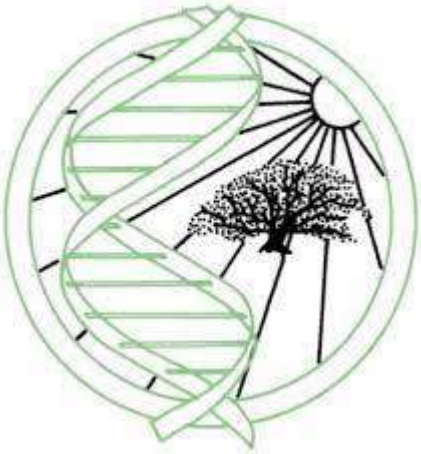
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W OLSZTYNIE

WYDZIAŁ BIOLOGII I BIOTECHNOLOGII



ROZPRAWA DOKTORSKA

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Wpływ wybranych leków przeciwdrobnoustrojowych na produkcję biogazu, rozpowszechnienie genów lekooporności oraz mikrobiom zaangażowany w procesy fermentacji metanowej gnojowicy bydlęcej

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Załączniki – publikacje stanowiące niniejszą rozprawę doktorską:

Załącznik 1. **Koniuszewska I,** Korzeniewska E, Harnisz M, and Czatzkowska M, "Intensification of biogas production using various technologies: A review". *International Journal of Energy Research.*, 2020; 2:1–19. https://doi.org/10.1002/er.5338; IF=4.672, MEiN=100.

Załącznik 2. **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. *Applied Sciences*., 2021; 11(1):369. https://doi.org/10.3390/app11010369; IF=2.838, MEiN=100.

Załącznik 3. **Koniuszewska I,** Harnisz M, Korzeniewska E, Czatzkowska M, Jastrzębski JP, Paukszto Ł, Bajkacz S, Felis E, Rusanowska P. The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure. *Energies*., 2021; 14(4):1125. https://doi.org/10.3390/en14041125; IF=3.252, MEiN=140.

Załącznik 4. **Wolak I,** Czatzkowska M, Harnisz M, Jastrzębski JP, 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(5):1920. https://doi.org/10.3390/en15051920; IF=3.252, MEiN=140.

Załącznik 5. **Wolak I**, Bajkacz S, Harnisz M, Stando K, Męcik M, Korzeniewska E. Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment. *International Journal of Environmental Research and Public Health.*, 2023; 20(3):2672. https://doi.org/10.3390/ijerph20032672; IF=4.614, MEiN=140.

Wpływ wybranych leków przeciwdrobnoustrojowych na produkcję biogazu, rozpowszechnienie genów lekooporności oraz mikrobiom zaangażowany w procesy fermentacji metanowej gnojowicy bydlęcej

Streszczenie

Wraz ze wzrostem zapotrzebowania na przyjazną dla środowiska energię alternatywną, fermentacja metanowa/ biometanizacja (ang. AD – anaerobic digestion) stała się popularną metodą stabilizacji materii organicznej z różnorodnych substratów. Instalacje takie jak biogazownie (ang. BP – biogas plants) są szeroko rozpowszechnione zarówno w Europie, jak również w Polsce, a ich liczba stale rośnie. Całkowita liczba inżynieryjnych projektów biogazowych na świecie wynosi 132 000, z czego 17 783 zlokalizowanych jest w Europie. W Polsce w 2021 r. działało już 128 biogazowni rolniczych.

Proces biometanizacji w warunkach beztlenowych jest atrakcyjną technologią stosowaną do higienizacji i stabilizacji odpadów organicznych oraz do produkcji energii przyjaznej środowisku jakim jest biogaz. Substraty jakimi są odpady organiczne poddawane procesowi AD nie ulegają całkowitemu rozkładowi, powstaje odpad jakim jest masa pofermentacyjna – poferment (ang. D – digestate). Poferment najczęściej wykorzystywany jest jako nawóz organiczny na nieużytki rolne. W celu zapewnienia bezpieczeństwa sanitarnego środowiska, istotna jest zawartość w pofermencie środków przeciwdrobnoustrojowych oraz produktów przemian tych związków, które trafiają tam z substratów AD. Ponieważ antybiotyki nie są w pełni metabolizowane przez organizm człowieka oraz zwierząt trafiają one do substratów AD, najczęściej z oczyszczalni ścieków oraz odchodów zwierzęcych. Wskutek powszechnego występowania antybiotyków w substratach, istnieje możliwość ich wpływu na mikrobiom zaangażowany w procesy AD, niekorzystne zmiany w społeczności drobnoustrojów, zahamowanie wzrostu i rozwoju metanogenów odpowiedzialnych za produkcję metanu (CH_4) oraz wzrost liczby genów lekooporności (ang. ARGs – antibiotic resistance genes) w pofermencie, a co się z tym wiąże szerzenie zjawiska antybiotykooporności (ang. AR – antibiotic resistance) w środowisku glebowym po nawożeniu jej pofermentem. W literaturze jest niewiele informacji na temat składu masy pofermentacyjnej. Pomimo, iż poferment zawiera składniki odżywcze i substancje organiczne, które mogą sprzyjać wzrostowi upraw, stosowanie go na glebach uprawnych może znacznie zwiększyć liczebność ARGs w mikrobiomie gleby. Dlatego nie można bagatelizować znaczenia badań dotyczących poziomu antybiotyków i ich obecności w pofermencie z biogazowni rolniczych, wpływu farmaceutyków na mikrobiom

zaangażowany w procesy AD oraz rozprzestrzenianie ARGs w masie pofermentacyjnej. Ważne jest poznanie bioróżnorodności mikrobiomu zaangażowanego w proces AD i odpowiedź społeczności drobnoustrojów na antybiotyki zawarte w substratach AD i masie pofermentacyjnej. Zarówno bakterie jak i *Archaea*, zaangażowane w proces AD mogą stanowić potencjalne źródło mobilnych elementów genetycznych (ang. MGEs – mobile genetic elements) i ARGs.

W ramach niniejszej rozprawy doktorskiej badano jednostkowy oraz synergistyczny wpływ wybranych leków przeciwdrobnoustrojowych na proces fermentacji beztlenowej prowadzonej w warunkach mezofilnych w układzie technologicznym na skalę laboratoryjną oraz bezpośrednio w biogazowniach rolniczych. W pracy badano obecność pozostałości antybiotyków należących do klasy beta-laktamów (amoksycylina, ampicylina), tetracyklin (tetracykliny, oksytetracykliny, doksycykliny, chlorotetracyklina), sulfonamidów (sulfametoksazol, sulfonamid), fluorochinolonów (enrofloksacyna, cyprofloksacyna), antybiotyków z grupy MLS (makrolidy – linkozamidy – streptograminy) i inhibitorów reduktazy dihydrofolianowej (trimetoprim) w pofermencie pobranym zarówno z układu technologicznego jak i bezpośrednio z wybranych biogazowni rolniczych w Polsce. W ramach niniejszej rozprawy doktorskiej badano także skład mikrobiomu masy pofermentacyjnej oraz zachodzące w mikrobiomie zmiany wśród społeczności drobnoustrojów należących zarówno do domeny *Bacteria* jak i *Archaea*. Celem niniejszych badań była również analiza dotycząca występowania ARGs niosących lekooporność na antybiotyki należące do klasy betalaktamów, tetracyklin, fluorochinolonów, sulfonamidów, grupy MLS, oraz genów integrazy klasy 1 oraz 2 w pobranych próbkach pofermentu. Zaplanowane badania pozwoliły uzyskać informacje odnośnie odpowiedzi drobnoustrojów na jednostkową oraz synergistyczną suplementację środków przeciwdrobnoustrojowych wprowadzanych do wsadu bioreaktorów AD, zmianach zachodzących w mikrobiomie zaangażowanym w proces AD, jakości mikrobiologicznej uzyskanego po procesie AD pofermentu oraz skuteczności procesu AD w eliminacji ARGs. Innowacyjne metody biologii molekularnej i metagenomiki pozwoliły uzyskać szeroki zakres informacji na temat jakości badanego pofermentu, zarówno w układzie technologicznym jak i bezpośrednio pochodzącym z procesu AD prowadzonego w biogazowniach rolniczych pracujących na skalę przemysłową w oparciu o różnorodne substraty. Na podstawie otrzymanych wyników badań podjęto próbę odpowiedzi na pytanie, czy fermentacja metanowa biomasy organicznej jest bezpieczna dla środowiska naturalnego w przypadku odprowadzania masy pofermentacyjnej do gleby oraz

czy proces ten może ograniczyć wprowadzanie środków farmaceutycznych do gleby i rozprzestrzenianie ARGs w pofermencie, glebie oraz szeroko pojętym środowisku. Wyniki niniejszej rozprawy doktorskiej pozwoliły określić, czy proces AD oprócz wytwarzania przyjaznej środowisku energii prowadzi także do całkowitego usunięcia ARGs i antybiotyków, niezależnie od składu substratowego poszczególnych próbek pofermentu. Badania prowadzone w ramach niniejszej pracy doktorskiej podzielono na trzy etapy.

W I etapie eksperymentu w układzie technologicznym AD, w warunkach laboratoryjnych zbadano indywidualny wpływ ośmiu środków przeciwdrobnoustrojowych należących do klasy beta-laktamów (AMO – amoksycylina, AMP – ampicylina), tetracyklin (OXY – oksytetracyklina, TET – tetracyklina, CHLOR – chlorotetracyklina), fluorochinolonów (ENRO – enrofloksacyna), sulfametoksazoli (SMX – sulfametoksazol) i pochodne nitroimidazolu (MET – metronidazol) na wydajność produkcji biogazu w procesie AD gnojowicy bydlęcej, który to proces prowadzono w warunkach mezofilnych. Zbadano mikrobiom zaangażowany w proces AD, obecność i liczbę genów *mcr*A, *MSC* i *MST* swoistych dla *Archaea* oraz liczebność genów oporności na antybiotyki w pofermencie pochodzącym z substratu eksponowanego na wybrane środki przeciwdrobnoustrojowe. Indywidualny dodatek antybiotyków do substratu w bioreaktorach procesowych AD istotnie obniżył produkcję biogazu. Zadawana jednostkowo do wsadu bioreaktorów AMO, należąca do klasy betalaktamów, spowodowała największy, 75% spadek produkcji CH_4 w porównaniu z próbkami kontrolnymi bez dodatku antybiotyku. Wprowadzane indywidualnie do bioreaktorów ENRO, TET, OXY i CHLOR także zmniejszyły ilość wytwarzanego biogazu odpowiednio o 36, 39, 45 i 53%. Wyniki wysokowydajnego sekwencjonowania 16S *r*RNA wykazały, że we wszystkich badanych próbkach pofermentu wśród mikroorganizmów dominowały te należące do domeny *Bacteria*. Ponadto, antybiotyki doprowadziły do zmniejszenia liczebności genów *mcr*A, *MSC*, *MST* charakteryzujących metanogeny należące do domeny *Archaea*. Środki przeciwdrobnoustrojowe indukowały również wzrost liczby genów oporności na tetracykliny. Po przeprowadzonym pierwszym etapie badań wywnioskowano, że antybiotyki zadawane indywidualnie do wsadu bioreaktorów zmniejszały wydajność procesu AD i znacząco obniżały objętość uzyskiwanego CH_4, jednocześnie stymulując wzrost liczby ARGs w próbkach pofermentu.

W II etapie eksperymentu AD prowadzonego w bioreaktorach beztlenowych układu technologicznego na skalę laboratoryjną zbadano długoterminowy (417 dni), synergistyczny

efekt środków przeciwdrobnoustrojowych na AD gnojowicy bydlęcej. Przy wykorzystaniu analiz metagenomicznych i bioinformatycznych zbadano rozpowszechnienie ARGs i zmiany w bioróżnorodności drobnoustrojów w pofermencie gnojowicy bydlęcej, którą to gnojowicę bydlęcą jako wsad bioreaktora eksponowano na mieszaninę leków wybranych na podstawie wyników uzyskanych w ramach pierwszego etapu, tj. AMO, ENRO oraz MET. Farmaceutyki zadawano do wsadu bioreaktorów w zmiennych, wzrastających stężeniach. W prowadzonym eksperymencie analizowano efektywność produkcji CH_4 a z wykorzystaniem analiz metagenomicznych badano mikrobiom zaangażowany w proces AD i zachodzące w nim zmiany w społeczności drobnoustrojów. Podobnie jak w pierwszym etapie prowadzonym w ramach niniejszej dysertacji badań, wśród drobnoustrojów dominowały mikroorganizmy należące do domeny *Bacteria*. *Bacteroidetes*, *Firmicutes* i *Actinobacteria* były najliczniejszymi grupami bakterii w próbkach. Domena *Archaea* reprezentowana była głównie przez metanogenne rodzaje *Methanothrix* i *Methanosarcina* oraz rząd *Methanomassiliicocales*. Ekspozycja na zadawaną do wsadu bioreaktorów mieszaninę antybiotyków hamowała wzrost i rozwój mikroorganizmów metanogennych we wsadzie bioreaktora AD. Antybiotyki wprowadzane synergistycznie w mieszaninie do wsadu bioreaktorów procesu AD wpłynęły również na obfitość i częstość występowania ARGs w próbkach pofermentu, w których zidentyfikowano i sklasyfikowano łącznie siedemnaście rodzajów ARGs. Najliczniej zidentyfikowano geny kodujące oporność na tetracykliny, antybiotyki MLS i aminoglikozydy, a także geny oporności wielolekowej. Antybiotyki synergistycznie oddziaływały na bakterie homoacetogenne i metanogeny oraz zmniejszały efektywność produkcji CH_4. Jednakże wywołany mieszaniną antybiotyków okresowy spadek produkcji CH_4 został zminimalizowany w obecności wysoce lekoopornych mikroorganizmów w bioreaktorach AD, które wykorzystały produkty metabolizmu leków jako źródło węgla jednocześnie produkując substraty do etapu metanogenezy w procesie AD, niezbędne drobnoustrojom metanogennym do produkcji CH_4.

W III etapie eksperymentu prowadzonego w warunkach in vivo w działających na skalę przemysłową biogazowniach rolniczych, poferment pochodzący z wybranych do badań biogazowni rolniczych pracujących w oparciu o różnorodne substraty tj. osad ściekowy, gnojowicę bydlęcą oraz substraty roślinne, badano pod kątem obecności wybranych 13 antybiotyków należących do klasy betalaktamów (AMO – amoksycylina), tetracyklin (TET, OXY, DOXY, CHLOR), sulfonamidów (SMX, SMD – sulfonamid), fluorochinolonów (ENRO, CIP – ciprofloksacyna), makrolidów (CLR – klarytromycyna), linkozamidów

(CLD – klindamycyna) i streptogramin oraz inhibitorów reduktazy kwasu dihydrofoliowego (TRIM – trimetoprim). W próbkach pofermentu badano także obecność genów niosących lekooporność na betalaktamy (*bla*$_{TEM}$, *bla*$_{OXA}$, *cfx*A), tetracykliny (*tet*A, *tet*M), sulfonamidy (*sul*1, *sul*2), fluorochinolony (*aac 6'-Ib-cr*, *qep*A) i grupę leków MLS (*erm*F). W pracy badano także geny integrazy klasy 1 oraz 2 (*intI*1 oraz *intI*2). Geny oporności na antybiotyki obecne w substratach przetwarzanych podczas AD w biogazowniach rolniczych mogą przedostać się do pofermentu, który jest używany jako nawóz w agrokulturze. Środki przeciwdrobnoustrojowe i ARGs mogą być przenoszone na grunty rolne, co może zwiększać ich koncentrację w środowisku. Na podstawie rezultatów uzyskanych w ramach przeprowadzonego trzeciego etapu badań stwierdzono, że środki przeciwdrobnoustrojowe nie zostały wyeliminowane podczas procesu AD. Ich stężenia różniły się w próbkach pofermentu otrzymanych z różnych substratów oraz we frakcji płynnej i stałej poszczególnych próbek. Ponadto próbki masy pofermentacyjnej otrzymane z substratów pochodzenia roślinnego charakteryzowały się wysokimi stężeniami ARGs. Wysokie stężenia genów integrazy rzędu do 10^7 kopii w 1 gramie pofermentu mogą również wskazywać, że MGEs są zaangażowane w rozprzestrzenianie oporności na antybiotyki. Wyniki powyższych badań sugerują, że ryzyko zanieczyszczenia gleby antybiotykami, i ARGs może być stosunkowo wysokie w gospodarstwach, w których poferment z biogazowni rolniczych jest używany jako nawóz.

Wyniki niniejszej dysertacji doktorskiej pozwalają określić, w jaki sposób środki przeciwdrobnoustrojowe wpływają na proces AD z różnorodnych substratów i na zmiany w różnorodności biologicznej drobnoustrojów oraz w koncentracji liczby genów funkcjonalnych i ARGs w pofermencie. Praca zawiera również technologiczne i praktyczne informacje na temat procesu AD, w którym substraty eksponowane są na określone stężenia leków przeciwdrobnoustrojowych.

Abstract

With the growing demand for environmentally friendly, alternative energy, anaerobic digestion / biomethanization (AD, anaerobic digestion) has become a popular method of stabilizing organic matter from a variety of substrates. Installations such as biogas plants (BP – biogas plants) are widespread both in Europe and Poland, and their number is constantly growing. The total number of engineered biogas projects in the world is 132,000, of which 17,783 are located in Europe. In 2021, 128 agricultural biogas plants were already operating in Poland.

The process of biomethanization in anaerobic conditions is an attractive technology used for the hygienization and stabilization of organic waste and for the production of environmentally friendly energy, which is biogas. Substrates such as organic waste subjected to the AD process are not completely decomposed, a waste is formed in the form of post-fermentation mass – digestate (D – digestate). Digestate is most often used as an organic fertilizer for farmlands. In order to ensure the sanitary safety of the environment, the content of antimicrobial agents and transformation products (TP) of these compounds in the digestate, which end up there from AD substrates, is important. Because antibiotics are not completely metabolized by the human and animal organisms, they end up in AD substrates most often from sewage treatment plants and animal faeces. Due to the widespread presence of antibiotics in substrates, there is the possibility of pharmaceuticals affecting the microbiome involved in AD processes, adverse changes in the microbial community, inhibition of the growth and development of methanogens responsible for methane (CH_4) production, and an increase in the number of antibiotic resistance genes (ARGs) in the digestate, and what is associated with it, spreading the phenomenon of antibiotic resistance (AR – antibiotic resistance) in the soil environment after fertilization with digestate. There is a few information in the literature on the composition of the digestate. Although digestate contains nutrients and organic matter that can promote crop growth, applying it to agricultural soils can significantly increase the abundance of ARGs in the soil microbiome. Therefore, the importance of research on the level of antibiotics and their presence in the digestate from agricultural biogas plants, the impact of pharmaceuticals on the microbiome involved in AD processes, and the spread of ARGs in the digestate cannot be underestimated. It is important to understand the biodiversity of the microbiome involved in the AD process and the response of the microbial community to the antibiotics contained in the AD substrates and digestate.

Both bacteria and *Archaea* involved in the AD process may be a potential source of mobile genetic elements (MGEs) and ARGs.

As part of this doctoral dissertation, the individual and synergistic effect of selected antimicrobial drugs on the process of AD carried out in mesophilic conditions in a technological system on a laboratory scale and directly in agricultural biogas plants was studied. The study examined the presence of residues of antibiotics belonging to the class of beta-lactams (amoxicillin, ampicillin), tetracyclines (tetracyclines, oxytetracyclines, doxycyclines, chlortetracycline), sulfonamides (sulfamethoxazole, sulfonamide), fluoroquinolones (enrofloxacin, ciprofloxacin), MLS antibiotics (macrolides – lincosamides –streptogramins) and dihydrofolate reductase inhibitors (trimethoprim) in digestate sampling from the technological system and directly from selected agricultural biogas plants in Poland. As part of this doctoral dissertation, the composition of the digestate microbiome and the changes taking place in the microbiome among the microbial communities belonging to both the *Bacteria* and *Archaea* domains were also studied. The aim of this study was also to analyze the presence of ARGs carrying drug resistance to antibiotics belonging to the class of beta-lactams, tetracyclines, fluoroquinolones, sulfonamides, MLS group, and class 1 and 2 integrase genes in the collected digestate samples. The planned research allowed to obtain information on the response of microorganisms to unitary and synergistic supplementation of antimicrobial agents introduced into the AD bioreactors, changes in the microbiome involved in the AD process, microbiological quality of the digestate obtained after the AD process, and the effectiveness of the AD process in eliminating ARGs. Innovative methods of molecular biology and metagenomics allowed to obtain a wide range of information on the quality of the studied digestate, both in the technological system and directly from the AD process carried out in agricultural biogas plants operating on an industrial scale based on various substrates. Based on the obtained test results, an attempt was made to answer the question of whether methane fermentation of organic biomass is safe for the natural environment in the case of discharging the digestate to the soil and whether this process can reduce the introduction of pharmaceuticals into the soil and the spread of ARGs in the digestate, soil and the broadly understood environment. The results of this doctoral dissertation allowed determining whether the AD process, apart from generating environmentally friendly energy, also leads to the complete removal of ARGs and antibiotics, regardless of the substrate composition of individual digestate samples. In the first stage of the experiment in the AD technological

system, the individual impact of eight antimicrobial agents belonging to the class of beta-lactams (AMO – amoxicillin, AMP – ampicillin), tetracyclines (OXY – oxytetracycline, TET – tetracycline, CHLOR – chlortetracycline), fluoroquinolones (ENRO – enrofloxacin), sulfamethoxazole (SMX – sulfamethoxazole) and nitroimidazole derivatives (MET – metronidazole) on the efficiency of biogas production in the AD process of cattle slurry, which was carried out in mesophilic conditions. The microbiome involved in the AD process, the presence and number of *Archaea*-specific *mcr*A, MSC, and MST genes, and the abundance of antibiotic-resistance genes in digestate derived from substrates exposed to selected antimicrobials were examined. The individual addition of antibiotics to the substrate in AD process bioreactors significantly reduced biogas production. AMO, belonging to the class of beta-lactams, applied individually to the batch of bioreactors, caused the largest, 75% decrease in CH_4 production compared to control samples without the addition of the antibiotic. Individually introduced into bioreactors ENRO, TET, OXY, and CHLOR also reduced the amount of biogas produced by 36, 39, 45 and 53%, respectively. The results of high-throughput 16S *r*RNA sequencing showed that in all digestate samples, those belonging to the Bacteria domain dominated among the microorganisms. In addition, antibiotics led to a reduction in the number of *mcr*A, MSC, and MST genes characterizing methanogens belonging to the Archaea domain. In addition, antimicrobials also induced an increase in the number of tetracycline resistance genes. After the first stage of the research, it was concluded that antibiotics applied individually to the bioreactor batch reduced the efficiency of the AD process and significantly reduced the volume of CH_4 obtained while stimulating an increase in the number of ARGs in the digestate samples.

