic microbiota, although the production of methane itself would indicate a distinct inhibition of the process by MET in the case of SS, and by AMO in the case of CS. Interestingly, SS OXY and SSC were in a common cluster (IIIc), as were CS OXY and CSC (cluster I). However, the exposure of both SS and CS to OXY was manifested by a significant reduction in the efficiency of methane production, in comparison to the proper control (Table 2). It is noticeable once again that the concentrations of the genes that are characteristic of methanogenic microorganisms, do not reflect the actual production of methane in bioreactors. In conclusion, while evaluating the effectiveness of methane fermentation, it is important to supplement the analysis of the process parameters with an additional analysis using molecular methods. Furthermore, the dominance of the SS digestate samples by the MST genes, and the CS digestate samples by MSC genes can be seen on the heatmap, as reflected also in Figure 2C–F. Considering the above, perhaps *Methanosaetaceae* dominant in SS is more sensitive to MET, while *Methanosarcinaceae* dominant in CS is more susceptible to inhibition by exposure to AMO.

These assays of the number of copies of the *MSC* and *MST* genes in CS and SS digestate samples revealed the biodiversity of microorganisms between the analyzed substrates. Sewage sludge which has trace quantities of drugs and products of their metabolism also contains microorganisms adapted somewhat to the present concentrations of drugs, which may explain differences in the number of gene copies between the SS and CS digestate samples. The differences in the number of gene copies between the two analyzed substrates could be a consequence of the high concentration of antibiotics in wastewater delivered to WWTPs, which can be seen as reservoirs of drug-resistant microorganisms [63–65]. The antibiotics fed to the bioreactor with SS as a substrate led to the diminishing populations of *Methanosarcinaceae* and *Methanosaetaceae*, but, irrespective of this, the microorganisms

adapted to unfavorable conditions remained in high numbers in the substrate until the AD process was terminated, which the high number of copies of the *MST* and *MSC* genes reflects well [66–69].