In the second stage of the AD experiment conducted in anaerobic bioreactors of the technological system on a laboratory scale, the long-term (417 days), synergistic effect of antimicrobial agents on the AD of cattle slurry was examined. Using metagenomic and bioinformatics analyses, the prevalence of ARGs and changes in microbial biodiversity in the digestate of cattle slurry were examined, which was exposed as a bioreactor feed to a mixture of drugs selected based on the results obtained in the first stage, i.e. AMO, ENRO, and MET. Pharmaceuticals were added to the bioreactors in a variable, increasing concentrations. In the conducted experiment, the effectiveness of CH_4 production was analyzed, and the microbiome involved in the AD process and the changes in the microbial community were studied using metagenomic analyses. As in the first stage of this research dissertation, microorganisms belonging to the Bacteria domain dominated among the microorganisms. *Bacteroidetes*,

Firmicutes, and *Actinobacteria* were the most numerous groups of bacteria in the samples. The *Archaea* domain was represented mainly by the methanogenic genera *Methanothrix* and *Methanosarcina* and the order *Methanomassiliicocales*. Exposure to the mixture of antibiotics added to the bioreactor batch inhibited the growth and development of methanogenic microorganisms in the AD bioreactor batch. Antibiotics introduced synergistically in the mixture to the bioreactors of the AD process also affected the abundance and frequency of ARGs in the digestate samples, in which a total of seventeen types of ARGs were identified and classified. Genes encoding resistance to tetracyclines, MLS antibiotics, and aminoglycosides, as well as multidrug resistance genes, were identified in the most numerous numbers. Antibiotics had a synergistic effect on homoacetogenic bacteria and methanogens and reduced the efficiency of CH_4 production. However, the antibiotic mixture-induced intermittent decrease in CH_4 production was minimized by the presence of highly drug-resistant microorganisms in AD bioreactors, which used the products of drug metabolism as a carbon source while producing substrates for the methanogenesis stage of the AD process, necessary for the methanogenic microorganisms to produce CH_4.

In the third stage of the experiment conducted in vivo in agricultural biogas plants operating on an industrial scale, digestate from selected agricultural biogas plants operating based on various substrates, i.e. sewage sludge, cattle slurry, and plant substrates, was tested for the presence of selected 13 antibiotics belonging to the class of beta-lactams (AMO, amoxicillin), tetracyclines (TET, OXY, DOXY, CHLOR), sulfonamides (SMX, SMD –sulfonamide), fluoroquinolones (ENRO, CIP – ciprofloxacin), macrolides (CLR – clarithromycin), lincosamides (CLD – clindamycin) and streptogramins and dihydrofolic acid reductase inhibitors (TRIM – trimethoprim).

In digestate samples, the presence of genes carrying drug resistance to beta-lactams (*bla*$_{TEM}$, *bla*$_{OXA}$, *cfx*A), tetracyclines (*tet*A, *tet*M), sulfonamides (*sul*1, *sul*2), fluoroquinolones (*aac 6'-Ib-cr*, *qep*A) and a group of MLS drugs (erm). The study also examined the class 1 and 2 integrase genes (*intI*1 and *intI*2). Antibiotic resistance genes present in substrates processed during AD in agricultural biogas plants can transfer to the digestate, which is used as fertilizer in agriculture. Antimicrobials and ARGs can be transferred to agricultural land, which may increase their concentration in the environment. Based on the results obtained in the third phase of the study, it was concluded that antimicrobials were not eliminated during the AD process. Their concentrations differed in digestate samples obtained from different

substrates and in the liquid and solid fractions of individual samples. In addition, samples of fermentation mass obtained from substrates of plant origin were characterized by high concentrations of ARGs. High concentrations of integrase genes of up to 10^7 copies per gram of digestate may also indicate that MGEs are involved in the spread of antibiotic resistance. The results of the above studies suggest that the risk of soil contamination with antibiotics and ARGs may be relatively high on farms where digestate from agricultural biogas plants is used as fertilizer.

The results of this doctoral dissertation allow us to determine how antimicrobials affect the AD process from various substrates and changes in microbial biodiversity and the concentration of functional genes and ARGs in the digestate. This work also contains technological and practical information on the AD process in which substrates are exposed to specific concentrations of antimicrobial drugs.

Wprowadzenie do tematyki badań

W związku z rosnącą ilością odpadów na całym świecie, jak również wraz ze wzrostem zapotrzebowania na energię alternatywną, przyjazną dla środowiska, fermentacja metanowa (ang. AD – anaerobic digestion) stała się popularną metodą stabilizacji materii organicznej z różnorodnych substratów [1]. Takie instalacje jak biogazownie są obecnie szeroko rozpowszechnione zarówno w Europie, jak również w Polsce, a ich liczba stale wzrasta. Obok biogazu, jednym z produktów końcowych procesu fermentacji metanowej jest poferment, szeroko wykorzystywany w rolnictwie do nawożenia upraw. Pierwsza biogazownia rolnicza w Polsce została uruchomiona w 2005 roku, natomiast z końcem 2021 r. na terenie Polski działało już 128 biogazowni rolniczych [2].

Substraty wykorzystywane w biogazowniach do procesu AD to najczęściej gnojowica bydlęca [2], osady ściekowe [3], flotat poubojowy [4], mączka mięsno-kostna [5], kiszonki roślinne takie jak kiszonka kukurydzy [6], kiszonka z buraków cukrowych [7], ścier ziemniaczany [8] czy odpady pochodzące z przemysłu cukierniczego [9]. Produkty te po procesie AD stanowią bogatą w substancje odżywcze masę pofermentacyjną, tzw. poferment. Poferment uzyskiwany po procesie AD złożony jest ze związków organicznych, mineralnych, azotu, makro i mikroelementów, a także co istotne, zasiedlany jest przez charakterystyczną dla pofermentu mikrobiotę czyli różnorodną społeczność drobnoustrojów biorących udział w procesie AD [10]. Poferment najczęściej wykorzystuje się w rolnictwie jako nawóz na pola uprawne [11], a nawożenie pofermentem jest powszechną praktyką mającą zastosowanie od wielu lat na całym świecie. Jednakże, w masie pofermentacyjnej, oprócz całego bogactwa różnorodnych substancji odżywczych wykorzystywanych przez rośliny do ich wzrostu, mogą występować także antybiotyki oraz produkty ich przemiany, jak również antybiotykooporne bakterie (ang. ARB – antibiotic resistance bacteria) [1]. Wykorzystanie pofermentu do nawożenia pól uprawnych może stanowić zatem wysokie ryzyko wprowadzenia do gleby obecnych w pofermencie mikrozanieczyszczeń [12]. W rolnictwie pomimo promowania zintegrowanego i ekologicznego systemu zarządzania, nadal dominuje system konwencjonalny, którego celem jest przede wszystkim intensyfikacja produkcji bez należytej dbałości o środowisko [13]. W samej hodowli zwierząt średnie roczne spożycie antybiotyków oszacowano na 172 mg kg^{-1} u świń, 148 mg kg^{-1} u kurcząt i 45 mg kg^{-1} u bydła na całym świecie [14].

Środki przeciwdrobnoustrojowe oraz lekooporne drobnoustroje obecne w pofermencie pochodzą z substratów wykorzystywanych w procesie AD. Wśród atrakcyjnych energetycznie substratów AD wyszczególnić można osad ściekowy pochodzący z oczyszczalni ścieków (ang. WWTP – Wastewater Treatment Pant), będącej rezerwuarem znacznej ilości leków i genów lekooporności (ARGs) [13]. Produkowany w WWTP osad ściekowy, poddawany procesowi AD w biogazowniach rolniczych może zawierać różnorodną pulę ARB oraz leków, albowiem większość leków nie jest w pełni metabolizowana w organizmach ludzkich. Odpady organiczne będące substratami w procesie AD, takie jak obornik czy gnojowica bydlęca pochodzące z rolnictwa również mogą zawierać antybiotyki oraz produkty przemian tych związków [3]. Antybiotyki takie jak np. amokysyclina (AMO) czy tetracyklina (TET) podlegają metabolizmowi tylko w 10–20%, podczas gdy inne, np. sulfametoksazol (SMX), w około 85% [4]. Autorzy licznych publikacji podają, iż każdego roku kilka tysięcy ton leków przeciwdrobnoustrojowych wraz z kałem zwierząt hodowlanych odprowadzanych jest do środowiska [5][6][7][8][9]. Te wydalane do środowiska związki przeciwdrobnoustrojowe mogą charakteryzować się bardzo wysoką toksycznością, a ich obecność w substratach poddawanych AD może hamująco wpływać na proces oraz wydajność produkcji biogazu [10]. Drobnoustroje metanogenne uczestniczące w procesie AD mają kluczowe znaczenie dla produkcji biogazu oraz są szczególnie wrażliwe na środki przeciwdrobnoustrojowe, których obecność w substratach poddawanych fermentacji może zmniejszyć zarówno tempo ich wzrostu, jak też ich aktywność metaboliczną [11].

Farmaceutyki w pofermencie mogą pochodzić także z odchodów zwierzęcych, flotatu poubojowego z rzeźni czy odpadów rolniczych, które są najczęściej poddawanymi procesowi AD substratami bogatymi w substancje mineralne oraz drobnoustroje. Wielu autorów dostarcza informacji o kilku tonach leków przeciwdrobnoustrojowych przedostających się do środowiska wraz z odchodami zwierzęcymi [14][15] czy osadem ściekowym [2]. W samym chowie przemysłowym zwierząt po zastosowaniu farmakoterapii od 30 do nawet 90% związków w postaci niezmienionej lub ich metabolitów pozostaje w odchodach zwierzęcych. W Chinach, które są największym globalnym producentem mięsa w 2013 roku całkowita ilość wydalonych antybiotyków w odchodach zwierzęcych wynosiła 45 000 ton, wśród których najczęściej występującymi antybiotykami były sulfonamidy i fluorochinolony [14]. Środki przeciwdrobnoustrojowe należące do klasy beta-laktamów, tetracyklin, sulfonamidów, fluorochinolonów oraz pochodnych nitroimidazoli są najczęściej stosowanymi lekami w medycynie weterynaryjnej na całym świecie. W ciągu ostatnich dziesięcioleci globalne

stosowanie antybiotyków ciągle wzrasta [13][14]. Europejska agencja ESVAC (ang . ESVAC – European Surveillance of Veterinary Antibacterial Consumption) podaje, iż w 2014 r. w Polsce w leczeniu zwierząt zostało zastosowanych 581,3 ton leków (http://www.ema.europa.eu). W poszczególnych etapach badań w ramach niniejszej rozprawy doktorskiej opisane wyżej środki przeciwdrobnoustrojowe zastosowano jako potencjalne inhibitory procesu AD.

Leki wprowadzane do środowiska wraz z pofermentem, jako nawozem suplementowanym do gleby, mogą wykazywać bardzo wysoką toksyczność wobec drobnoustrojów, wskutek czego ich presja selekcyjna może być przyczyną wysokiego ryzyka wzrostu poziomów ARGs w glebie i sprzyjać szerzeniu zjawiska lekooporności (ang. AR – Antibiotic Resistance) na szeroką skalę [9]. Co więcej, w Europie środki przeciwdrobnoustrojowe należące do tej samej klasy są stosowane u zwierząt służących do produkcji żywności oraz w farmakoterapii zakażeń u ludzi. Przykładem są fluorochinolony i sulfonamidy stosowane w medycynie weterynaryjnej w Chinach. W Europie stanowią one odpowiednio 2.2% i 9.2% wszystkich sprzedawanych środków przeciwdrobnoustrojowych znajdujących zastosowanie zarówno w farmakoterapii ludzi, jak i zwierząt. Popularnie stosowanymi lekami przeciwdrobnoustrojowymi w medycynie weterynaryjnej w Europie są także antybiotyki należące do grup betalaktamów i tetracyklin [15][16][17]. Co więcej, istnieją doniesienia o wykryciu w produktach rolno-spożywczych makrolidów [18], linkozamidów [19] czy inhibitorów reduktazy kwasu dihydrofoliowego (trimetoprim). Ze względu na tak bogaty wachlarz szeroko stosowanych leków, rozwój lekooporności na jeden wybrany antybiotyk może prowadzić do oporności na całą pokrewną klasę leków [20][21].

Tempo degradacji chemioterapeutyków w glebie może się różnić zależnie od temperatury, tekstury gleby, zawartości węgla organicznego w glebie czy cech oporności bytujących tam mikroorganizmów [14]. Utrzymujące się w glebie farmaceutyki mogą być przenoszone wraz ze spływami powierzchniowymi do zbiorników wodnych, które są często wykorzystywane jako miejsca pojenia zwierząt [15], a stamtąd wraz ze spożywaną wodą do łańcucha pokarmowego i organizmów tych zwierząt. Następnie, w cyklu produkcji rolnej leki obecne w produktach spożywczych pochodzenia zwierzęcego mogą być przenoszone do organizmów ludzkich [16]. Świeże produkty spożywane przez konsumentów mogą być źródłem narażenia na pozostałości środków przeciwdrobnoustrojowych, ARB i ARGs o znaczeniu klinicznym [24]. Stwarza to ogromne ryzyko wystąpienia wśród konsumentów zakażeń ARB, a co się z tym wiąże, ograniczonymi możliwościami farmakoterapii z powodu

rosnącej w organizmach ludzkich i zwierzęcych lekooporności bytujących tam drobnoustrojów. Dlatego tak ważne jest zrozumienie skali rozpowszechnienia farmaceutyków w produkcie pofermentacyjnym, aby ograniczyć możliwości rozpowszechniania leków w środowisku w chwili suplementacji gleb pofermentem, w celu zmniejszenia ich presji selekcyjnej na bytujące w środowisku drobnoustroje i zachodzące w mikrobiomie AD oraz gleby zmiany [16].

Należy także zwrócić uwagę na możliwość występowania zjawiska antybiotykooporności w bioreaktorach AD na skutek powszechnego stosowania antybiotyków w medycynie weterynaryjnej oraz rolnictwie [12]. Antybiotyki obecne w środowisku mogą pośredniczyć w rozprzestrzenianiu ARGs i populacji ARB w glebie, stymulując powstawanie nowych typów ARGs lub podnosząc ich obecną koncentrację w danym środowisku [16]. Innym możliwym ryzykiem jest szybkie rozprzestrzenianie ARGs wśród społeczności mikroorganizmów środowiskowych poprzez horyzontalny transfer genów (ang. HGT – horizontal gene transfer) ze względu na obecność ruchomych elementów genetycznych (ang. Mobile Genetic Elements – MGEs) takich jak np. integrony u mikroorganizmów obecnych w substracie poddanym AD oraz w pofermencie [17].

Do chwili obecnej niewielu autorów prowadziło tak szerokie, wieloetapowe badania obejmujące kolejno indywidualny oraz synergistyczny wpływ antybiotyków na produkcję biogazu, występowanie ARGs oraz bioróżnorodność konsorcjów drobnoustrojów podczas procesu AD gnojowicy bydlęcej i zmian zachodzących w społeczności tych drobnoustrojów. Co więcej, badanie w ramach niniejszej pracy prowadzono zarówno w układzie technologicznym w warunkach laboratoryjnych, jak i warunkach in vivo, tj. w biogazowniach rolniczych działających na skalę przemysłową. Ponadto, niewielu autorów opisuje zależność między AD w obecności antybiotyków a szerzeniem AR. Przetwarzanie gnojowicy bydlęcej w procesie biometanizacji na biogaz w procesach AD zyskuje w ostatnich latach na znaczeniu. Jednakże, z uwagi na stale rosnącą na całym świecie konsumpcję antybiotyków, można spodziewać się wzrostu stężenia środków przeciwdrobnoustrojowych w gnojowicy bydlęcej oraz pofermencie. Cykl wytwarzania biogazu jest zintegrowanym systemem wykorzystywania zasobów naturalnych, unieszkodliwiania odpadów organicznych, dystrybucji składników nawozowych oraz produkcji energii odnawialnej, co przynosi zarówno korzyści energetyczne jak i środowiskowe. Jednakże, w celu optymalizacji prowadzonego w biogazowniach procesu AD, niezbędna jest zarówno współpraca

naukowców z technologami, jak też monitorowanie warunków procesu zarówno pod kątem mikrobiologicznym jak i technologicznym.

Cel i hipotezy badawcze

Cel naukowy badań

Głównym celem naukowym pracy doktorskiej było określenie wpływu wybranych środków przeciwdrobnoustrojowych na wydajność procesu AD, produkcję biogazu, zmiany w bioróżnorodności mikrobiomu zaangażowanego w procesy AD oraz na liczbę kopii ARGs w pofermencie.

Hipotezy badawcze

1. Leki przeciwdrobnoustrojowe zadane do wsadu bioreaktorów AD jednostkowo oraz synergistycznie, należące do klasy beta-laktamów, tetracyklin, sulfonamidów, fluorochinolonów oraz pochodne nitroimidazoli, wpływają hamująco na wydajność procesu AD oraz produkcję biogazu.
2. Skład jakościowy i ilościowy drobnoustrojów obecnych w systemach AD jest modyfikowany poprzez obecność środków przeciwdrobnoustrojowych we wsadzie bioreaktorów AD, zarówno w przypadku farmaceutyków zadawanych do układu technologicznego jednostkowo, jak i synergistycznie.
3. Farmaceutyki, zadawane do wsadu bioreaktorów jednostkowo lub synergistycznie, a także te obecne w substratach poddawanych AD, wpływają istotnie na wzrost liczby kopii ARGs wśród społeczności drobnoustrojów odpowiadających za proces biometanizacji.
4. Pochodzenie substratu i skład materii organicznej w substracie AD wpływa na obecność środków przeciwdrobnoustrojowych oraz ARGs w masie pofermentacyjnej uzyskiwanej po procesie AD w biogazowniach rolniczych.

Zakres i metodyka pracy

Zakres pracy

1. Określenie indywidualnego i synergistycznego wpływu wybranych leków przeciwdrobnoustrojowych oraz ich stężeń w układzie technologicznym w eksperymencie laboratoryjnym na efektywność produkcji biogazu i wydajność procesu AD gnojowicy bydlęcej.
2. Badanie indywidualnego i synergistycznego wpływu wybranych antybiotyków oraz ich stężeń na zmiany zachodzące w konsorcjach mikroorganizmów zaangażowanych w proces AD gnojowicy bydlęcej. Określenie składu ilościowego i jakościowego mikroorganizmów w oparciu o wyniki sekwencjonowania o wysokiej przepustowości (Illumina).
3. Określenie rozpowszechnienia ARGs w masie pofermentacyjnej procesu AD oraz określenie współzależności pomiędzy stężeniem leków a liczbą kopii ARGs w fermentowanym oborniku bydlęcym z dodatkiem kiszonki kukurydzy, osadach ściekowych oraz mieszaninie kiszonki kukurydzy, flotatu poubojowego, pulpy ziemniaczanej i masy cukierniczej w oparciu o wyniki badań molekularnych z wykorzystaniem metody ilościowej reakcji łańcuchowej polimerazy (ang. qPCR – quantitaive polymerase chain reaction).
4. Określenie stężeń wybranych leków w pofermencie pochodzącym z eksperymentu prowadzonego w warunkach in vivo, w biogazowniach rolniczych pracujących w warunkach mezofilnych w oparciu o różnorodne substraty organiczne tj., obornik bydlęcy z dodatkiem kiszonki kukurydzianej, osady ściekowe oraz mieszaninę kiszonki kukurydzianej, pulpy ziemniaczanej, masy cukierniczej i flotatu poubojowego.

Omówienie wyników badań

Farmaceutyki obecne w substratach poddawanych AD., tj gnojowicy bydlęcej, kiszonce kukurydzy, flotacie poubojowym, osadzie ściekowym, zielonym odpadzie roślinnym mogą hamować skuteczność i wydajność procesu poprzez bezpośrednie oddziaływanie na bakterie oraz metanogeny **(Załącznik 1)**. Mikroorganizmy biorące udział w tym procesie mają różne wymagania metaboliczne i istnieje między nimi ścisła zależność – syntrofia **(Załącznik 2)**. Środki przeciwdrobnoustrojowe obecne w substratach poddawanych AD mogą hamować replikację DNA, transkrypcję RNA, odpowiedź SOS lub wytwarzanie ATP w organizmach drobnoustrojów **(Załącznik 3)**. Farmaceutyki mogą również zakłócać podział komórek, translację białek i syntezę ściany komórkowej lub biosyntezę nukleotydów. Chlorotetracyklina należąca do klasy antybiotyków tetracyklinowych w procesie AD może całkowicie hamować acetoklastyczne metanogenne *Archaea* **(Załącznik 4)**. Antybiotyki, takie jak sulfametoksazol i tetracyklina, zapobiegają wzrostowi bakterii, wiążąc się nieodwracalnie z podjednostkami odpowiednio 50S i 30S bakteryjnego rybosomu. Sulfonamidy działają jako konkurencyjne inhibitory enzymu syntazy dihydropteranianowej, który katalizuje konwersję para-aminobenzoesanu do dihydropteranianu, prekursora syntezy kwasu foliowego. Kwas tetrahydrofoliowy bierze udział w syntezie kwasów nukleinowych, a tym samym zapobiega rozwojowi bakterii. Sulfametoksazol na etapie kwasogenezy i acetogenezy może wspomagać degradację substratów np., poprzez niszczenie błon komórkowych komórek roślinnych, poprawiając w ten sposób solubilizację, ale jednocześnie zwiększając produkcję lotnych kwasów tłuszczowych (LKT), których kumulacja może również prowadzić do obniżenia wydajności biogazu lub zahamowania tego procesu. W AD większość procesów hydrolizy i kwasogenezy zależy od metabolizmu bakterii, co wpływa na jej stabilność **(Załącznik 5)**. Pogłębienie wiadomości na temat procesu AD gnojowicy bydlęcej w obecności antybiotyków, ich wpływu na produkcję biogazu i mikrobiom zaangażowany w procesy AD skutkowało opracowaniem pracy przeglądowej na ten temat (**Załącznik 1**). Praca ta stanowiła podwaliny do przygotowania metodyki badań umożliwiających weryfikację postawionych w prezentowanej dysertacji doktorskiej czterech hipotez badawczych. Do opracowania metodyki wykorzystanej w dysertacji doktorskiej przyczyniły się również badania wstępne opisane w **Załączniku 2**. Wyniki tych badań pozwoliły na uzyskanie informacji na temat odpowiedzi mikroorganizmów na poszczególne antybiotyki co pozwoliło na wybranie określonych antybiotyków do dalszych etapów badań **(Załączniki 3, 4 oraz 5)**.

Etap I

W pierwszym etapie badań prowadzonych w ramach niniejszej rozprawy doktorskiej w warunkach laboratoryjnych zbadano indywidualny wpływ ośmiu środków przeciwdrobnoustrojowych należących do klasy beta-laktamów (AMO – amoksycylina, AMP – ampicylina), tetracyklin (OXY – oksytetracyklina, TET – tetracyklina, CHLOR – chlorotetracyklina), fluorochinolonów (ENRO– enrofloksacyna), sulfametoksazol (SMX) i pochodnych nitroimidazolu (MET – metronidazol) na wydajność produkcji biogazu w procesie AD gnojowicy bydlęcej, w układzie technologicznym w warunkach laboratoryjnych. W ramach prac prowadzonych w tym etapie zbadano mikrobiom zaangażowany w proces AD, obecność i liczbę genów *mcr*A, *MSC* i *MST* charakteryzujących *Archaea* oraz liczebność ARGs w pofermencie. Ponadto zbadano degradację środków przeciwdrobnoustrojowych podczas biometanizacji gnojowicy bydlęcej. Przeprowadzony I etap badań pozwolił na uzyskanie informacji na temat odpowiedzi mikroorganizmów zaangażowanych w proces AD i wyselekcjonowanie antybiotyków zmniejszających efektywność procesu AD, które suplementowano w kolejnym etapie badań.

Etap II

W drugim etapie badań określono synergistyczny wpływ wybranych leków przeciwdrobnoustrojowych tj. AMO, ENRO oraz MET, i ich zmiennych, wzrastających stężeń w układzie technologicznym AD w eksperymencie laboratoryjnym na efektywność produkcji biogazu i wydajność procesu AD gnojowicy bydlęcej. Leki suplementowano do wsadu bioreaktorów AD w mieszaninie, w zmiennych, wzrastających stężeniach. W badaniach prowadzonych w ramach tego etapu analizowano również zmiany zachodzące w konsorcjach mikroorganizmów zaangażowanych w proces AD. Określono także skład ilościowy i jakościowy mikroorganizmów w oparciu o wyniki sekwencjonowania o wysokiej przepustowości (Illumina). W niniejszym etapie określono także rozpowszechnienie ARGs w masie pofermentacyjnej procesu AD oraz określono współzależność pomiędzy synergistycznym stężeniem leków a liczbą kopii ARGs w fermentowanej gnojowicy bydlęcej w oparciu o wyniki badań molekularnych z wykorzystaniem metody qPCR oraz wyniki analiz metagenomicznych (Illumina).

Etap III

W trzecim etapie badań określono stężenia wybranych środków przeciwdrobnoustrojowych w pofermencie pochodzącym z eksperymentu prowadzonego w warunkach in vivo, w biogazowniach rolniczych pracujących w warunkach mezofilnych, w oparciu o różnorodne substraty organiczne. W tym etapie badań prowadzonych w ramach niniejszej dysertacji doktorskiej zbadano także rozpowszechnienie ARGs w pofermencie uzyskanym z procesu AD prowadzonego w obecności antybiotyków pochodzących z substratów wprowadzonych do układu technologicznego. W ramach tego etapu badane próbki pofermentu pobrano z trzech różnych biogazowni rolniczych pracujących w oparciu o gnojowicę bydlęcą z kiszonką kukurydzy, osady ściekowe oraz mieszaninę flotatu poubojowego, ścieru ziemniaczanego i roślinnych odpadów zielonych.

Weryfikacja I hipotezy badawczej

Leki przeciwdrobnoustrojowe zadane do wsadu bioreaktorów AD jednostkowo oraz synergistycznie, należące do klasy beta-laktamów, tetracyklin, sulfonamidów, fluorochinolonów oraz pochodne nitroimidazoli, wpływają hamująco na wydajność procesu AD oraz produkcję biogazu.

Pierwsza hipoteza badawcza została zweryfikowana w badaniach przedstawionych w następujących artykułach naukowych:

Załącznik 2. **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. *Applied Sciences.* 2021; 11(1):369. https://doi.org/10.3390/app11010369.

Załącznik 3. **Koniuszewska I,** Harnisz M, Korzeniewska E, Czatzkowska M, Jastrzębski JP, Paukszto Ł, Bajkacz S, Felis E, Rusanowska P. The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure. *Energies*. 2021; 14(4):1125. https://doi.org/10.3390/en14041125.

Załącznik 4. **Wolak I,** Czatzkowska M, Harnisz M, Jastrzębski JP, 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(5):1920. https://doi.org/10.3390/en15051920.

W przedstawionych wyżej pracach naukowych określono indywidualny i synergistyczny wpływ wybranych leków przeciwdrobnoustrojowych oraz ich stężeń w układzie technologicznym w eksperymencie laboratoryjnym na efektywność produkcji biogazu i wydajność procesu AD gnojowicy bydlęcej. W pracach tych potwierdzono przedstawioną w niniejszej dysertacji hipotezę I, w której założono, że leki przeciwdrobnoustrojowe należące do klasy beta-laktamów, tetracyklin, sulfonamidów, fluorochinolonów oraz pochodne nitroimidazoli podane jednostkowo jak i synergistycznie do wsadu bioreaktorów AD wpływają hamująco na wydajność procesu oraz produkcję biogazu **(Załączniki 2, 3 oraz 4)**.

Pierwszym etapem badań weryfikujących hipotezę I były analizy wydajności procesu AD oraz produkcji biogazu w obecności określonych stężeń środków przeciwdrobnoustrojowych zadawanych indywidualnie do wsadu bioreaktorów procesowych **(Załącznik 2)**. Zastosowano automatyczny system testowania potencjału metanu (ang. AMPTS II – Authomatic Methane Potential Test System) (Bioprocess Control, Lund, Szwecja) do pomiaru ilości wyprodukowanego metanu (CH_4). Jakość biogazu mierzona była za pomocą chromatografu gazowego połączonego z detektorem przewodnictwa cieplnego (GC-TCD) (Agillent 7890 A, Lund, Szwecja). W próbkach przed i po fermentacji oznaczano zawartość lotnych kwasów tłuszczowych (LKT), pH, stosunek FOS/TAC (FOS/TAC to stosunek stężenia lotnych kwasów tłuszczowych (FOS) do zasadowości (tj. pojemności buforowej) (TAC) w komorze fermentacyjnej), oznaczono całkowitą zawartość ciał stałych (ang. TS – total solids) i suchą masę organiczną (ang. VS – volatile solids) oraz azot całkowity (ang. TN – total nitrogen) i fosfor całkowity (ang. TP – total phosphorus). Następnie analizowano obecność stężenia antybiotyków takich jak metronidazol (MET), amoksycylina (AMO), ampicylina (AMP), enrofloksacyna (ENR), sulfametoksazol (SMX), oksytetracyklina (OXY), chlorotetracyklina (CHLOR) i tetracyklina (TET), w próbkach pofermentu gnojowicy bydlęcej. Stężenia wybranych do badań farmaceutyków analizowano z wykorzystaniem metody chromatografii cieczowej — spektrometrii mas (ang. LCMS
– Liquid Chromatography Mass Spectrometry).

Antybiotyki zadawane indywidualnie do bioreaktorów znacząco wpłynęły na produkcję LKT, co kolejno miało bezpośredni wpływ na ilość produkowanego biogazu. Spośród badanych LKT najwyższym stężeniem charakteryzował się kwas octowy ($16{,}11 \pm 2{,}09$ g L^{-1}).

Jednak stężenia innych kwasów były również wyższe w porównaniu z próbką kontrolną. Wyższe stężenia LKT zwiększyły również nieznacznie wskaźnik FOS/TAC. Wskaźniki AD (np. stosunek FOS/TAC czy pH) nie uległy zmianie w bioreaktorach z AMO, OXY, ENRO, CHLOR i TET. W niniejszej pracy, stężenie OXY przekraczało 1 g L^{-1} i co interesujące zaobserwowano zahamowanie produkcji CH_4, które mogło być spowodowane obecnością leku we wsadzie bioreaktora. Po dodaniu antybiotyków do substratu stwierdzano akumulację LKT w bioreaktorach, co doprowadziło do zahamowania produkcji CH_4 **(Załącznik 2)**.

W ramach weryfikacji założonej hipotezy I w niniejszej pracy badano również los antybiotyków zadawanych indywidualnie do wsadu bioreaktorów AD. AMO nie było wykrywalne w żadnej frakcji pofermentu gnojowicy bydlęcej po procesie AD – ani we frakcji ciekłej, ani w fazie stałej próbek. Ampicylinę wykryto jedynie w fazie ciekłej próbek, natomiast obecność MET stwierdzano jedynie w fazie stałej próbek (stężenie nie przekraczało 0,02 μg g_D^{-1}). Inne związki były wykrywalne w próbkach po obróbce AD, zarówno w fazie ciekłej, jak i stałej. Wyniki pozwoliły nam oszacować wartości parametrów współczynnika degradacji leku (K_d), które są miarą powinowactwa sorpcji na stałych (zawieszonych) cząstkach badanej próbki. Po obliczeniu wartości parametrów K_d wywnioskowano, że żaden z testowanych antybiotyków nie wykazywał bardzo silnego powinowactwa do frakcji stałej pofermentu. Najwyższe wartości tych współczynników zaobserwowano dla TET ($\log K_d$=1,6), chociaż wskazują one jedynie na umiarkowane powinowactwo do sorpcji na frakcji stałej. Wartości współczynników K_d wyznaczone w tym eksperymencie wskazywały na znacznie większe powinowactwo badanych związków farmaceutycznych do cieczy niż do fazy stałej. Prawdopodobnie adsorpcja tych substancji mogła być ograniczona dostępną powierzchnią frakcji stałej inokulum i gnojowicy bydlęcej, tym bardziej, że uwodnienie próbek było bardzo wysokie i przekraczało 93% **(Załącznik 2)**.

W eksperymencie jednostkowego wpływu środków przeciwdrobnoustrojowych na proces AD gnojowicy bydlęcej stwierdzono, że farmaceutyki ulegały degradacji na różnych poziomach, od 57% (OXY) do 100% (MET, AMO, AMP i SMX). Jednak nawet w przypadku farmaceutyków, dla których zaobserwowano całkowity rozkład, produkty degradacji tych leków były obecne w fazie stałej przefermentowanej gnojowicy bydlęcej. Ze względu na charakter przeprowadzonych eksperymentów, produkty uboczne transformacji zostały zidentyfikowane jedynie jakościowo, na podstawie porównania danych z wcześniej opublikowanej literatury oraz z uwzględnieniem masy dysocjacyjnej wywołanej kolizją

otrzymanej w różnych trybach pracy spektrometru mas, takie jak: jon prekursorowy, jon(y) produkcyjny i strata obojętna. W przypadku antybiotyków takich jak SMX, AMO, AMP, obserwowano powstawanie produktów ubocznych o tej samej masie cząsteczkowej (ale różnym czasie retencji). Produkty te zostały wstępnie zidentyfikowane jako 4-amino-N-(5 metylo-1,3-oksazol-2-ilo)benzenosulfonamid lub 4-amino-N-(5-metylo-1,2-oksazol-3(2H) ylideno)benzenosulfonamid, diketopiperazyna amoksycylina i diketopiperazyna ampicylina. Jednoznaczne potwierdzenie struktur wstępnie zidentyfikowanych ubocznych produktów transformacji wymaga dalszych badań polegających na porównaniu jonów prekursora i produktu oraz uzyskanych czasów retencji dla wstępnie zidentyfikowanych produktów ubocznych o tych samych parametrach wzorców analitycznych tych substancji **(Załącznik 2)**.

W drugim etapie badań, prowadzonym w celu weryfikacji hipotezy I, badano długoterminowy, synergistyczny efekt środków przeciwdrobnoustrojowych na proces AD gnojowicy bydlęcej. Gnojowicę poddawano AD w komorach fermentacyjnych o pojemności 2 L, pracujących w warunkach dynamicznych, półciągłych. Komorę procesową zasilano substratem zawierającym mieszaninę AMO, ENRO i MET. W bioreaktorze procesowym biomasę poddano AD w obecności mieszaniny AMO, ENRO i MET. W kontrolnym bioreaktorze w warunkach beztlenowych fermentowano biomasę bez dodatku mieszaniny antybiotyków. W bioreaktorze procesowym AD przeprowadzono w siedmiu seriach doświadczalnych, które różniły się stężeniami antybiotyków dodawanych do biomasy. Stężenia antybiotyków stopniowo zwiększano w kolejnych seriach doświadczalnych. Hydrauliczny czas retencji (ang. HRT – hydraulic retention time) wynosił 28 dni. Komory fermentacyjne były wyposażone w mieszadła mechaniczne, systemy podawania i odprowadzania oraz były połączone jak w pierwszym etapie badań z AMPTS II, który mierzył produkcję CH_4. Eksperyment trwał 417 dni, każdą serię eksperymentalną prowadzono w dwóch powtórzeniach. W celu monitorowania procesu AD próbki fermentowanych substratów pobierano dwa razy w tygodniu w celu określenia ich pH, stosunku FOS/TAC oraz zawartości TS, VS, TN i TP **(Załącznik 4)**.

Zawartość LKT oznaczono za pomocą chromatografu gazowego (Brüker, 450-GC) z detektorem płomieniowo-jonizacyjnym (FID). Stosunek FOS/TAC określono za pomocą TitraLab serii AT1000 Titrator (Hatch). Zawartość TS i VS w próbkach biomasy oznaczono według APHA. Zawartość TN i TP w próbkach zmineralizowanych oznaczono w testach

kuwetowych HachLange. Wszystkie pomiary wykonano w dwóch powtórzeniach **(Załącznik 4)**.

Podsumowując, zbadano indywidualny i synergistyczny wpływ ośmiu środków przeciwdrobnoustrojowych na wydajność produkcji biogazu w procesie AD gnojowicy bydlęcej. Farmaceutyki indywidualnie zadawane do podłoża istotnie obniżyły produkcję biogazu i ilość uzyskiwanego CH_4. Amoksycylina wprowadzana jednostkowo do wsadu bioreaktora spowodowała 75% spadek produkcji CH_4 w porównaniu z próbkami kontrolnymi. Enrofloksacyna, tetracyklina, oksytetracyklina i chlorotetracyklina również wprowadzane jednostkowo do wsadu bioreaktorów zmniejszyły ilość wytwarzanego biogazu odpowiednio o 36, 39, 45 i 53%. Antybiotyki zadawane synergistycznie do wsadu bioreaktorów wywołały niewielki i tymczasowy spadek wydajności CH_4 zależny od stężenia leków we wprowadzanej do bioreaktorów mieszaninie. Spadek ten mógł wynikać z ograniczonej dostępności substratów do produkcji CH_4, obecności substancji toksycznych w bioreaktorze, zmian w społeczności mikroorganizmów oraz przejściowych zakłóceń parametrów fizykochemicznych procesu AD.

Na podstawie przedstawionych powyżej wyników badań potwierdzono, iż leki suplementowane do biomasy fermentacyjnej znacząco zmniejszają ilość produkowanego biogazu, jednakże wpływ środków przeciwdrobnoustrojowych zależny jest od klasy antybiotyków, ich dawkowania oraz sposobu w jaki są one wprowadzane do wsadu bioreaktorów (tj. bezpośrednia suplementacja do bioreaktora jednostkowo, w mieszaninie lub jako macierzysta zawartość gnojowicy bydlęcej oraz innych stosowanych w AD substratów).

Weryfikacja II hipotezy badawczej

Skład jakościowy i ilościowy drobnoustrojów obecnych w systemach AD jest modyfikowany poprzez obecność środków przeciwdrobnoustrojowych we wsadzie bioreaktorów AD, zarówno w przypadku farmaceutyków zadawanych do układu technologicznego jednostkowo, jak i synergistycznie.

Druga hipoteza badawcza została zweryfikowana w badaniach przedstawionych w następujących artykułach naukowych:

Załącznik 1. **Koniuszewska I,** Korzeniewska E, Harnisz M, and Czatzkowska M, “Intensification of biogas production using various technologies: A review”.

International Journal of Energy Research., 2020:2:1–19. https://doi.org/10.1002/er.5338.

Załącznik 2. **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. *Applied Sciences*. 2021; 11(1):369. https://doi.org/10.3390/app11010369.

Załącznik 3. **Koniuszewska I,** Harnisz M, Korzeniewska E, Czatzkowska M, Jastrzębski JP, Paukszto Ł, Bajkacz S, Felis E, Rusanowska P. The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure. *Energies*. 2021; 14(4):1125. https://doi.org/10.3390/en14041125.

Załącznik 4. **Wolak I,** Czatzkowska M, Harnisz M, Jastrzębski JP, 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(5):1920. https://doi.org/10.3390/en15051920.

W przedstawionych wyżej publikacjach naukowych **(Załączniki 1, 2, 3 oraz 4)** określono indywidualny i synergistyczny wpływ wybranych leków przeciwdrobnoustrojowych oraz ich stężeń w układzie technologicznym w eksperymencie laboratoryjnym na skład jakościowy i ilościowy drobnoustrojów obecnych w systemach AD. Stwierdzono, że mikrobiom zaangażowany w procesy AD gnojowicy bydlęcej jest modyfikowany poprzez obecność środków przeciwdrobnoustrojowych w biomasie fermentacyjnej dodanej do bioreaktorów, zarówno farmaceutyków zadawanych do układu technologicznego AD jednostkowo jak i synergistycznie.