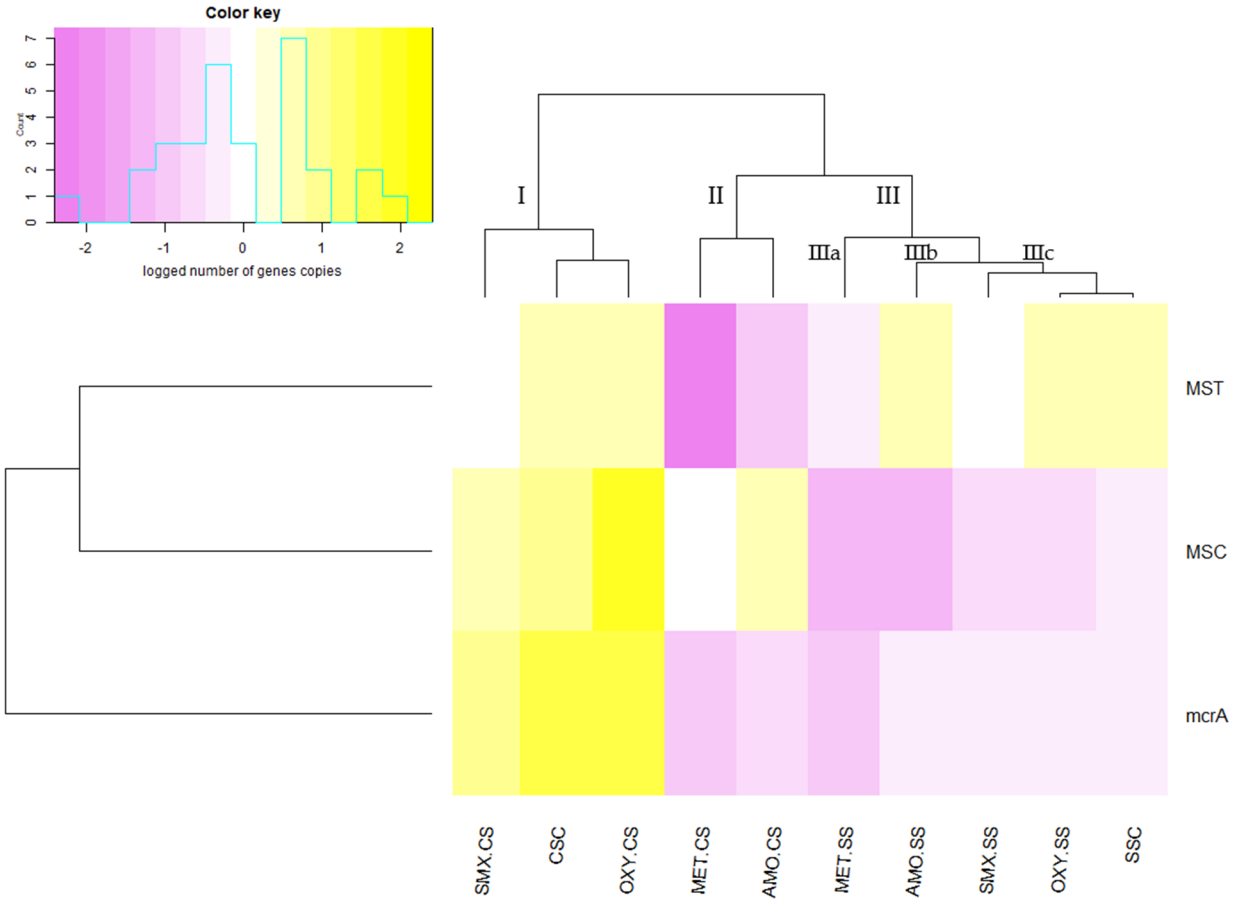

Figure 4. Heatmap with the logged concentrations (number of copies in g_D^{-1}) of *MST*, *MSC* and *mcr*A genes in process and control bioreactors with sewage sludge (SS) and cattle slurry (CS). (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry.

Differences in concentrations of the *MSC* and *MST* genes in cattle slurry samples may be due to the origin and type of substrate as well as the industrial character of the farm they were obtained from [70]. Cattle slurry undergoing AD in this experiment came from a farm where pharmaceuticals are used on a much smaller scale than in industrial production of farm animals. Another significant aspect is the fact that slurry is considered to be a less drug contaminated product than sewage sludge from WWTPs. In industrial production, animals, especially young ones, are administered drugs to promote their growth and prevent bacterial diseases, hence there is no constantly increasing influx of pharmaceuticals, which in turn is typical of WWTPs and sewage sludge [71].

4. Conclusions

Exposure of SS and CS to the antimicrobials chosen for this study resulted in differences in the composition of microbiota engaged in the AD process. It was observed that antimicrobial substances produced different effects on this process. The presence of drugs in substrates undergoing AD may manifest itself through the accumulation of particular VFAs in bioreactors, diminished amounts of generated biogas or lower CH_4 content of biogas. Drugs and products of their transformations present in substrates can also have an adverse influence on changes in the composition of microbiota that are characteristic of a given substrate, which was observed in this study. The microorganisms present in

digestate samples differed in their sensitivity to the particular antimicrobial substances, and the prevalence of one of the two analyzed families from the domain *Archaea* in SS or CS (*Methanosarcinaceae* and *Methanosaetaceae*, respectively) could have resulted in the different CH_4 production potential determined for the two tested substrates.

The above results confirm the assumption that the presence of medicinal substances in SS and CS may hinder the degradation of these substrates in the AD process. The research also confirm the claim that antimicrobial substances have a substantial influence on the efficiency of AD, in a manner dependent on the concentration of these drugs in substrate and on the type of substrate. Despite exposure of SS and CS digestate samples to the same concentration of a given antimicrobials, changes in the number of copies of genes that are characteristic of *Archaea* as well as changes in AD parameters followed different trends.

The results obtained in the course of this study are not sufficient to clearly identify the degree to which the tested drugs affect the selected part of the methanogenic community of microorganisms in SS or CS substrates. It is essential to pursue this research further, including such aspects as an assessment of abundance and activity of other groups of microorganisms engaged in this process, as well as analyses of changes in the number of the *mcrA* gene transcript. Studies based exclusively on the presence and abundance of certain groups of methanogens are insufficient to monitor their activity in substrates undergoing AD. Compared to an analysis of the number of the *mcrA* gene copies, expression of the gene might be a more accurate instrument for determination of the activity of methanogens, as it provides a better insight into the dynamics of microbial metabolism during an AD process.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/1/369/s1, Table S1: Oligonucleotide primers and PCR reaction profile, and Figure S1: Average values of methane production in bioreactors during anaerobic digestion of substrates such as (A) sewage sludge (SS) with MET, AMO, OXY, SMX supplementation, and control reactor (SSC), and (B) cattle slurry (CS) with MET, AMO, OXY, SMX supplementation, and control reactor (CSC).

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