W pierwszym etapie badań prowadzonym w celu weryfikacji postawionej hipotezy II, wykonano przegląd literatury w celu określenia wpływu środków przeciwdrobnoustrojowych na proces AD i określenia inhibitorów oraz intensyfikatorów procesu **(Załącznik 1)**. Następnie w badaniach analizowano indywidualny wpływ wybranych leków przeciwdrobnoustrojowych oraz ich stężeń na mikrobiom zaangażowany w proces AD gnojowicy bydlęcej. W badaniach określono skład jakościowy i ilościowy drobnoustrojów i stwierdzono, że mikrobiom AD jest modyfikowany poprzez obecność antybiotyków wprowadzonych jednostkowo do wsadu bioreaktorów. Przeprowadzona analiza bioinformatyczna sekwencji genu 16S rRNA uzyskanych z masy pofermentu wykazała obecność 324 OTUs (ang. OTUs – operational taxonomic units). Wysokowydajna analiza

sekwencjonowania wykazała znaczne zmiany w populacji mikroorganizmów w pofermencie w zależności od dodanego indywidualnie antybiotyku do wsadu bioreaktora. Wśród zidentyfikowanych OTUs w analizowanych próbkach pofermentu najliczniej stwierdzano obecność mikroorganizmów typu *Bacteroidetes* i *Firmicutes*, które potrafią rozkładać LKT, tolerują wahania temperatury, pH i dostępności tlenu oraz charakteryzują się potencjalną zdolnością do hydrolizy i kwasogenezy wodorowej. Oprócz degradacji białek, większość bakterii typu *Bacteroidetes* wytwarza enzymy lityczne i kwas octowy i są szeroko rozpowszechnione w AD. Wcześniejsze badania wykazały również, że stosunek *Firmicutes* do *Bacteroidetes* może służyć jako wskaźnik stabilności procesu AD. W pofermencie z dodatkiem MET lub AMP bakterie typu *Bacteroidetes* stanowiły prawie 40% zbiorowisk drobnoustrojów. W pofermencie z AMO, ENRO, OXY lub TET *Bacteroidetes* stanowiły blisko 30% wszystkich zidentyfikowanych mikroorganizmów. *Bacteroidetes* i *Firmicutes* stanowiły kolejno po 22% społeczności drobnoustrojów w pofermencie uzupełnionym CHLOR. Próbka pofermentu zawierająca SMX wykazała najbardziej rozległe zmiany w zbiorowisku drobnoustrojów w stosunku do próbki kontrolnej. Liczne rodzaje *Bacteroides* i *Paludibacter* należące do typu *Bacteroidetes* stwierdzono we wszystkich próbkach pofermentu pobranych z bioreaktorów zawierających biomasę eksponowaną na środki przeciwdrobnoustrojowe. Wśród rodzaju *Firmicutes* dominowały drobnoustroje należące do klas *Bacilli* i *Clostridia*. W większości próbek pofermentu odnotowano liczne odczyty przypisane do rodzajów *Bacillus, Rumellibacillus, Enterococcus, Turicibacter*, należące do klasy *Bacilli.* Jednak liczba odczytów na próbkę, pobranego z wsadu bioreaktora eksponowanego na leki była znacznie niższa w porównaniu z próbką kontrolną. Typy *Bacteroidetes* i *Firmicutes* stanowiły po 10% każdego ze zbiorowisk mikroorganizmów w pofermencie z SMX. Jest wysoce prawdopodobne, że w trakcie procesu transformacji SMX produkty degradacji antybiotyków spowodowały spadek liczebności mikroorganizmów. Mikroorganizmy typu *Proteobacteria* stanowiły 11,8% OTU w pofermencie z MET, 5,99% w pofermencie z ENRO i prawie 4% w próbkach pofermentu z AMO i AMP. Tymczasem *Proteobacteria* stanowiły nieco ponad 1% społeczności drobnoustrojów w produktach pofermentacyjnych eksponowanych na TET. Wśród mikroorganizmów należących do typu Proteobacteria dominowały klasy *Alpha-*, *Beta-* i *Gammaproteobacteria.* Dominujący rodzaj wśród *Alphaproteobacteria* należał do rzędu *Rhizobiales*. Wśród nich dominował rodzaj *Oligella* należący do rzędu *Burkholderiales klasa Betaproteobacteria*. Wśród *Gammaproteobacteria* odnotowano liczne rodzaje *Pseudomonas* należące do rzędu

Pseudomonadales. Proteobacteria odgrywają bardzo ważną rolę w AD, zużywając glukozę, octany i propioniany, które nagromadzone w dużych ilościach mogą powodować obniżenie pH, co najczęściej prowadzi do pewnego zaburzenia lub zahamowania tego procesu. Inne ważne typy mikroorganizmów to *Spiralles* (ponad 1% w próbkach z AMO, ENRO lub OXY), rodzaje *Treponema* i *Tenericutes* (ponad 2% w pofermencie z MET, AMO, AMP i 1% w próbkach z TET), rodzaje *Acholeplasma* (ponad 1% w próbkach z MET, AMO, AMP, ENR, OXY, CHLOR i TET). Sekwencjonowanie ujawniło kilka odczytów dla domeny *Archaea*. Najczęściej identyfikowano rodzaje *Methanosarcina* i *Methanosaeta* (ponad 1% sekwencyjnie w próbkach z MET, AMP, SMX, OXY, CHLOR, TET oraz w próbkach z MET, AMP, ENRO, OXY, CHLOR, TET), co jest zgodne z analizą qPCR przeprowadzoną w tym badaniu, ukierunkowaną na geny *MSC* i *MST*, specyficzne dla rodzin *Methanosarcinaceae* i *Methanosaetaceae* **(Załącznik 2)**.

W drugim etapie w celu weryfikacji hipotezy II badano synergistyczny wpływ wybranych leków przeciwdrobnoustrojowych oraz ich stężeń na mikrobiom zaangażowany w proces AD gnojowicy bydlęcej. W badaniach określono skład jakościowy i ilościowy drobnoustrojów. Założono, że struktura zbiorowisk drobnoustrojów w próbkach pofermentu będzie zbliżona do mikrobiomu jelitowego bydła, na co istotny wpływ ma m.in. obecność farmaceutyków w paszach oraz aplikowanych podczas terapii farmaceytycznej lekach. Czas trwania eksperymentu był kolejnym czynnikiem, który indukował zmiany w składzie konsorcjów drobnoustrojów w AD. W rezultacie bakteryjne OTUs w pofermencie różniły się znacznie w zależności od dawki wprowadzanego do mieszaniny antybiotyku i długości trwania procesu AD. Osiem typów bakterii zidentyfikowano w produktach pofermentacyjnych podczas trwania procesu AD i pod koniec 417-dniowego eksperymentu. Były to: *Bacteroidetes* (klasa *Bacteroidia*), *Firmicutes* (klasy *Bacilli*, *Clostridia*, *Mollicutes* i *Erysipelotrichia*), *Proteobacteria* (klasy *Alphaprotebocateria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria* i *Epsilonproteobacteria*), *Acidobacteria* (klasa *Blastocatellia*), *Spirochaetes, Synergistetes* (klasa *Synergistia*) *Actinobacteria* i *Tenericutes*. *Firmicutes* i *Bacteroidetes* były dominującymi typami podczas AD. *Bacteroidetes* są dobrze znanymi bakteriami proteolitycznymi, podczas gdy *Proteobacteria* należą do kluczowych konsumentów utleniających długołańcuchowe LKT podczas AD. *Bacteroidetes* stanowiły 89% bakterii w pofermencie pozyskanym z serii I przeprowadzonego eksperymentu, z najniższą zadawaną dawką środków przeciwdrobnoustrojowych, i ponad 70% bakterii w pofermencie pozyskanym z serii II eksperymentu, w której zwiększono stężenie

zadawanych leków. W porównaniu z próbami doświadczalnymi z serii I i II udział *Bacteroidetes* zmniejszył się do 50% w pofermencie pozyskanym z serii III eksperymentu. *Bacteroidetes* stanowiły również 50% bakterii w pofermencie z próbki kontrolnej z serii I eksperymentu. Udział *Bacteroidetes* określono również na 50% w dwóch próbkach pobranych w trakcie VII serii doświadczenia. Obfitość *Bacteroidetes* w próbkach serii doświadczalnej IV i VI nie przekraczała 30%. W pozostałych próbkach doświadczalnych i kontrolnych liczebność *Bacteroidetes* oszacowano na 20%. Co ciekawe, udział procentowy *Bacteroidetes* w większości próbek eksperymentalnych wahał się od 30% do 50%, jednakże w próbkach kontrolnych określono go na poziomie zaledwie 20%. Ta obserwacja sugeruje, że gatunki *Bacteroidetes* były wysoce oporne na badane środki przeciwdrobnoustrojowe. Co więcej, *Bacteroidetes* szeroko kolonizują przewód pokarmowy bydła i są wszechobecne w gnojowicy bydlęcej **(Załącznik 4)**.

Żyjące w glebie *Actinobacteria,* zidentyfikowane w niniejszym badaniu rozkładają różne związki organiczne i ksenobiotyki, podczas gdy *Acidobacteria* są bezwzględnymi beztlenowcami, które fermentują związki aromatyczne i octany. *Synergistetes* i *Spiralles* przekształcają produkty takie jak propionian, maślan, izomaślan, kwas walerianowy, kwas izowalerianowy i etanol w octan, H_2 i CO_2. Powstałe produkty są wykorzystywane przez metanogeny do produkcji CH_4. W próbkach doświadczalnych pofermentu pozyskanego w I serii doświadczeń *Acidobacteria* stanowiły od 1% do 3% zidentyfikowanych bakterii, podczas gdy ich udział w próbkach kontrolnych bez dodatku antybiotyku przekraczał 11%. Udział gatunków *Acidobacteria* w przefermentowanej biomasie wzrastał w kolejnych tygodniach doświadczenia, przekraczając 50% w próbce serii II. Udział *Acidobacteria* sięgał nawet 60% w próbkach serii II, III i IV. W próbce kontrolnej z serii IV *Acidobacteria* reprezentowały około 60% społeczności drobnoustrojów. W serii V udział *Acidobacteria* w próbce gwałtownie spadał do około 20%, a trend ten utrzymywał się w próbkach doświadczalnych zawierających mieszaninę AMO, ENRO i MET do końca trwania procesu AD. W próbce kontrolnej pobranej w ostatnim etapie procesu AD w serii VII *Acidobacteria* stanowiły 64% społeczności drobnoustrojów. Wyniki te sugerują, że *Acidobacteria* były oporne tylko na niższe stężenia badanych leków **(Załącznik 4)** .

Bakterie z rodzaju *Firmicutes* stanowiły od 4% do 8% mikroorganizmów w próbkach pofermentu z serii doświadczalnej I. W próbce kontrolnej pobranej z serii I ponad 17% zbiorowiska bakterii reprezentowały gatunki *Firmicutes*. W seriach II i III udział *Firmicutes*

w mikrobiomie próbek pofermentu wahał się od 9% do 12%. Udział *Firmicutes* oszacowano od 12% do około 20% w próbkach pobranych w seriach IV, V i VI. Co ciekawe, w próbkach doświadczalnych pozyskanych w VII serii doświadczalnej *Firmicutes* stanowiły 23% mikroorganizmów, ale zaledwie 8% w próbce kontrolnej. *Firmicutes* mogą łatwo rozkładać złożone substraty polisacharydowe i białkowe w warunkach beztlenowych i syntetyzować octan z aminokwasów wytwarzanych podczas hydrolizy białek. Ten typ bakterii dominuje w przewodzie pokarmowym ludzi i zwierząt, a także jest wszechobecny w nieoczyszczonych ściekach, co sugeruje, że gatunki *Firmicutes* są wysoce oporne na środki przeciwdrobnoustrojowe. *Firmicutes* prawdopodobnie przechowują i przenoszą ARGs w środowisku, w tym w ekosystemach wodnych. Według danych literaturowych *Firmicutes* są głównymi nosicielami ARGs w glebach rolniczych. Inni autorzy wykazali we wcześniejszych badaniach, że bakterie mogą nabywać oporność na wybrane środki przeciwdrobnoustrojowe (tetracyklinę, streptomycynę czy sulfonamidy) bez kontaktu z tymi lekami. Problemem jest również to, że oporne bakterie i ARGs mogą utrzymywać się przez lata w środowisku, nawet przy krótkotrwałej podaży antybiotyków. Ponadto warto zauważyć, że ARGs mogą być przenoszone poziomo między drobnoustrojami za pośrednictwem MGEs. Transfer ARGs w wyniku HGT może wystąpić między drobnoustrojami niepatogennymi, patogennymi, a nawet pomiędzy odlegle spokrewnionymi organizmami **(Załącznik 4)**. Ostatnie badania wykazały specyficzne przypadki przenoszenia ARGs z udziałem HGT w jelitach ludzi [3] i zwierząt [4], glebie [5][6], osadach [7] i wodzie [8].

Liczebność w obrębie gatunku *Euryarchaeota* w próbkach pofermentu różniła się znacznie w seriach doświadczalnych w procesie AD. W większości próbek dominującymi metanogenami były mikroorganizmy z rodzajów *Methanothrix* i *Methanosarcina* oraz z rzędu *Methanomassiliicocales.* Rodzaj *Methanothrix* składa się z acetoklastycznych metanogenów, które są wrażliwe na niekorzystne warunki środowiskowe, podczas gdy *Methanosarcina* spp. jest w stanie wykorzystywać różnorodne substraty, w tym octan, H_2 i związki metylowe **(Załącznik 4)**.

Methanothrix soehngenii, *Methanosarcina mazei* i *Methanomassiliicocales archaeonRumEnM2* były dominującymi gatunkami zarówno w eksperymentalnych, jak i kontrolnych próbkach pofermentu gnojowicy bydlęcej. *Methanobrevibacter millerae* dodatkowo zidentyfikowano w pięciu próbkach doświadczalnych z serii I, II, III oraz VII, zawierających mieszaninę AMO, ENRO i MET oraz w dwóch próbkach kontrolnych serii IV

i VII nie suplementowanych antybiotykami. W próbkach doświadczalnych pobranych z serii I, II i III wykryto *Methanosarcina thermophila.* Jednakże wzrost *Methanosarcina thermophila* został całkowicie zahamowany w kolejnych seriach eksperymentalnych prawdopodobnie z powodu niesprzyjających warunków podczas przedłużającej się AD oraz wrażliwości bakterii na zastosowaną kombinację leków. W seriach tych stężenie antybiotyków było wyższe, co sugeruje, że przedłużona ekspozycja na środki przeciwdrobnoustrojowe może zmniejszyć różnorodność zbiorowisk metanogennych w biomasie. *Methanosarcina mazei* była również liczna w próbkach doświadczalnych pobranych w seriach I–III, ale jej częstość występowania gwałtownie malała w odpowiedzi na wyższe stężenie antybiotyków w seriach IV i utrzymywała się na niskim poziomie do końca procesu AD **(Załącznik 4)**.

Podsumowując, społeczność drobnoustrojów reprezentowana była głównie przez *Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes* i *Tenericutes,* co jest zgodne z wynikami eksperymentów przeprowadzonych przez innych badaczy [9][10][11][12][13][11][14]. Zidentyfikowane mikroorganizmy charakteryzują się zdolnością hydrolityczną, a produkty hydrolizy służą jako substrat dla mikroorganizmów biorących udział w kwasogenezie w celu wytworzenia LKT. *Bacteroidetes, Firmicutes* i *Proteobacteria* mogą rozkładać LKT i wykorzystywać je jako źródło węgla dla w swojej aktywności metabolicznej. W przeciwieństwie do próbek kontrolnych, większość analizowanych próbek pofermentu (z wyjątkiem próbki z AMO i TET) nie zawierała LKT. Jest wysoce prawdopodobne, że mikroorganizmy obecne w pofermencie, wykorzystując dostępne substraty jako źródło węgla, zmniejszyły podaż LKT dla mikroorganizmów acetogennych, które z kolei przy braku wystarczającej ilości substratu zmniejszyły tempo ich produkcji jako substratu dla mikroorganizmów metanogennych. Doprowadziło to do zaburzenia szlaku metanogenezy i spadku wydajności produkcji CH_4 we wszystkich próbkach pofermentu pochodzących ze wsadu bioreaktora eksponowanego na leki. Wyniki analiz bioinformatycznych w oparciu o sekwencjonowanie 16S rRNA sugerują, że zidentyfikowane *Bacteroidetes* i *Firmicutes* mogą odgrywać znaczącą rolę w degradacji i usuwaniu TS. Na podstawie przedstawionych wyników badań można stwierdzić, że bakterie typu *Bacteroidetes* i *Firmicutes* w obecności antybiotyków nie dostarczały odpowiednich ilości substratów dla mikroorganizmów metanogennych wytwarzających biogaz. Na społeczność mikroorganizmów zaangażowanych w AD mogły wpływać zmiany warunków środowiskowych, które z kolei mogły prowadzić do zmniejszenia wydajności bioreaktora lub

do błędów technologicznych, takich jak nadmiernie obniżone pH powodujące zakwaszenie środowiska i zmniejszenie uzysku CH_4, co z różnym stopniem nasilenia, zaobserwowano we wszystkich analizowanych próbkach pofermentu **(Załącznik 4)**. W związku z powyższym wykorzystanie pofermentu w postaci nawozu może zaburzać naturalną mikrobiotę glebową oraz sprzyjać przenoszeniu ARB w środowisku glebowym.

Weryfikacja III hipotezy badawczej

Farmaceutyki, zadawane do wsadu bioreaktorów jednostkowo lub synergistycznie, a także te obecne w substratach poddawanych AD, wpływają istotnie na wzrost liczby kopii ARGs wśród populacji drobnoustrojów odpowiadających za proces biometanizacji.

Trzecia hipoteza badawcza została zweryfikowana w badaniach przedstawionych w następujących artykułach naukowych:

Załącznik 2. **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. *Applied Sciences*. 2021; 11(1):369. https://doi.org/10.3390/app11010369.

Załącznik 3. **Koniuszewska I,** Harnisz M, Korzeniewska E, Czatzkowska M, Jastrzębski JP, Paukszto Ł, Bajkacz S, Felis E, Rusanowska P. The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure. *Energies*. 2021; 14(4):1125. https://doi.org/10.3390/en14041125.

Załącznik 4. **Wolak I,** Czatzkowska M, Harnisz M, Jastrzębski JP, 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(5):1920. https://doi.org/10.3390/en15051920.

Załącznik 5. **Wolak I,** Bajkacz S, Harnisz M, Stando K, Męcik M, Korzeniewska E. Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment. *International Journal of Environmental Research and Public Health*. 2023; 20(3):2672. https://doi.org/10.3390/ijerph20032672.

W pierwszym etapie badań prowadzonych w celu weryfikacji hipotezy III badano jednostkowy wpływ ośmiu różnych środków przeciwdrobnoustrojowych na obecność i liczbę ARGs w próbkach pofermentu pochodzącego z bioreaktora, w którym jako substrat w procesie AD zastosowano gnojowicę bydlęcą. Na skutek przeprowadzonych badań stwierdzono, że antybiotyki zadawane indywidualnie do wsadu bioreaktorów AD stymulowały wzrost liczby ARGs w masie pofermentacyjnej. Odnotowano istotne statystycznie (p<0,05) różnice w całkowitej liczbie kopii genów (ang. AAs – absolute abundances) *tet* (rzędu 1,0 × 10^8 g_D^{-1}) pomiędzy próbkami pofermentu zależnie od suplementowanego indywidualnie do wsadu bioreaktora antybiotyku. W większości badanych próbek pofermentu, w porównaniu z próbką kontrolną, odnotowano liczne ARGs niosące oporność na tetracykliny. W niniejszych badaniach prowadzonych w ramach rozprawy doktorskiej, wprowadzane jednostkowo do podłoża bioreaktora stężenia antybiotyków tetracyklinowych powodowały presję selekcyjną i prawdopodobnie wpłynęły na liczbę i rozprzestrzenianie ARGs w pofermencie pochodzącym z gnojowicy bydlęcej. Geny oporności na tetracykliny są szeroko rozpowszechnione w gnojowicy bydlęcej, a antybiotyki suplementowane do biomasy w systemach AD dodatkowo indukowały wzrost oporności na te antybiotyki, co wyjaśnia ich dużą liczebność w próbkach pofermentu pobranych z wsadów bioreaktorów eksponowanych na farmaceutyki. Możliwe także, że geny *tet* są stabilną częścią genomów fizjologicznej mikrobioty przewodu pokarmowego zwierząt i dlatego są przekazywane z jelit do odchodów zwierzęcych, gdzie AR może być przenoszona przez mechanizmy HGT **(Załączniki 2 oraz 3)**.

AAs genów *bla* w $1g_D^{-1}$ wahały się w granicach rzędu 10^4-10^7 kopii genu. W porównaniu z próbką kontrolną zaobserwowano istotne statystycznie (p<0,05) różnice w wartościach AAs dla genu bla_{TEM}. Liczba kopii genu bla_{TEM} była o jeden rząd wyższa w 1 g próbki pofermentu z dodatkiem AMP, ENRO lub CHLOR. Ponadto zaobserwowano wyraźny wzrost liczby kopii genu *cfx*A o jeden rząd w 1 g pofermentu w obecności AMP w stosunku do próbki kontrolnej. Gen bla_{TEM} to najczęściej występujący gen wśród genów *bla* w próbkach środowiskowych. W większości analizowanych próbek pofermentu nie zaobserwowano obecności genu bla_{OXA}. Degradacja antybiotyków beta-laktamowych w procesie AD mogła być powodem, dla którego gen bla_{OXA} był nieobecny. Bakterie wykazują oporność na antybiotyki beta-laktamowe poprzez wytwarzanie beta-laktamaz, odpowiedzialnych za tolerancję na te leki. Drobnoustroje reagując na obecność beta-laktamów wytwarzają beta-laktamazy, umożliwiające bakteriom oporność na te antybiotyki.

Jednak niestabilność pierścienia beta-laktamowego w antybiotykach beta-laktamowych może spowodować ich szybką degradację w środowisku, a tym samym obniżyć możliwość lub wręcz uniemożliwić bakteriom obecnym w tym otoczeniu uzyskanie lekooporności na te antybiotyki **(Załączniki 2 oraz 3)**.

W porównaniu z próbką kontrolną (liczba kopii genu *sul*1 rzędu 10^7 g_D^{-1}) stwierdzano istotne statystycznie (p> 0,05) różnice w liczbie genów *sul*1 w próbkach pofermentu pochodzących z biomasy suplementowanej różnymi antybiotykami. W próbkach pofermentu z AMP, TET i CHLOR stwierdzano wzrost liczby genu *sul*1 o jeden rząd wielkości (10^8 kopii genu g_D^{-1}). Powstawanie produktów przemiany podczas AD zależało od rodzaju antybiotyku i jego poziomu suplementacji do biomasy w bioreaktorze. W badaniach tych zaobserwowano całkowitą degradację SMX, choć możliwe jest, że produkty przemian tego związku np. zmiana w obrębie pierścienia izoksazolowego, mogą pojawiać się i pozostawać w masie pofermentu z gnojowicy bydlęcej. Epimeryzacja antybiotyków wydaje się być pierwszym etapem transformacji w warunkach beztlenowych procesu AD. Jest wysoce prawdopodobne, że suplementacja SMX do substratu spowodowała presję selekcyjną i zwiększoną liczbę ARGs na ten lek. Na poziomie genetycznym AR skutkuje obecnością ARGs w chromosomach i/lub w MGEs, takich jak integrazy, których znaczną ilość kopii odnotowano w większości próbek pofermentu analizowanych w prezentowanych badaniach. Wspomniane wyżej czynniki, takie jak presja selekcyjna czy obecność genów integrazy, są ściśle związane z obfitością ARGs w procesie AD, sprzyjającym powstawaniu mutacji u drobnoustrojów i nabywaniu przez nie nowych cech, w tym AR **(Załączniki 2 oraz 3)**.

Wartość AAs genów *erm*F, *lin*A, *mef*A mieściła się w zakresie od 10^5 do 10^8 kopii genu w $1g_D^{-1}$. W stosunku do próbki kontrolnej odnotowano istotne statystycznie różnice w liczbie genu *mef*A pomiędzy próbkami pofermentu z dodatkiem antybiotyków. W porównaniu z próbką kontrolną (liczba kopii rzędu 10^6 g_D^{-1}), gen *mef*A występował licznie w pofermencie z biomasy suplementowanej MET, AMO, AMP, CHLOR i TET. W próbkach pofermentu z AMP w porównaniu z próbką kontrolną odnotowano wzrost liczby kopii genu *mef*A o dwa rzędy wielkości. W próbkach pofermentu z dodatkiem MET, AMO, CHLOR lub TET gen *mef*A był obecny w ilościach rzędu 10^7 kopii g_D^{-1}. W pofermencie po procesie AD wykryto niewielkie ilości MET i znaczne ilości leków tetracyklinowych. Antybiotyki obecne w pofermencie mogły wywołać wzrost AR w kierunku tych środków farmaceutycznych. Możliwe jest również, że niektóre rodzaje metanogennych *Archaea*, w tym *Methanosaeta*

licznie odnotowane w próbkach pofermentu, są potencjalnymi nosicielami ARGs, które odpowiedzialne są za oporność na antybiotyki należące do grupy MLS **(Załączniki 2 oraz 3)**.

W porównaniu z próbkami kontrolnymi szczególnie duże i istotne statystycznie różnice (ANOVA Kruskal-Wallis; $p<0,05$; $N= 6$) w liczbie genów *qep*A ($1,0 \times 10^9$ kopii genu g_D^{-1}) stwierdzano dla próbek pofermentu pochodzącego z biomasy jednostkowo eksponowanej na AMO, AMP i OXY. Fluorochinolony są związkami silnie adsorbującymi się do materii organicznej obecnej w gnojowicy bydlęcej, dzięki czemu ich degradacja jest znacznie słabsza niż innych antybiotyków, np. beta-laktamów. W niniejszych badaniach spadek liczby genów *qep*A i *aac 6'-Ib-cr* zaobserwowano jedynie w pofermencie pochodzącym z biomasy z dodatkiem MET **(Załączniki 2 oraz 3)**.

Wartość AAs analizowanych ARGs w większości próbek pofermentu pobranych z wsadu AD z dodatkiem antybiotyków wahała się w granicach od 10^6 do 10^8 kopii genu g_D^{-1}. Liczba genów w próbce kontrolnej oscylowała wokół 10^6 do 10^7 kopii genu g_D^{-1}. Wysoka ilość kopii genów *intI*1 i *intI*2 w próbkach pofermentu pobranych z wsadu AD z dodatkiem antybiotyków oraz w próbce kontrolnej może świadczyć o możliwości rozprzestrzeniania ARGs w procesie AD poprzez HGT. Obfitość genów *intI*1 i *intI*2 może zatem służyć jako wskaźnik procesów HGT. W niniejszych badaniach koncentracja kopii genów *intI*1 i *intI*2 w większości próbek pofermentu z dodatkiem antybiotyków wzrosła o jeden rząd w porównaniu z próbkami kontrolnymi. Podobna liczba kopii badanych genów integraz w procesie AD może świadczyć o przystosowaniu się mikroorganizmów do stężeń antybiotyków stosowanych na początku procesu AD. Presja selekcyjna wywierana przez antybiotyki może zwiększać liczbę kopii genów integrazy. Antybiotyki mogą indukować AR, a także wzrost i rozprzestrzenianie ARGs na szeroką skalę w środowisku. Jednak to właśnie HGT pozostaje głównym mechanizmem rozprzestrzeniania ARGs w środowisku, co stwarza ogromne zagrożenie dla zdrowia ludzi i zwierząt z powodu AR, a także ogranicza możliwości jego kontrolowania **(Załączniki 2 oraz 3)**.

Wśród odnotowanych ARGs liczba kopii genu *erm*F silnie korelowała z liczbą kopii genów *MST* ($R= 0,8153$, $p< 0,05$), *mcrA* i *MSC* ($R= 0,8158$, $p< 0,05$). Wystąpiła również wysoka korelacja między liczbą genów *sul*1 i integrazy, przy czym korelacja z *intI*1 i z *intI*2 sięgała $R= 0,8184$ ($p< 0,05$). Wysoka, istotna statystycznie korelacja między liczbą kopii badanych genów może sugerować, że mikroorganizmy metanogenne obecne w pofermencie mogły być lekooporne, a presja selekcyjna wywierana przez antybiotyki spowodowała ich wzrost i rozprzestrzenianie AR w procesie AD gnojowicy bydlęcej. Geny *intI*1 i *intI*2

wykazywały statystycznie istotne ($p< 0,05$) dodatnie korelacje z większością analizowanych ARGs (R = od 0,60 do 0,82), co sugeruje istotną rolę tych genów w HGT. Zwiększone koncentracje antybiotyków w procesie AD mogą zmniejszać różnorodność biologiczną drobnoustrojów zaangażowanych w ten proces **(Załączniki 2 oraz 3)**.

W drugim etapie badań prowadzonych w celu weryfikacji III hipotezy badawczej postawionej w niniejszej rozprawie doktorskiej badano długoterminowy, synergistyczny efekt środków przeciwdrobnoustrojowych na rozpowszechnienie ARGs w procesie AD i pofermencie na bazie gnojowicy bydlęcej. Gnojowicę bydlęcą poddano AD w komorach fermentacyjnych o pojemności 2 L, pracujących w warunkach dynamicznych, półciągłych. Komorę procesową zasilano substratem zawierającym mieszaninę AMO, ENRO i MET. W kontrolnym bioreaktorze fermentowano biomasę bez antybiotyków. AD przeprowadzono w bioreaktorach procesowych w siedmiu seriach doświadczalnych, które różniły się stężeniami antybiotyków dodawanych do biomasy. Stężenia farmaceutyków stopniowo zwiększano w kolejnych seriach doświadczalnych. Środki przeciwdrobnoustrojowe znacząco wpłynęły na całkowitą obfitość ARGs w pofermencie. Łącznie 401 podtypów ARGs należących do 17 typów ARGs, które kodują oporność na bleomycynę, kasugamycynę, trimetoprim, polimyksyny, fosfomycyny, chinolony, ryfamycynę, fosmidomycynę, chloramfenikol, beta-laktamy, sulfonamidy, wankomycynę, bacytracynę, aminoglikozydy, makrolidy-linkozamidy-streptograminy (MLS) i tetracykliny, a także geny odpowiedzialne za oporność wielolekową zidentyfikowano w produktach pofermentacyjnych na bazie gnojowicy bydlęcej suplementowanej mieszaniną AMO, ENRO oraz MET w zmiennych, wzrastających stężeniach **(Załącznik 4)**.

Najbardziej rozpowszechnionymi podtypami ARGs kodującymi oporność na leki należące do grupy MLS były *erm*F, *lnu*B, *lsa*, *mac*A, *mac*B i *mef*A. Pod koniec 417-dniowego eksperymentu zaobserwowano niewielki wzrost liczebności kopii genu *erm*F. Gen *erm*F koduje specyficzne dla adeniny N-6-metylotransferazy 23S rRNA, które metylują bakteryjny 23S *r*RNA. Metylacja zapobiega wiązaniu się leków należących do grupy MLS z rybosomami bakteryjnymi i w konsekwencji koduje oporność na tę grupę antybiotyków. Gen *erm*F jest jednym z najbardziej rozpowszechnionych ARGs nadających oporność na antybiotyki MLS, a także jednym z głównych nabytych genów oporności u bakterii. Wzrost liczebności *erm*F w pofermencie może przyczynić się do rozprzestrzeniania innych ARGs w środowisku wskutek HGT, ponieważ oprócz genu *erm*F, geny *tet*X1 i *tet*X2 zostały również

zidentyfikowane na koniugacyjnym transpozonie CTnDOT. Dominującymi genami oporności na tetracyklinę były *tet*32, *tet*36, *tet*44, *tet*M, *tet*O, *tet*P, *tet*Q, *tet*T, *tet*W, *tet*X2 i *tetracycline_resistance_protein*. Zidentyfikowane w niniejszym badaniu podtypy ARGs kodują dwa mechanizmy swoistej oporności na tetracykliny. Pierwszy mechanizm kodują geny *tet*32, *tet*36, *tet* 44, *tet*M, *tet*O, *tet*P, *tet*Q, *tet*T i *tet*G, które są połączone z rybosomalnymi białkami ochronnymi (RPP), podczas gdy drugi mechanizm oporności na tetracykliny tj., wydzielanie enzymu rozkładającego tetracyklinę koduje gen *tet*X2. Mechanizm RPP jest wszechobecny w środowisku, co wyjaśnia obecność zidentyfikowanych genów oporności na tetracykliny w próbkach analizowanych w niniejszych badaniach. Wielu badaczy donosi, że gnojowica bydlęca jest rezerwuarem ARGs kodujących RPP [15][16]. Dlatego proces AD tego substratu przyczyniać się może do rozprzestrzeniania genów *tet* w biomasie fermentowanej w bioreaktorach, a następnie wskutek nawożenia pofermentem na polach uprawnych. Powyższe stwierdzenia potwierdzają również dane literaturowe [17][18][19] wskazujące na liczne geny oporności na tetracykliny w próbkach wierzchniej warstwy gleby pobranych z pól uprawnych **(Załącznik 4)**.

W pofermencie pobranym z bioreaktorów z wsadem eksponowanym na mieszaninę leków w zmiennych, narastających stężeniach, najbardziej rozpowszechnionymi i licznymi genami oporności na aminoglikozydy były *aad*(6), *aad*A, *aad*E, *ant(9)-I*, *aph(3''')-III*, *aph(3'')-I* i *aph(6)-I*. Zidentyfikowane geny kodują *O*-nukleotydylotransferazy, tj. enzymy modyfikujące aminoglikozydy [20]. Ten enzymatyczny proces modyfikacji jest jednym z najczęstszych mechanizmów AR w środowisku. Postępujący AR i rozprzestrzenianie ARGs zmniejszają skuteczność aminoglikozydów. W doniesieniach literaturowych [21][22][23] w procesie fermentacji mezofilowej ARGs na glikozydy były jednymi z 4 najliczniej obecnych, natomiast w procesie prowadzonym w warunkach termofilowych odnotowano wzrost ARGs na aminoglikozydy, co stwarza ogromne zagrożenie rozpowszechniania tych ARGs na szeroką skalę i prawdopodobieństwo ich nieskuteczności w przypadku szczepów klinicznych, gdyż leki te często stosowane są w farmakoterapii szpitalnej **(Załącznik 4)**.

Najpowszechniej występującymi genami oporności wielolekowej były *Tol*C, *ade*J, *ade*K, *mex*B, *mex*T, *multidrug_ABC_transporter*, *multidrug_transporter* i *opr*M. Gen *ade*J jest odpowiedzialny za rozpoznawanie leków i wytwarzanie protonowej siły napędowej, która zapewnia energię komórkową wymaganą do transportu leku [24]. *Tol*C należy do rodziny transporterów oporności (RND) i nadaje oporność na chloramfenikol, aminoglikozydy,

makrolidy, akryflawinę, doksorubicynę, erytromycynę, puromycynę i beta-laktamy. Dwa z wykrytych genów, *mex*B i *mex*T, są najczęściej identyfikowane w izolatach klinicznych, co daje poważny powód do niepokoju. Obecność tych genów w pofermencie z gnojowicy bydlęcej wskazuje, że *mex*B i *mex*T mogą przedostawać się ze środowiska szpitalnego do środowiska naturalnego i rolnictwa **(Załącznik 4)**.

Co ciekawe, pomimo tego, że jednym z suplementowanych do wsadu bioreaktorów AD antybiotyków był AMO należący do klasy beta-laktamów, geny *cfx*A i bla_{OXA} niosące oporność na tę klasę leków były jedynymi genami oporności na beta-laktamy w próbkach pofermentu. Beta-laktamy mają niestabilny pierścień i nie są bardzo trwałe w środowisku, co mogłoby tłumaczyć niską liczebność genów kodujących oporność na tę klasę antybiotyków **(Załącznik 4)**.

Łączna liczba sekwencji charakterystycznych dla zidentyfikowanych ARGs była wyższa w próbkach eksperymentalnych zawierających mieszaninę antybiotyków, jednakże liczne sekwencje ARGs zidentyfikowano również w próbkach kontrolnych. W badanych próbkach pofermentu pobranych z bioreaktora, w którym wsad eksponowano na mieszaninę leków obfitość genów oporności na beta-laktamy została zmniejszona o 50%. Jednak częstość występowania genów kodujących oporność na tetracykliny, aminoglikozydy i leki MLS znacznie wzrosła w badanych próbkach pofermentu pobranych z bioreaktorów z biomasą eksponowaną na mieszaninę leków. Obfitość ARGs w próbkach doświadczalnych (z dodatkiem AMO, ENRO i MET) oraz kontrolnych jest niepokojąca, ponieważ może wskazywać, że rozprzestrzenianie ARGs w gnojowicy bydlęcej jest procesem, który rozpoczął się przed AD. Jednak eksperymentalne i kontrolne próbki pofermentu z gnojowicy różniły się znacznie profilami ARGs, co wskazuje, że ARGs są przenoszone różnymi drogami w obecności antybiotyków. Analizowane próbki różniły się częstością występowania podtypów ARGs. Zarówno liczebność, jak i różnorodność ARGs były znacznie wyższe w próbkach eksperymentalnych pobranych z wsadu bioreaktorów, który eksponowano na mieszaninę antybiotyków, w porównaniu do próbek kontrolnych. W badaniu oceniającym wpływ leków na AD inni autorzy [25][26][27] również donoszą o wzroście liczebności poszczególnych ARGs podczas AD i wnioskują, iż zbiorowiska drobnoustrojów i ruchome elementy genetyczne odgrywają kluczową rolę w przenoszeniu ARGs i wzroście ich liczebności. Szczególnie niepokojący jest fakt, że ARGs mogą docierać do innych patogennych mikroorganizmów poprzez HGT, co może zagrozić skuteczności dostępnych

antybiotyków, zwiększając w ten sposób częstość występowania zakażeń wywołanych przez patogeny, które nie reagują na farmakoterapię **(Załącznik 4)**.

Zaobserwowano silną i istotną korelację (p<0,05*;* ANOVA) między liczebnością genów kodujących oporność na tetracykliny i aminoglikozydy (R = 0,77). Liczba genów oporności na tetracyklinę była istotnie skorelowana z liczebnością *Fermentiomonas caenicola* (R = 0,75); *Proteiniclasticum ruminis* (R = 0,73) i *Methanosarcina thermophila* (R = 0,75). Te korelacje sugerują, że powyższe mikroorganizmy mogą być potencjalnymi nosicielami genów oporności na tetracykliny. Stwierdzano silną i istotną korelację między liczebnością *Proteiniclasticum ruminis* i *Methanosarcina thermophila* (R = 0,87). Zaobserwowano również silne wzajemne korelacje między liczebnością *Erysipelothrix* i *Methanomassiliicocales archaeon RumEnM2* (R = 0,78) oraz *Methanothrix soehngenii* i *Syntrophus aciditrophicus* (R = 0,71). Obecność wzajemnych korelacji między mikroorganizmami wskazuje, że współpracują one i wchodzą w interakcje syntroficzne podczas procesu AD, ponieważ hydroliza, kwasogeneza i metanogeneza są kluczowymi fazami procesu AD, który to proces obejmuje zróżnicowaną grupę społeczności drobnoustrojów **(Załącznik 4)**.

Wyniki badań przedstawione w niniejszym opracowaniu wykazały, że suplementacja biomasy rosnącymi stężeniami AMO, ENRO i MET w mieszaninie wywierała przejściowy wpływ na produkcję CH_4. Na podstawie otrzymanych wyników analizy metagenomicznej przeprowadzonej w ramach niniejszej rozprawy doktorskiej wywnioskowano, że długotrwała mezofilna AD gnojowicy bydlęcej w obecności wszystkich trzech badanych antybiotyków może modyfikować profil opornościowy i zwiększać względną obfitość poszczególnych ARGs w pofermencie. Istnieje jednak również ryzyko rozprzestrzeniania ARGs w środowisku, w którym geny mogą być przenoszone z pofermentu na pola uprawne, do wód powierzchniowych, do gleby, części roślin i dalej do łańcucha pokarmowego zwierząt gospodarskich i ludzi. Obfitość genów nadających odporność na tetracykliny, aminoglikozydy i leki MLS znacznie wzrosła w próbkach pofermentu. Uzyskane wyniki wskazują, że przy obecnym poziomie rozwoju technologicznego ARGs nie są usuwane podczas procesu AD, co może upośledzać funkcje mikroorganizmów glebowych na polach nawożonych pofermentem. Powyższe stwarza bezpośrednie zagrożenie zdrowotne zarówno dla ludzi, jak i zwierząt gospodarskich żywiących się roślinami pastewnymi uprawianymi na polach nawożonych pofermentem. Dlatego też niezbędne są skuteczne strategie zarządzania procesem AD

i powstającą po procesie masą pofermentacyjną, aby zmniejszyć obfitość ARGs w przefermentowanej biomasie i ograniczyć ich dalsze rozprzestrzenianie w środowisku **(Załącznik 4)**.

Weryfikacja IV hipotezy badawczej

Pochodzenie substratu i skład materii organicznej w substracie AD wpływa na obecność środków przeciwdrobnoustrojowych oraz ARGs w masie pofermentacyjnej uzyskiwanej po procesie AD.

Czwarta hipoteza badawcza została zweryfikowana w badaniach przedstawionych w następujących artykułach naukowych:

Załącznik 5. **Wolak I,** Bajkacz S, Harnisz M, Stando K, Męcik M, Korzeniewska E. Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment. *International Journal of Environmental Research and Public Health*. 2023; 20(3): 2672. https://doi.org/10.3390/ijerph20032672.

W tym etapie badań prowadzonych w celu weryfikacji IV hipotezy badawczej postawionej w niniejszej rozprawie doktorskiej porównano liczebność genów ARGs kodujących oporność na beta-laktamy (*bla*$_{\text{TEM}}$, *bla*$_{\text{OXA}}$, *cfx*A), tetracykliny (*tet*A, *tet*M), sulfonamidy (*sul*1, *sul*2), fluorochinolony (*aac6'-Ib-cr*, *qep*A) i antybiotyki z grupy MLS (*erm*F) oraz geny integrazy (*intI*1 i *intI*2) w pofermencie pochodzącym z procesu AD prowadzonego w warunkach in vivo, tj. w biogazowniach. W powyższych próbkach porównano także stężenia środków przeciwdrobnoustrojowych, a także skład mikrobiomu zaangażowanego w procesy AD różnorodnych substratów. Pobrane próbki pofermentu pochodziły z trzech różnych biogazowni rolniczych zlokalizowanych w Polsce, pracujących w tych samych warunkach temperaturowych tj. mezofilnych. Co więcej, wybrane do badań próbki różniły się składem substratowym w celu porównania różnic w obecności środków przeciwdrobnoustrojowych, liczebności i różnorodności ARGs oraz bioróżnorodności mikrobiomu w masie pofermentacyjnej i określenia, czy wartości te zależne są od ich składu substratowego. Badaniom poddano próbki pofermentu pochodzącego z procesu AD prowadzonego in vivo, w biogazowniach rolniczych na bazie osadu ściekowego, mieszanki gnojowicy bydlęcej i kiszonki z kukurydzy oraz mieszaniny flotatu poubojowego, kiszonki kukurydzy, masy cukierniczej oraz ścieru ziemniaczanego.

Geny oporności na antybiotyki określono ilościowo z wykorzystaniem metody qPCR w aparacie LightCycler®system (Roche Diagnostics, Mannheim, Niemcy). W próbkach pofermentu identyfikowano i określano ilościowo 11 ARGs nadających oporność na pięć klas antybiotyków, jak również geny integrazy klasy 1 oraz 2, które promują rozprzestrzenianie się AR poprzez HGT z udziałem mobilnych elementów genetycznych takich jak plazmidy czy integrony. Geny oporności na antybiotyki wykryto we wszystkich próbkach pofermentu w stężeniach od 1,16 x 10^3 do 2,25 x 10^6 kopii g_D^{-1} pofermentu. Masa pofermentacyjna z substratów pochodzenia roślinnego z dodatkiem flotatu poubojowego charakteryzowała się wysokimi stężeniami ARGs (w zakresie od 5,73 × 10^2 kopii g_D^{-1} *cfx*A do 2,98 × 10^9 kopii g_D^{-1} *sul*1). Wyniki potwierdziły, że poferment jest rezerwuarem ARGs (od 5,73 × 10^2 do 8,89 × 10^{10} kopii g_D^{-1}). Liczebność ARGs była najwyższa w pofermencie z osadów ściekowych oraz pofermencie pochodzącym z mieszanki gnojowicy bydlęcej i kiszonki z kukurydzy z procesu AD prowadzonego in vivo w biogazowniach rolniczych. Niezależnie od składu substratowego próbek masy pofermentacyjnej odnotowano wysokie stężenia genów integrazy (10^5 do 10^7 kopii g_D^{-1}) co wskazuje, że mobilne elementy genetyczne mogą być zaangażowane w rozprzestrzenianie oporności na antybiotyki bez względu na rodzaj i pochodzenie przetwarzanego w AD substratu. ARGs w substratach przetwarzanych podczas AD w biogazowniach rolniczych mogą dostać się do pofermentu, który jest używany jako nawóz. Środki przeciwdrobnoustrojowe i ARGs mogą być zatem przenoszone na grunty rolne, co zwiększa ich stężenie w środowisku. Wyniki badań przeprowadzonych w ramach niniejszej dysertacji sugerują, że ryzyko zanieczyszczenia gleby ARGs jest wysokie w gospodarstwach, w których poferment jest używany jako nawóz **(Załącznik 5)**.

Środki przeciwdrobnoustrojowe najliczniej odnotowano w próbkach masy pofermentacyjnej na bazie osadów ściekowych. Antybiotyki są powszechnie spożywane na całym świecie i nie są w pełni metabolizowane w organizmach ludzkich i zwierzęcych, dlatego w osadach ściekowych obecne są wysokie stężenia tych farmaceutyków. Ponadto, zgodnie z literaturą, w wybranych porach roku nasila się zażywanie antybiotyków, a także związany z tym wzrost ich ilości wprowadzanych do oczyszczalni wraz ze ściekami [28]. Dlatego też znaczne ilości środków przeciwdrobnoustrojowych, które nie ulegają biodegradacji w procesie oczyszczania ścieków, mogą być przenoszone do osadów ściekowych, które są wykorzystywane jako substraty poddawane fermentacji metanowej w bioreaktorach AD, co tłumaczy ich obecność i wysokie stężenia w badanych próbkach pofermentu [29]. Należy również zauważyć, że farmaceutyki, w tym antybiotyki, są często

nadużywane i stosowane bez wskazówek lekarza, co może znacznie zwiększyć ich stężenie w osadach ściekowych **(Załącznik 5)**.

Podsumowanie i wnioski

Przeprowadzone badania poszerzają wiedzę o procesie AD oraz wpływie środków przeciwdrobnoustrojowych na wydajność tego procesu i produkcję biogazu, mikrobiom zaangażowany w proces i zachodzące w społeczności drobnoustrojów zmiany, a także na występowanie i koncentrację farmaceutyków w procesie i ich wpływ na liczebność oraz rozpowszechnienie ARGs w bioreaktorach AD i szeroko pojętym środowisku. Przeprowadzone w niniejszej rozprawie doktorskiej badania pozwoliły zweryfikować postawione w pracy hipotezy badawcze i wykazać, że:

1. Antybiotyki znacząco wpływały na proces AD, bez względu na sposób ich suplementacji do wsadu bioreaktorów. Farmaceutyki wprowadzane do biomasy fermentacyjnej w bioreaktorach zarówno indywidualnie jak i synergistycznie znacząco wpływały na efektywność produkcji AD i CH_4, zmiany w społeczności drobnoustrojów AD oraz na wzrost i rozpowszechnienie ARGs. Środki przeciwdrobnoustrojowe wykazywały wysoce prawdopodobną toksyczność wobec mikroorganizmów, co bezpośrednio ograniczało produkcję biogazu.

2. Proces AD w obecności antybiotyków stymuluje również wzrost liczby ARGs i ich rozprzestrzenianie się w próbkach pofermentu. Antybiotyki testowane w prezentowanych badaniach działały prawdopodobnie inhibująco nie tylko na bakterie uczestniczące w poszczególnych etapach procesu AD, ale także na drobnoustroje metanogenne, odpowiedzialne bezpośrednio za produkcję CH_4.

3. Antybiotyki niezależnie od sposobu suplementowania do wsadu bioreaktora, zarówno indywidualnie jak i synergistycznie spowodowały zmianę proporcji liczebności bakterii w stosunku do *Archaea* w masie pofermentacyjnej gnojowicy bydlęcej. W celu zmniejszenia toksyczności antybiotyków w komorach fermentacyjnych przetwarzających gnojowicę bydlęcą zalecana jest kofermentacja, czyli biometanizacja gnojowicy bydlęcej z dodatkiem np. kiszonki kukurydzy, który jest lokalnie dostępnym i łatwo biodegradowalnym substratem. Ta metoda może znacznie poprawić stabilność procesu AD w porównaniu z rozkładem pojedynczego substratu. Co więcej, zalecane

jest także minimum kilkutygodniowe kompostowanie substratów AD, w celu większej degradacji środków przeciwdrobnoustrojowych obecnych w biomasie.

4. Zależnie od składu substratowego masy pofermentacyjnej antybiotyki wpłynęły na zmiany w koncentracji antybiotyków oraz ARGs. W masie pofermentacyjnej na bazie gnojowicy bydlęcej i kiszonki kukurydzy oraz w próbkach masy pofermentacyjnej pochodzącej z biogazowni pracującej w oparciu o mieszaninę flotatu poubojowego, kiszonki kukurydzy, masy cukierniczej oraz ścieru ziemniaczanego odnotowano najniższą koncentrację antybiotyków oraz liczne ARGs.

5. Koncentracja antybiotyków była najwyższa w próbkach masy pofermentacyjnej z biogazowni pracującej w oparciu o osad ściekowy. Stężenia leków należących do różnych klas antybiotyków znacznie różniły się pomiędzy próbkami pofermentu pobieranymi w poszczególnych porach na bazie osadów ściekowych. Ponadto, frakcje płynne i stałe pofermentu także różniły się obecnością i stężeniem analizowanych leków. Antybiotyki identyfikowano częściej we frakcji stałej próbek pofermentu. Obecność leków w pofermencie z osadów ściekowych może być przypisywana zanieczyszczeniu substratu przed procesem AD.

Wyniki przeprowadzonych badań dostarczają informacji na temat wpływu określonych stężeń antybiotyków na wydajność procesu AD i produkcję biogazu. Uzyskane wyniki badań w ramach prezentowanej dysertacji określają także dynamikę zmian ARGs w bioreaktorach AD z dodatkiem wybranych antybiotyków i wskazują implikacje dla środowiska jakie mogą się w przyszłości pojawić, na skutek braku kontroli nad procesem AD i braku optymalizacji procesu. Badania dostarczają także odpowiedzi jakie dokładnie drobnoustroje uczestniczą w procesie fermentacji metanowej oraz czy instalacje takie jak biogazownie stanowią wektor rozprzestrzeniania ARGs. Wdrożenie fermentacji beztlenowej w pełnowymiarowych biogazowniach zlokalizowanych w dużych gospodarstwach rolnych może być rozwiązaniem przyjaznym dla środowiska, zapewniającym bezpieczeństwo energetyczne gospodarstwa, pozwalającym na obniżenie kosztów transportu i zarządzania. Jednak warunkiem osiągnięcia tych korzyści jest racjonalne gospodarowanie antybiotykami i ograniczenie ich podaży zwierzętom.

Podsumowując, konieczne jest monitorowanie procesu AD w biogazowniach, jak również pofermentu otrzymanego z tego procesu oraz środowiska, do którego taki poferment jest wprowadzany. Niniejsza rozprawa doktorska wzbogaca literaturową bazę danych na

temat procesu AD, a także wskazuje praktyczne wskazówki które mogą wspomóc pracę technologów w biogazowniach oraz projektowanie i uruchamianie procesu AD w pełnej skali.

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Załącznik 1

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Intensification of biogas production using various technologies: A review

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Summary

Anaerobic digestion (AD) is an organic matter conversion technology which offers a wide range of options for production of biogas from organic biomass, providing an excellent opportunity to convert abundant bioresources into safe, eco-friendly, renewable energy. Important factors in the process of AD are the biodiversity of microorganisms, chemical load of oxygen demand, and content of water and total solids. A challenge for the future is to find technologies that will maximally enhance biogas production and to find pathways for biogas to supersede well-established technologies and practices in the contemporary heavily fossil fuel-based energy system. Current studies on technologies of biogas production indicate a number of possibilities of using appropriate biological and physicochemical additives, like added enzymes or fruit pomace, as well as immobilizing microorganisms on biofilters. Anaerobic biorefinery is an emerging concept that generates not only bioenergy but also high-value biochemical products from the same feedstock. This study is a review of articles describing the intensification of biogas production by using various technologies.

KEYWORDS

anaerobic digestion, biogas, eco-friendly fuel, methane fermentation, organic biomass

1 | INTRODUCTION

For years, people have been increasingly aware of the need to protect the environment, reduce quantities of waste, and limit the consumption of fossil fuels.[1-5] The high level of carbon dioxide emission to the environment and its consequence such as global warming call for the implementation of alternative sources of energy.[3,6-8] Over the recent years, the production of waste, including municipal solid waste, has increased. Despite the enormous global potential of biogas production, currently widespread solutions such as direct application of livestock manure on fields and open-fire combustion of organic biomass, for example, straw, grass, and crop residues and agricultural waste, are contributing to significant emission to the atmosphere.[7,9] In addition, industrial production entails the consumption of large amounts of fuels used to power industrial plants and facilities. In order to diminish the dependence on fossil fuels and ensure the state's energy safety, extensive research has been conducted for many years pertaining to the production of biogas in the methane fermentation process under anaerobic digestion (AD) conditions.[10-13] Methane fermentation is an organic matter conversion technology, which offers a wide range of options for production of biogas, a safe and eco-friendly fuel. This process, performed by a microbial consortium, depends on a number of factors such as pH, temperature, hydraulic

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retention time (HRT), C/N ratio, and so on.[8] Methane fermentation is used to recycle harmful liquid or semi-liquid animal waste, sewage sludge, and various types of organic solid fraction. AD is a process that occurs naturally in the environment, but which for industrial purposes is conducted in closed chambers under controlled conditions. Generation of eco-friendly energy which requires low investment inputs is a key to the improvement of people's quality of life, especially in developing countries where agriculture and industries are dominant.[12,14] A challenge for the future is to find pathways for biogas to supersede well-established technologies and practices in the contemporary heavily fossil fuel-based energy system.[11]

During methane fermentation, products of metabolism of some microorganisms become substrates for others, and mutual interactions between microorganisms can be both direct and indirect in character. Microorganisms degrade organic substrate owing to specific enzymes which catalyze biochemical reactions of organic matter decomposition.[15] During the process of methane fermentation, both exogenous and endogenous enzymes degrading specific substrates are needed to decompose molecules and colloid substrates with molecules of different sizes.[16-18] An essential factor in the process of methane fermentation is the biodiversity of microorganisms because each type of microbes has a different set of enzymes and different capacity of degrading organic matter, depending on the substrate supplied for digestion.[19] Both the source of a bacterial inoculum and the content of solid particles in the substrate are responsible for the rapid occurrence and multiplication of various populations of microorganisms in the digestion chamber, which in consequence leads to more rapid activation of the digestion process, as well as more efficient reduction of the chemical load of oxygen demand (COD), and eventually to greater intensification of biogas production.[20-22] Another important parameter in AD is the content of water in the substrate. The process is divided into three types with regard to the water content. Thus, "wet," "semi-dry," or "dry" substrates can be submitted to methane digestion. A wet methane fermentation technology is employed in wastewater treatment plants (WWTPs) due to the high content of water in sewage slurry, where the share of solids is less than 10%. In municipal waste recycling plants, where the content of solids in waste is between 10% and 20%, the applied technologies belong to semi-dry AD. However, when the solids content exceeds 20%, a dry digestion technology is implemented.[23,24] The literature recommends semi-dry or dry technologies owing to lower demand for water during the process and a smaller size of the reactor's chamber, which lowers the costs of production. Abbassi-Guendouz et al[1] determined that the methanogenic activity of microorganisms strongly depends on the total solids (TS) content, and a higher production output of methane can be achieved at a higher content of water in biomass. The actual average amount of generated methane decreased from 141 to 129 mL/1 g TS when the content of TS increased from 10% to 25%. Vergara-Fernandez et al[24] observed a similar relationship in their experiments. They obtained more methane at a shorter digestion time when the content of dry matter equaled 20%, but noted that methane productivity decreased at TS equal 30%. Over the past few years, the applicability of the AD method has risen considerably, a wide range of substrates used in this process has been developed, and this has given rise to the search for improved research methods aiming at the optimization of this process, to make it economically efficient and environmentally safe. Scientists have been paying particular attention to the course of this process, trying to optimize it depending on the applied substrate.[25]

Biogas produced from organic waste is counted among the most promising renewable energy resources.[26-29] Methane and carbon dioxide, which are the main components of biogas, add to the greenhouse gas effect if released from landfills, WWTPs or farms. This is the reason why it is essential that biogas be obtained under controlled conditions and used to generate thermal energy and electricity.[30,31] This article contains a review of literature published recently (Figure S1), and an overview of various technologies employed in biogas production. This study describes both biological and physicochemical factors that can influence the process of biogas production. The purpose of this review article is to present different methods and the factors that stimulate the process of biomethanization and intensification of biogas production.

2 | AD AS A REVOLUTIONARY PROCESS IN ENERGY GENERATION

Biogas is a cell gas which appears in the process of methane fermentation owing to the activity of methanogenic microorganisms under anaerobic conditions. The biological process during methane AD takes place in a reactor's chamber, which is tightly sealed, insulated, and has no oxygen inflow. This creates perfect conditions for the development of methanogenic microorganisms, which are capable of decomposing organic matter by converting it into biogas.[1,2,6] Biogas is a mixture composed of mostly methane and carbon dioxide, but it also contains smaller concentrations of hydrogen, nitrogen, ammonia, oxygen, hydrogen sulfide, carbon oxide, and water vapor. The percentages of particular chemical components depend on the type

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of substrate submitted to digestion and on the parameters of the technological process (Table 1).[32-35]

The most common measures applied to specify concentrations of biogas in waste or sewage are biological oxygen demand and COD, expressed in mg O_2/L, and the content of volatile solids (VS) expressed in mg/L or %.[36] For years, researchers have undertaken efforts to employ various raw materials and microorganisms in this process, looking for both substrates and microbes which would be distinguished by the highest, economically speaking, efficiency of methane production compared with fossil fuels (Table 2).[37,38]

The productivity of AD is dependent on the investment outlays, maintenance costs of a biogas production plant, and the optimal volume of methane produced. The methane production maximum efficiency is particularly important in the methane fermentation of plants from dedicated energy crop plantations. The use of animal waste or biomass, which are by-products of technological processes in industries or agriculture, does not entail costs incurred by the production of these materials. The costs of producing energy crops, on the other hand, must be covered by the amount of methane obtained from the methane fermentation process. Thus, the efficiency of methane production per km^2 must be maximized in the methane fermentation of energy crops.[37-40] The quality of energy crops used in the AD process is controlled directly on field. Crop plantators determine the plant content of substances needed for methane generation, which depends, for example, on the plant development stage.[37]

The chemical composition of substrates and their degree of degradability have a significant effect on biogas production (Table 3).[41] Biogas generation from different substrates in the process of methane fermentation depends significantly on adequately set and optimized physicochemical parameters such as hydraulic time of retention, appropriately chosen for specific substrates, and the optimal load of organic biomass fed to the reactor's chamber. Also, the temperature inside the digester strongly affects the biogas production process.[42] When designing the course of a methane fermentation process, it is necessary to eliminate inhibitors like antibiotics or plant protection chemicals, which can substantially decrease the efficiency of the process.[43,44]

TABLE 1 Methane yield from different algae strains[32,33]

Algae strains	Methane yield (dm^3/kg total solids)
Laminaria sp.	270
Gracilaria sp.	340
Sargassum sp.	155
Laminaria digitata	500
Ulva sp.	200

TABLE 2 Energy efficiency of 1 m^3 of biogas of the calorific value equal 26 MJ/m^3 compared with other fuels, according to Arbon[37]

The selected fuel equivalent	The calorific value of fuel (MJ/m^3)	The volume of fuel (m^3 or kg)
Natural gas	33.5	0.77
Carbon	23.4	1.1
Firewood	13.3	2

3 | STAGES OF AD

Methane fermentation consists of four biological and chemical stages, which engage particular groups of mutually interacting microorganisms (Figure 1).[45,46] AD is a slow process, and microorganisms need at least 3 weeks to adapt to a new environment whenever a substrate, temperature, or other environmental factors change.[47] In AD process, microbial interactions are complex, and a lack of equilibrium between consortia of microorganisms responsible for different stages in methane production affects the rate of the reaction, in addition to which it can induce the accumulation of inhibitory substances.[48] It is necessary to achieve a symbiotic relationship between microorganisms at all stages and between acetogenic microorganisms

TABLE 3 Amount of methane from 1×10^3 kg of selected substrates and estimated percentage (%) of methane in biogas depending on the substrate used to generate methane

Organic substrates	The volume of methane from 1×10^3 kg of substrates (m^3/Gg)	Methane content in biogas (%)	References
Municipal waste	396	50–60	6
Sludge from wastewater treatment plants	310	60-75	20
Wastes from livestock	32	60–75	3
Plant waste from the agro-food industry	85	68	6

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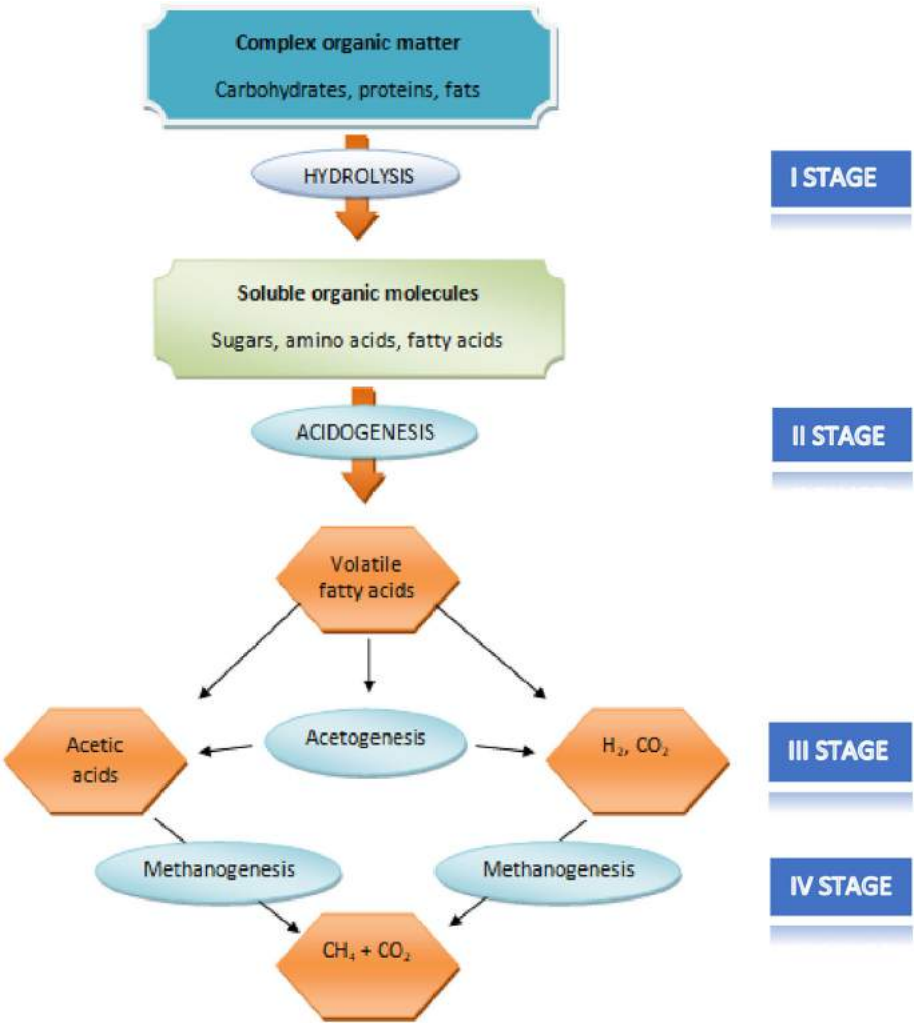


FIGURE 1 Stages and products of anaerobic digestion [Colour figure can be viewed at wileyonlinelibrary.com]

producing hydrogen and methanogens consuming hydrogen at the final, key stage of the production.[49]

3.1 | Hydrolysis

The first stage (I STAGE, Figure 1) comprises hydrolysis, where insoluble and high-molecular compounds such as proteins, lipids, and carbohydrates, in a process mediated by saprophytic bacteria with the participation of extracellular enzymes, are converted to simple, low-molecular organic compounds, mostly volatile fatty acids (VFAs) (formic, propionic and butyric acids) as well as alcohols, aldehydes, CO_2, and H_2.[26,47,48] The products of decomposition are then used as a source of energy and carbon for microorganisms found in the decomposed organic matter, which mediate the subsequent stage, that is, acidogenesis.[49] This stage is participated by relatively anaerobic bacteria *Micrococcus*, strictly anaerobic *Fusobacterium*, *Clostridia*, *Bacteroides*, *Butyrivibrio*, and facultative bacteria *Streptococcus* and *Selenomonas*, which secrete extracellular hydrolyzing enzymes, including cellulase, cellobiose, xylanase, amylase, protease, lipase, and so on, essential to conduct this process.[50] During hydrolysis, hydrolytic bacteria colonize the surface of solid waste molecules and release enzymes on the surface of the material undergoing decomposition, which enable the degradation of organic molecules under the influence of bacteria.[51] Organic waste submitted to hydrolysis is a rich source of energy required to produce biogas, as the main components of such waste are lignins, hemicellulose, and cellulose.[51-53] The main substrates of this type are the biomass from the agricultural, wood, and food processing industries, which is both readily available and abundant in the environment, in addition to which it is one of the least expensive sources of carbohydrates.[42] In order to improve the hydrolysis of larger molecules of organic matter, a pretreatment stage can be implemented. It can employ technologies divided into three groups: physical methods (thermal pretreatment, sonification, manipulation of physicochemical parameters), chemical ones (acid, alkaline, or enzymatic processing), and ensiling.[54-57] Acceleration of

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the first stage facilitates the course of the subsequent stage and has a significant effect on the efficiency of the process and the amount of biogas yield. Slow degradation of substrates submitted to AD, particularly substrates with a high content of solids in the suspension, may even lead to the complete inhibition of the process.[50] This explains why it is so important to employ an appropriate method to accelerate hydrolysis and improve the overall efficiency of an AD process. Acidification is the stage where micromolecules produced during hydrolysis can be taken up by acidogenic microorganisms and then transformed into VFA. Higher production of VFA means more acetic acid, which is a direct substrate for the metabolism of methanogens, and in consequence the production of methane becomes more efficient.[51]

3.2 | Acidogenesis

During acidogenesis (II STAGE, Figure 1), acidogenic bacteria convert products of hydrolysis and water-soluble compounds into alcohols such as methanol or ethanol, into aldehydes, carbon dioxide, and hydrogen short-chain organic acids, for example, formic, propionate, butyric, pentane, and acetic acid.[53] Compounds like long-chain fatty acids or amino acids, produced through hydrolysis, serve as a substrate for fermentation microorganisms, which include *Streptococcus*, *Lactobacillus*, *Bacillus*, *Escherichia coli*, and *Salmonella*.[58,59] During the decomposition of proteins, the resultant amino acids and peptides can be harvested for energy by anaerobic microorganisms. Depending on microbial populations present at the stage of acidogenesis, the process can proceed in two directions, and therefore be divided into hydrogenation and dehydrogenation, which lie at the foundation of any technology of carbon conversion into liquid fuels. Hydration and dehydration are reactions of adding or distributing hydrogen on the carbon skeleton. Most reactions that occur in the industry are carried out in the presence of catalysts. Nowadays, catalysis is one of the most important technologies applied in environmental protection. It is particularly helpful in preventing the emission of toxic substances, including methane released from landfills or carbon dioxide to the environment. The basic pathway of biochemical transformations involves such compounds as acetates, carbon dioxide, hydrogen and other compounds, which play less important roles. On the pathway of these transformations, methanogenic microorganisms can use the newly synthesized products as substrates or as a source of energy.[58,59] New products must be first converted by bacteria producing hydrogen at the stage of acidogenesis, and the subsequent products can be used directly by methanogenic strains. Products that appear during acidogenesis are ammonia and hydrogen sulfide, which are responsible for the appearance of an intensive and unpleasant odor at this stage of the process.[60] Acetic acid is a key intermediate product, which significantly affects the efficiency of methanogenesis, hence increased generation of acetic acid is essential in the process of methane fermentation.[61] Moreover, treatments that lead to the improvement of acidification of hardly degradable substrates are beneficial to the metabolic reactions of methanogenic microorganisms. Results presented in numerous publications confirm that production of acetic acid at the stage of fermentation is the main driver of general methane production.[62]

3.3 | Acetogenesis

During acetogenesis (III STAGE, Figure 1), acetic bacteria of the genera *Syntrophomonas* and *Syntrophobacter* convert the products, that is, propionate to acetate, glucose to acetate and ethanol to acetate, which can then be used by methanogenic microorganisms as a substrate. The microorganisms of the genus *Methanobacterium* decompose pentane acid to propionic acid, while *Methanobacterium propionicum* decompose propionic acid to acetic acid—the final product of acetogenesis. During this process, hydrogen molecules are released, having a toxic effect on the microorganisms which carry out this stage, and this explains the importance of the symbiosis of acetic bacteria with autotrophic methane microorganisms, as the latter can use hydrogen as a substrate. Nearly 70% of methane, which is the main component of biogas, are produced in the course of acetogenesis, during the reduction of acetates, and consequently acetates are considered to be the key intermediate product of methane fermentation.[2,15]

3.4 | Methanogenesis

In the fourth stage called methanogenesis (IV STAGE, Figure 1), methanogenic microorganisms convert hydrogen, carbon, and acetic acid dioxide to methane. This stage is mediated by *Methanosarcina* spp., *Methanothrix* spp., *Methanobacterium*, *Methanococcus* as well as numerous other methanogenic strains.[61-65] Acetic acid is direct substrate for methanogenic microorganisms, and therefore the improved conversion of acetic acid can strengthen this stage of methanogenesis. The implementation of appropriate methods enhancing the AD process is also beneficial in terms of generating the maximum amount of methane.[66-68] Being highly sensitive to pH changes, methanogens prefer a slightly alkaline environment. When the pH falls below 6, methanogenic microorganisms die, which explains why appropriate optimization of the physicochemical

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parameters of the process is so important. The pH of a suspension changes at different stages of AD. At the second stage, that is, acidogenesis, the pH equals approximately 6 and a large amount of CO_2 is released. Over the next 2-3 weeks, the pH increases as VFAs are digested and CH_4 is generated. To ensure a constant influx of CO_2, it is necessary to maintain an appropriate pH level in the reactor's chamber. The digestate is most often buffered if its pH is within 6.5 and 7.5. In this range of pH, microorganisms are highly active, and biodegradation is very effective. However, if the pH falls below 6.5 or increases above 7.5, the environment becomes unfavorable or even hostile to the growth of methanogenic microorganisms. It is therefore crucial to prevent any rapid changes in pH in the reactor's chamber, as this may lead to the lack of balance in populations of methanogens. Methanogenic microorganisms are strict anaerobes and even the smallest amount of oxygen can cause their death. Due to such extreme sensitivity of these microorganisms to the presence of oxygen and changes in the environment, the biochemistry, physiology, and ecology of methanogenic microorganisms still await better recognition. Methanogens live in an environment with a pH range close to neutral. that is, from 6.8 to 7.2.[41] They are classified as chemolithotrophic microorganisms because they are able to use CO_2 as a source of carbon.[68] These microorganisms are a very important group of microbes in the environment, where their capabilities are employed to carry out anaerobic degradation of wastewater and sewage, as a stage in a wastewater treatment process. The digestate generated in the process can be additionally used as natural nutrient-rich fertilizer.[69] Methanogens also attract attention of the pharmaceutical industry because these microorganisms can serve as a valuable source of vitamin B_{12}.[70]

4 | FEEDSTOCKS, TECHNOLOGIES, AND FACTORS DESIGN TO INTENSIFY BIOGAS PRODUCTION

Methane production from organic substrates chiefly depends on their content of substances degradable to CH_4 and CO_2. Composition and biodegradability are crucial for the methane yield obtained from energy crops and animal manure.[8] Crude protein, crude fat, crude fiber, cellulose, hemi-cellulose, starch, and sugar markedly influence methane formation. Biomethane production can also make use of organic substrates such as plant waste from the agricultural sector, for example, maize silage,[2,39] energy crops or plant residue left after harvest, microalgae,[68,70] lignocellulose biomass,[71-75] waste from farm animals, including liquid bovine manure and swine slurry, waste from the industrial sector or from forestry and timber production as well as sewage sludge.[76-79] Large amounts of biogas are generated in solid municipal landfills, where they can be used for controlled biogas collection and biogas conversion to energy. The research results published by Wuebbles and Hayhoe[78] demonstrate that the process of methane production during anaerobic degradation of waste on landfills or sludge in wastewater treatment plants is a source of considerable methane emission to the atmosphere, representing 13% of the annual production for landfills and around 10% for WWTPs. Methane produced in a controlled way at WWTPs or landfills can be collected and converted to energy.[78] While producing biogas, harmful waste and noxious odor are neutralized, and valuable fertilizer is obtained.[80] Good biogas production efficiency is achieved from fermentation of liquid solution of swine slurry or sewage sludge. The results reported by Korzeniewska et al[36] prove the high efficiency of the above substrates. The quantity of biogas produced from liquid bovine manure oscillated around 9.4 mL/1 g of the fed feedstock, while for sewage sludge the resulting amounts were from 6.1 to 9.9 mL/1 g of the supplied substrate. The AD of slurry is also affected by the diet of animals. Amon et al[2] achieved the highest methane productivity from manure obtained from medium-yielding cows fed a well-balanced diet. Refined biogas in the form of biomethane can serve to generate electricity, thermal energy,[8] or to power engine vehicles.[26-28] Harun et al[33] tested experimentally the usefulness of six dominant species of microalgae as alternative substrates in biogas production. These authors showed that the biogas production potential was significantly dependent on the chosen species of microalgae and on the pretreatment of a substrate. Fermentation of green algae *Chlamydomonas reinwardtii* led to the production of 587 mL of biogas per g of VS from 60 mL of fermented sludge, while fermentation of *Scenedesmus obliquus* yielded no more than 287 mL of biogas. In this experiment, the researchers used fresh or dried algal cells as substrates for methane fermentation. The results showed that the pretreatment consisting of drying algae decreased the production of biogas to ca 80%. The content of methane in biogas from microalgae was by 7%-13% higher than in biogas from maize silage. The authors investigated the application of microalgae as a substrate in the production of biogas in a two-step process. In the first step, they analyzed the content of produced hydrogen when green microalgae *C. reinwardtii* were processed. The second step comprised AD of biomass of selected species of microalgae. The results justified the conclusion that the productivity of biogas obtained from algae following the hydrogen production cycle improved by 123% compared to fresh algal biomass processing. The reason could be that such compounds as starch and lipids with high fermentation potential are stored in cells in response to an increased concentration of hydrogen in the bioreactor.[33] Harun et al[32] maintain that microalgae contain hardly any

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lignin and less cellulose; therefore, they ensure good process stability and high conversion efficiency for AD. Roj-Rojewski et al[81] tested the biogas and methane production from wetland plant species harvested in a river. In their experiment, the authors used the aerial biomass of five species: reed canary grass (*Phalaris arundinacea*), common reed (*Phragmites australis*), reed sweet-grass (*Glyceria maxima*), tufted sedge (*Carexelata*), and woolly fruit sedge (*Carex lasiocarpa*). *P. australis* proved to be the most promising energy species.[81] Despite the low methane yield per kg of VS, common reed achieved quite high methane production per area (1.16×10^8 L/kg of VS) owing to its large emergent biomass The research results implicate that biomass of reed rushes can become a substantial source of renewable energy used for biogas generation, which will make it possible to use wetlands for economic purposes while preserving their natural functions and services. These researchers also confirmed that selected species of algae could be good substrates for the production of biogas, and AD should be seriously considered as the final step in future concepts of biorefineries where algae serve as substrates (Table 4).

There are many technological solutions and factors that support the production of biogas and improve it is efficiency (Table 5).

4.1 | Biological technologies

4.1.1 | Addition of cosubstrate

Codigestion in anaerobic fermentation has been widely used to improve biogas production. Organic substances are characterized by different rates of decomposition and amounts of biogas produced through methane digestion.[29] The anaerobic co-digestion (AcoD) can ensure stable pH value by preventing extreme acidification. The pH value of mixed raw materials in AcoD is more stable and easier to maintain within the optimum pH range during the digestion process compared to single-substrate digestion.[87] Bougrier et al[25] improved the efficiency of biogas generation by adding fruit waste such as pressed pulp to slurry. Braun et al[29] conducted an experiment in which the biogas production efficiency was higher when a 30% of the feedstock fed to a bioreactor consisted of cosubstrate. The cited researchers used sewage sludge as a cosubstrate and bovine manure as the basic feedstock. Based on the results, it was concluded that, depending on the composition and amount of a cosubstrate, the intensification of biogas production in digestion chambers could be raised by as much as 80%-400%. It was found out that an addition of 5%-15% of cosubstrate was optimal, as it did not cause any negative changes in the course of AD.[26,42] The researchers suggested that methane fermentation could be carried out on the main substrate (eg, liquid manure) and an appropriately selected cosubstrate, for example, pressed fruit pulp or animal fat, which raised the diversity of microorganisms participating in the digestion processes.[26,29]

4.1.2 | Bioaugmentation

AD is a process whose course largely depends on the presence of microorganisms in systems of reactors.[88-90] To avoid stress affecting microorganisms present in a bioreactor induced by high concentrations of inhibitors (nitrates, heavy metals, sulfates, access of oxygen, hardly biodegradable lignocellulose compounds), a possible solution applied to intensify biogas production is to supplement the substrate fed to a bioreactor with microorganisms tolerant to unfavorable conditions, which can support the efficiency of methane fermentation.[91]

The latest studies[86,92] report that the most popular technologies are the ones which use biological additives

TABLE 4 Potential of biogas yield from selected substrates submitted to AD depending on the dry matter (TS) content

Type of substrate	TS content (%) in 1×10^3 kg substrate	Organic substance content in TS (%)	The yield of biogas production (dm^3/kg TS)	References
Cattle manure	8	86	280	2
Slurry	2	60	223	6
Fruit pomace	45	62	400	6
Municipal waste	60	55	396	6
Silage maize	32	95	600	2
Silage from cereal plants	40	95	520	15
Corn on the cob, shredded	65	98	680	15
Grains of cereals	86	98	700	34

Abbreviations: AD, anaerobic digestion; TS, total solids.

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TABLE 5 Selected technologies design of intensify biogas production

Kind of technology	Stimulators		Biogas yield (mL/g VS)	References
Biological	Addition of cosubstrate	Olive mill waste	251	6, 14, 48
		Fruit and vegetable waste	249-396	
		Fish waste	398	
		Sugar beet waste	353	
	Pretreatments	Addition of enzymes	732-819	1, 43, 82, 83
		Fungi	587	
		Partial composting and silage	513	
Physicochemical	Addition microelements Cu, Zn, Mo, Co		170-370	6, 44
	Addition macroelements N, P, K, Mg, Fe, Ca, Na		292	6
	Addition ashes from waste incineration		193	48
	Addition Fe^0		351	47
	Sewage separation		160	34, 73
	Manipulation of physicochemical parameters		160-550	6
	Immobilization of biofilms/biofilters		353	28
	Chemical pretreatments	Acid pretreatment	184	27, 28, 62
		Alkaline pretreatment	166-202	
		Calcium hydroxide and sodium hydroxide	120-170	
	Oxidative pretreatment	Hydrogen peroxide	247.3-260	6, 27
		Ozone	246	
		Wet oxidation	320	
	Ionic liquids		170.92	84
Mechanical	Pretreatments	Sieving	120-240	28
		Mixing and grinding	148.4-252.8	43
		Ultrasound	177-194	85
		Thermal	504	72, 74, 86

with a bacterial inoculum or with an added cosubstrate, which enlarge the microbial population and enhance the diversity of the microbiome responsible for AD. By selecting, introducing, and increasing the counts of appropriate microorganisms in biodigesters, it is also possible to increase the production of methane in biogas. Manipulation of microorganisms in the environment is known as bioaugmentation, where selected strains in adequate quantities are introduced to the environment in order to improve, enhance, or purify the environment.[93] In biogas production, owing to bioaugmentation, the diversity and abundance of microorganisms in systems increase, which leads to the intensification of biogas production.[84,85,94] These microorganisms collaborate in strict synthesis during four mutually dependent stages, and the maintenance of equilibrium between groups of microorganisms is the main condition for the stability of a bioreactor, which results in more intensive biogas production. The metabolic efficiency of the work done by microorganisms is crucial for the success of the whole process, and this requires the presence of various microorganisms capable of performing hydrolysis, acidogenesis, acetogenesis, generation of hydrogen, and production of the final product of methane fermentation, that is, methane. A study on strains of methanogenic *Archaea* demonstrated that a high (23%) content of hydrogenotrophic methanogenic microorganisms in an inoculum was conducive to the acceleration of the activation of AD processes. It seems probable that hydrogen is used by microorganisms during the stages of hydrolysis and acidogenesis.[95]

Another biological additive test consisted of an inoculum with methanogenic *Methanococcoides burtonii*.[16,36,38,82] Poszytek et al[46] achieved very good results using the bacterial strain *Ochrobactrum* sp. POC9 applied to intensify the process of neutralization of sewage sludge. In biotechnology, it is also possible to use more broadly the strain

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Ochrobactrum sp. VA1, which enabled researchers to obtain very good effects in the neutralization of sewage sludge.[15] Kovács et al[17] concluded that in order to intensify biogas production, organic biomass should be inoculated with thermophilic acetogenic bacteria able to produce hydrogen, for example, *Caldicellulosiruptor saccharolyticus*. The scientists added the inoculum to the biomass both before and during the digestion process. The tested method allowed them to obtain a higher percentage of methane in biogas.[17] Nkemka et al[44] completed an experiment where they examined the efficiency and effectiveness of bioaugmentation achieved by using anaerobic fungi of the species *Piromyces rhizinflata* YM600. Bioaugmentation was implemented in an anaerobic, two-stage system for digestion of maize silage and cattail (*Typha latifolia*). Comparable productivity of methane, equal 328.8 ± 16.8 and 295.4 ± 14.5 mL/g as well as hydrogen at 59.4 ± 4.1 and 55.6 ± 6.7 mL/g, was achieved for maize silage not exposed to bioindication and submitted to this process, respectively. Similar methane productivity reaching 104 ± 19.1 and 101.0 ± 4.8 mL/g and a low hydrogen yield H_2 (<1 mL/g) were achieved from maize silage with and without bioaugmentation, respectively. Bioaugmentation led to a faster rate of CH_4 and H_2 generation, but it also accelerated the decomposition of VFAs, both in maize silage and common cattail.[43]

The cited authors claim that bioaugmentation is a prospective tool to be used on a broad scale in order to improve the digestion of lignocellulose substrates used for production of biogas and biohydrogen.[43] Martin-Ryals et al[88] investigated the influence of bioaugmentation on anaerobic fermentation of cellulose waste. In order to improve the hydrolysis of a substrate and to increase the total methanogenic efficiency, they employed cellulolytic organisms for bioaugmentation. It was discovered that bioaugmentation improved the efficiency of the hydrolytic process, increased the level of acetic acid, and raised the efficiency of methane generation. In their experiment conducted in a bioreactor, owing to the implementation of multiple bioaugmentation, the COD increased by 25% and the yield of methane rose by 15% compared to a single application of bioaugmentation. In a laboratory-scale continuous system, bioaugmentation effectively increased COD by 29%-68% and acetic acid by 31%-34% in the acid phase, which was beneficial for the processes of hydrolysis and acidogenesis, and eventually led to a permanent rise in methane production by 56% compared to the control treatment without bioaugmentation. Dębowski et al[71] also confirmed the positive impact of bioaugmentation on intensification of biogas production. These authors completed an experiment whose aim was to verify whether psychrophilic bacteria could be employed for the generation of hydrogen and biogas in biofermentation of whey. The tests were carried out in anaerobic reactors of the capacity of 500 mL, at a temperature of 20°C. The initial organic load in the fermentation chambers reached 10 g COD/L. Depending on an experimental variant tested, the following psychrophilic bacteria, isolated from groundwater and from benthic waters of surface water bodies, were tested: class *Gammaproteobacteria-Rahnella aquatilis* (nine strains) and species *Firmicutes*: *Carnobacterium maltaromaticum*, *Trichococcus collinsii*, and *Clostridium algidixylanolyticum*. The experiment revealed varied and strain-specific biogas productivity, ranging from 126.48 to 477.72 mL/g of bacterial biomass. The highest H_2 in biogas, between 65.15% and 69.12%, and H_2 yield from 1587.47 to 3087.57 mL/g, were achieved when strains of *R. aquatilis* isolated from a lake's benthic waters were applied. The lowest H_2 in gaseous metabolites, from 15.46% to 20.70%, was recorded for bacteria from the genus *Firmicutes*.[71] Braun et al[29] conducted continuous fermentation tests for a large variety of potential cosubstrates, for example, animal fat, waste edible oil, blood plasma. All tests were inoculated using 25% standardized digested sewage sludge from municipal sewage treatment. Diluted substrates were added at the onset of the experiments and continuously increased portions were added depending on the course of digestion. Most pure and mixed cosubstrates like blood plasma proved to be well suited for AD, resulting in biogas yields up to 1.36 m^3/kg of VS added.[82]

Bioaugmentation is a promising technology, which can solve practical problems in wastewater treatment plants and which creates opportunities to intensify the production of biogas in AD processes. By implementing important achievements in microbiology, molecular biology, techniques of immobilization of microorganisms on carriers, and techniques of advanced design of bioreactors, it is possible to improve the capabilities of bioaugmentation, and to assess them on the basis of data collected from in-field implementation projects.[96]

4.1.3 | Pretreatment

Feedstocks like organic substrates sometimes require pretreatment in order to improve the methane yield in an AD process.[97] Pretreatment decomposes a complex organic structure into simpler molecules, which then undergo microbial degradation more readily. The main sources of methane in biogas substrates are sugars and other small molecules. Plant substrates rich in lignocellulose (a biopolymer) are composed mainly of three fractions: starch, cellulose, and hemicellulose. Small molecules originate from the breakdown of these fractions. While starch undergoes biological decomposition relatively easily and quickly, cellulose and hemicellulose, whose role is to maintain the structure of the plant, break down slowly and with difficulty. Other reasons why the breakdown of cellulose

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and hemicellulose is complicated are bonds between different cellulose chains and the presence of lignin, another polymer that retards the breakdown process. Pectin also affects lignocellulosic biomass degradation. Pectins combine cellulose fibrils with each other and bind plant cells together.[98] The degradation of the lignocellulose complex using pretreatment is extremely important to enhance biogas production and its efficiency.[99] In general, the following pretreatment methods can be distinguished: mechanical, thermal, chemical, physical, and biological ones as well as their combinations. Biological methods typically consist of ensiling, partial composting, fungal, or enzyme application.

Addition of enzymes

A complex microbial consortium is engaged in the hydrolysis and fermentation of organic material. Bacteria degrade substrate through the use of enzymes, which facilitate the decomposition of organic matter with high molecular weight.[1] Microorganisms break down the substrate via enzymes. There are two types of enzymes involved in the degradation of the substrate: endoenzymes and exoenzymes. Endoenzymes are secreted by all bacteria, but only some can secrete exoenzymes. Enzymes can work in a wide range of environmental conditions and stay active even if these conditions change rapidly.[100] By being smaller, more soluble and more mobile in sludge biomass than microbes, enzymes can reach the substrate more easily. Significant improvement in biogas production has been reported after using crude and commercial enzymes to condition complex organic matter. For example, Davidsson et al[101] showed a positive impact of microbial enzymes applied in batch digestion tests of sewage sludge on AD (60%), in addition to which it was demonstrated that the prehydrolysis stage with the addition of enzymes at controlled temperature for 4 hours significantly increased the methane yield.[101] Akao et al[102] reported that pretreatment of citrus peels with fungal enzymes doubled the limit load for AD. Bruni et al[103] reported that the combination of steam treatment with NaOH and subsequent enzymatic treatment increased the methane yield by 34%. Gerhardt[104] concluded that the addition of hydrolytic enzymes could facilitate the decomposition of structural polysaccharides, resulting in biogas yield increased by up to 20%. The enzymatic treatment of biomass from sewage sludge can improve its solubilization, anaerobic digestibility, dehydration, and hygienization. However, a complex biomass hydrolysis is a multienzyme process that requires the combination of appropriate enzymes, dosage strategies, optimal pH, and temperature conditions.[105]

Use of fungi

During the last decade, several groups of fungi, especially brown, white, and soft mushrooms, have been tested for selective degradation of lignin and hemicelluloses,.[106] Several fungi, for example, brown-, white-, and soft-rot fungi, have been applied in order to make cellulose and hemicelluloses more accessible for further enzymatic hydrolysis. Mushrooms can be grown in biomass to facilitate the degradation of the substrate by increasing the size of pores pervaded by mycelium, and to contribute to the breaking up of bonds between polysaccharides, lignin, and cellulose.[107,108] Fungi release oxidative hydrolytic enzymes involved in ligninolysis: peroxidase and laccase, to digest the external carbon source and absorb rare nutrients such as nitrogen and iron.[109-111] The advantages of using white-rot fungi are as follows: improved biodegradability of lignocellulose owing to larger pores when penetrated by mycelium, ruptured bonds between polysaccharides and lignin, removal of lignin, released cellulose, and a reduced degree of cellulose polymerization. The process proceeds under mild conditions, hence by-products of the biological pretreatment do not have an inhibitory effect.[112] Microorganisms able to convert polysaccharides to monosaccharides include cellulase-producing *Trichoderma reesei* and *Trichoderma viride*. Among other strains that secrete the tested hydrolytic enzymes, the *Penicillium restrictum* fungus is especially promising. When grown in cheap solid substrates consisting of agroindustrial waste, *P. restrictum* releases a number of hydrolases able to break down the most complex organic compounds. This degradation enables a significant increase in the removal efficiency of organic matter, which results in obtaining high quality wastewater in the next stage of biological treatment.[113] Srilatha et al[114] obtained a 33% methane yield increase from orange processing waste which had been treated with selected fungi strains. Similarly, Taherzadeh and Karimi[115] reported improved enzymatic hydrolysis (94% sugar recovery) when treating office paper with selected aerobic bacteria. *Pleurotus sajorcaju*, an edible mushroom grown on different agricultural residues, can degrade cellulose, hemicelluloses, and lignin components. Afterward, biogas can be produced from the degraded agricultural waste, with the biogas yield being twice as high from fungus (*Pleurotus* sp. *florida*) treated straw than from untreated straw. Twenty-two basidiomycetes, mostly white rot fungi, were cultivated on wheat straw, and it was found that the oyster mushroom caused the most rapid delignification of straw. "Myco-straw" was found to be better hydrolyzed and converted to biogas than untreated straw. After biological lignin removal, the straw cellulose was more accessible to AD. A procedure that includes microbial delignification and biogas production creates opportunities to recycle and remove residual wheat straw in an entirely biological manner, generating two commercial products: mushrooms first and methane next. Among white-rot fungi, there are many edible mushrooms; some grow on compost, for example, *Agaricus* spp., while others grow on wood, for

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example, *Pleurotus* spp. and *Lentinula edodes*.[116] The very well-known *Agaricus bisporus* is one waste dumpster whose degradation mechanisms are able to hydrolyze both cellulose and lignin.[109] Nevertheless, ideal fungi for industrial cultivation are *Pleurotus* species and other xylophagous fungi because they can grow on various agricultural residues, such as cereal straws, sawdust, cotton stalks, and almost any other substrate that has a significant cellulose component, and this solution does not require any composting process.[116] There is a need to optimize levels of the essential factors that affect the performance of biogas production, namely, the type of organic biomass, amount of VS, optimal proportion of substrates, and the inoculum in mono-digestion or co-digestion, adequate selection of fungal species. The literature implicates that fungi are a promising alternative to a mono-digestion or co-digestion for generation of pure energy in AD.[117]

Partial composting and silage

Partial composting or ensiling are techniques employed to preserve various feedstocks. Feedstocks like ensiled corn used in an industrial-scale AD process are sometimes enriched with other energy-rich substrates in order to obtain an adequate organic loading or to improve economic feasibility.[20] There are reports on codigestion of manure with different lignocellulosic materials.[118,119] Amon et al[2] conducted an experiment to produce biogas from ensiled maize biomass and obtained the maximum methane yield per km^2 ranging between 7.1×10^5 and 9.0×10^5 m^3 CH_4/km^2 from late cultivars of maize. Maize should be ensiled before undergoing anaerobic fermentation because, as evidenced by these authors, this improves the efficiency of methane production. It is also important to note than the maximum methane productivity was achieved from ensiled whole maize plants. When silage was made from cobs, grain, or mixed cobs, the methane productivity per km^2 was lower by 43%-70% than that produced from silage made from whole maize plants.[2] Carrerè et al[43] researchers achieved the highest biogas production intensity in an experiment where maize silage underwent methane fermentation. Tišma et al[108] studied the use of white-rot fungus *Trametes versicolor* for corn silage pretreatment and investigated the effect of pretreatment on biogas productivity. The results of this study reveal a positive influence of the application of fungal-based, solid-state pretreated corn silage on pH stability and higher biogas productivity. Arici and Koçar[120] showed that anaerobic codigestion of animal manure with maize silage increased total biogas and methane formation, in addition to the improved performance of digestion in waste utilization. Herrmann et al[121] noted that ensiling showed favorable effects on methane yield, raising it up to 11%, and this effect may be dependent on organic acids and alcohols progressively formed during ensiling. However, despite the positive outcome of this experiment, methane digestion of maize silage is not economically viable, and this type of feedstock is not used on any broader scale, neither in biotechnology nor in the power generation industry.[43]

4.2 | Physicochemical technologies

4.2.1 | Addition of macroelements and microelements

An important contributor to enhancement of the AD process is the increased bioavailability of trace elements and controllability of the process of their addition. Inorganic additives are also used in biogas production, for example, macroelements and microelements or combustion ash. Among the inorganic additives, there are macroelements, for example, P, N, and S, and microelements, for example, Co, Fe, Ni, Mn, Ca, W, and Se, which provide nutrition for microorganisms participating in the process of methane fermentation. Hijazi et al[122] studied the impact of trace elements on methanogenesis, concluding that cobalt strongly stimulated acetogenesis in the initial operational phase and methanogenesis in subsequent stages. Pobeheim et al[123] stated that limitations of nickel as well as cobalt lead to process instability. Similarly, Evranos and Demirel[124] reported that the supplementation of Ni, Co, and Mo, either singly or mixed in different proportions, seemed to improve the methane yields. Abdelsalam et al[125] investigated the impact of nanoparticles (NPs) of trace metals such as Co, Ni, Fe, and Fe_3O_4 on biogas and methane production by AD of livestock manure, and they found that the biogas yield increased significantly, by 1.7-, 1.8-, 1.5-, and 1.7-fold, respectively, to the mentioned metals, in comparison with the control. Karlsson et al[126] concluded that addition of trace elements improves AD. Trace metals, such as Ni, Co, and Fe, have been shown to be stimulatory to anaerobic treatment of different types of wastewater,[127] while Kida et al[128] report that trace metals are engaged in enzymatic activities during acidogenesis and methanogenesis.

Effect of NPs in biogas production

Nanotechnology is a branch of technology and science which deals with matter on a nanoscale (1-100 nm), that is, on the level of single atoms and molecules. The essence of nanotechnology lies in the controlled formation and application of materials, structures, devices, and systems in nanoscale dimensions.[129,130] Nanotechnology

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is considered as one of the greatest achievements of science and technology in the past decade.[130] Nanotechnology offers a wide range of innovative materials, processes, and devices with unique activity in terms of environmental pollutants which are difficult to decompose. Nanoscale matter is composed of NPs. The nanosize allows them to interact with biological structures as NPs are able to permeate through cellular membranes, which means it is easier for them to pass through the biological barrier, and hence they can interact with the immune system, with the uptake, absorption, and distribution of substances and with the metabolism of a living organism.[124] Owing to their unique properties, such as an exceptionally small size, high reactivity, and chemical stability, NPs draw an increasing attention among researchers and have more and more applications in industry.[131] Moreover, NPs are a very good carrier because they possess a very high surface to volume ratio, which increases their activity owing to a greater number of available sites. For example, during methanogenesis, NPs can stimulate the stage of hydrolysis by facilitating the adherence of organic matter particles to NPs.[129] Addition of NPs also changes the structure of a microbial community and the counts of prevalent microorganisms in reactors, which improves the efficiency of the system and methanogenesis. Furthermore, NPs can stimulate the efficiency of bioreactors by acting as electron donors or acceptors as well as cofactors of various enzymes.[132] Kaushal and Baitha[129] studied the impact of graphene oxide NPs on intensification of biogas and methane production in the AD of $Ca(OH)_2$ suspension mixed with wheat straw. They observed that the application of NPs doubled the volume of methane relative to the control sample. Farghali et al[133] examined the effect of TiO_2 NPs on the efficiency of AD of bovine slurry and recorded, in comparison with the control, 1.5-fold higher biogas production efficiency after TiO_2 had been added to bioreactors.[133] Khalid et al,[134] when using Fe_3O_4 NPs in an AD process, recorded an increase by 33% in the efficiency of biogas production from rice straw.[134] Hassanein et al[130] noticed that a concentration of 12 mg/L NPs of Ni can raise the production of methane by 38.4%, while NPs of Fe, Co, and Fe_3O_4 can increase methane production by over 25%.[130] Nanotechnology and NPs are attracting increasing attention among researchers owing to their unique characteristics. NPs are used in various branches of science and industry, also to improve the productivity of biogas.[124,130,134-136] However, further studies are needed to understand fully the impact of NPs on activity of microorganisms, and to determine optimal conditions for an AD process. It is also recommended to conduct research into the profitability of using NPs in industrial-scale biogas production.

Addition of Fe^0

Regarding inorganic additives, the available research data indicate that the application of iron in the form Fe^0 and Fe^{III} leads to very good results. In the AD process, iron is mainly a donor of electrons and a cofactor of enzymatic activities, which are extremely important in a properly conducted process.[6,21,22] Over the past few years, the effect of added iron has been studied, and the results prove that addition of this element is among the most effective technologies of AD. Owing to its low price and very good conductivity, iron substantially improves the efficiency of this process.[61] Zotos et al[127] submitted to processing wastewater with an addition of nitrogen dye in an upflow anaerobic sludge blanket (UASB) reactor and found out that Fe^0 (zerovalent iron) had a beneficial influence on the growth of methanogenic microorganisms, which were then able to achieve a high rate of degradation of the organic material at low temperatures (25°C), and the hydraulic retention time could be reduced to 12 hours. Liu et al[75] investigated the use of Fe^0 in combination with an electric field in a UASB reactor. Owing to the implemented modifications, the voltage was raised to 1.4 V, the granulation of the sludge was more rapid, and the COD removal in the reactor increased from 60% to 91% in 4 days. The electric field meant that zerovalent iron could buffer the environment in the bioreactor more efficiently, in addition to which it maintained acidity and a relatively low potential of oxygenation reduction in the reactor. Moreover, the electric field caused a considerable increase in the process of leaching ferrous ions and producing extracellular polymeric substances. These changes favored the process of methanogenesis.[75] Rashed and Tori,[135] having researched the removal of hydrogen sulfide (H_2S) from biogas using zerovalent iron concluded that this method reduced high operation costs and risk to the system. The solution is highly recommended because of environmental benefits such as potentially lower air pollution by H_2S/SO_2 emission and lesser impact on human health. Krongthamchat et al[83] reported that the addition of iron as a trace metal could reduce the lag time of a mixed culture. Liu et al[75] stated that iron ions released from the dissolution of magnetic NPs can stimulate bacterial activities. Fe additive could increase the yield of methane owing to the enhanced generation of acetate, a suitable substrate for methanogenesis, in the presence of Fe additives. Furthermore, Fe could act directly as an electron donor for reducing CO_2 into CH_4 through autotrophic methanogenesis, thus improving methane generation.[136-138]

Manipulation of physicochemical parameters

Intensification of biogas production can be also achieved by dewatering wastewater and sewage, optimization of physicochemical parameters such as pH, temperature, HRT, size of molecules submitted to fermentation,

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submitted to fermentation, configuration of a bioreactor, C/N ratio, intensity of the mixing process, and by employing immobilized biofilms or biofilters.[139,140]

pH

pH is a key parameter in the fermentation process because of its direct influence on the digestion of substrates. Methanogenic microorganisms are highly sensitive to changes in pH in bioreactors. Excessively low pH can cause inhibition of AD, while very high pH can lead to the production of free ammonia, which is toxic to methanogenic microorganisms.[133,139,140] The microorganisms participating in the process of methane fermentation, that is, hydrolytic, acidogenic, and methagenic microorganisms, have optimal pH ranges in which the maximum reaction efficiency is obtained. Hydrolytic bacteria, responsible for the first stage of methanogenesis, have a broad pH range within 4-8.5.[141] A similar pH range is characteristic for microorganisms converting products to organic acids. Methanogenic microorganisms possess a much narrower range of pH, which should be comprised within 6.8-7.2. The difference in environmental requirements is a frequent argument for selecting a two-stage system of reactors for conducting an AD process.[142] The pH value of the suspension also depends on a stage of the process. At the beginning of the process, during the stage of production of acids and release of large amounts of carbon dioxide, pH is around 6.[133] In the subsequent weeks of the AD process, pH slowly increases, methanogenic microorganisms are active and methane is produced. In the pH range of 6.5-7.5, microorganisms are very active and biodegradation occurs much more efficiently. The amounts of carbon dioxide and VFAs generates during the AD process affects the pH of a bioreactor's contents. For AD to proceed normally, the concentration of VFAs, acetic acid especially, should be less than 2000 mg/L.[8] If pH in biodigestors exceeds the optimal range values, conditions become unfavorable to methanogenic microorganisms, and the process slows down and is less efficient.[130,134]

Temperature. The temperature inside the digester has a major impact on the AD process. It directly affects the metabolic activity of microorganisms involved in methanogenesis.[125] Any deviation from the optimal temperature ranges may lead to a depressed activity of microorganisms, and therefore result in a low efficiency of the fermentation chamber.[134] There are three temperature ranges for AD: psychrophilic (<30°C), mesophilic (30°C-40°C), and thermophilic (50°C-60°C), but the optimal temperature for anaerobes is in the range of 30°C-60°C. Weiland[19] reported that the majority of wet fermentations are operated at mesophilic temperatures in the range of 38°C and 42°C. Biogas plants are very rarely set at thermophilic conditions between 50°C and 55°C because the ultimate methane yield from organic matter is not increased although higher temperatures accelerate the degradation, hence hydraulic retention times are shorter and smaller reactor volumes are required.

Immobilization of biofilms/biofilters

With improved analytical methods, we are now able to determine morphological properties of a biofilm which accumulates on a carrier selected for immobilization. The development of biological biosensors and advanced techniques of microbial detection allow us to identify the degree of concentration of microorganisms on a biological membrane, which is of key importance to the formation of the biofilm.[143] Ramm et al[144] analyzed novel magnetic foam glass particles, able to act as biofilm carriers and to retain active microbial biomass used in the production of biomethane. Magnetic foam glass particles evidently stabilized the methane production process, mostly owing to the beneficial effect on methanogenic microorganisms, which accounted for 49% of the population settled on magnetic carriers. Weib et al[145] concluded that there were considerable differences among physiological characteristics of scattered bacterial cells vs cells immobilized on a carrier. Immobilized microorganisms demonstrate a much higher metabolic activity compared to cells which are suspended freely in the environment. Quite good results were achieved when charcoal served as a carrier, also because it had sorptive characteristics, which contributed to the removal of hardly degradable pollutants. All these characteristics of a carrier may help reduce the hydraulic retention time.[145]

Chemical/oxidative pretreatments

In biogas plants, the chemical pretreatment of feedstocks is usually combined with thermal pretreatment because of the higher biogas yields obtained. Different types of alkalis and acids can be used for enhancement of biogas production in thermochemical pretreatment.[146] The thermochemical pretreatment at a temperature below 160°C gives very promising results regarding the improved biogas production efficiency, but it must be calculated whether the energy obtained gives a profit compared to the financial contribution incurred, and for that reason this technology is rarely used. In the initial oxidative treatment, hydrogen peroxide or ozone are most commonly used. According to the current knowledge, thermochemical and oxidation treatments, due to high costs, are very rarely used in biogas plants. Organic materials subjected to oxidative treatment with hydrogen peroxide or ozone are much easier to be decomposed.

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Ionic liquids/ultrasounds

In environmental engineering, ionic liquids are mainly used for the purification of sewage sludge where the presence of aggregated clumps consisting of microorganisms (the so-called flocks) is a factor interfering with the process of AD. The addition of ionic liquids to feedstocks like sewage sludge causes the breakdown of flocks which are being formed on the surface of sewage sludge. The influence of ionic liquids on the AD process is not well understood and requires further research to determine its effect. Ultrasound frequencies around 20 kHz cause cavities in the cell wall, followed by disintegration of the cells, which facilitates the access of cellulose to hydrolytic enzymes and accelerates the hydrolysis of biomass.[142] Ultrasound pretreatment is considered to be an eco-friendly process increasing the biodegradability of organic matter during AD.[85] Cesaro et al[146] investigated the effect of ultrasonic pretreatment on AD of organic solid waste under mesophilic conditions and reported that biogas production from the sonicated mixture was 24% higher than that from untreated biomass. Ultrasound pretreatment is a simple, low-cost technology that can have a positive effect on enhancing biogas production in fermenters fed with a hardly decomposable plant substrate.

4.3 | Mechanical technologies

4.3.1 | Intensification of biogas production in a two-stage reactor

Two major groups participating in AD can be distinguished, that is, acidogenic and methanogenic microorganisms.[131] The conditions needed for the growth and development of these two groups are quite different, which is why these microorganisms need different technological and environmental conditions to be maintained during the methane fermentation process. Striving to achieve the highest possible efficiency of the AD process, a two-stage methane fermentation, known as the two-stage AD (TSAD) technology has been developed.[132] The TSAD system is controlled by a number of factors, such as the type of inoculum, working temperature, and pH, time of hydraulic retention and organic loading rate (OLR). The TSAD process makes it possible to separate such stages as hydrolysis and acidogenesis from the stage of methanogenesis. The separation into two stages was designed in order to improve the efficiency of AD, to intensify the production of acetic acid as well as methanogenesis, and ultimately to increase the quantity of produced biogas. Hydrolysis conducted in a separate stage can be more efficient and can therefore result in lower levels of inhibiting factors in each consecutive stage of AD.[147] In the two-stage system, when stage 1 is separated, it can be carried out under extreme temperature and pH regimes so as to maximize its efficiency, while ensuring specific conditions for the demanding methanogenic microorganisms. The acidification stage is a quick process, which usually takes place at a low-HRT value and in the pH range of 5.0-6.0. Methanogenesis is a slower process, taking much longer and occurring in the pH range of 6.0-8.0, at HRT typically lasting 20-30 days, which enables the multiplication of slow-growing methanogenic microorganisms.[148] The two-stage methane fermentation process makes it possible to improve the efficiency of the process, increase amounts of produced biogas, remove VS more effectively, achieve better stability and energy recovery.[149] In a one-stage methane fermentation process, a problem appears due to sedimentation of heavy fractions and the appearance of foam, which can interfere with the course of the process, unlike in a two-stage process, where methanogenesis can run more smoothly and efficiently[150,151] TSAD is a promising technology to achieve better stability and improved productivity of biogas in a reactor processing highly biodegradable substrates at a higher OLR.[145-147,152]

4.3.2 | Sieving/mixing/grinding/thermal

Mechanical pretreatment includes processes such as sieving, mixing, milling, and temperature manipulation. Mechanical pretreatment makes substrates, especially lignocellulose, more accessible to enzymes, which considerably strengthens enzymatic degradation. Each type of physical pretreatment is helpful to attain better substrate availability for enzymes, but Schell and Harwood[151] recommend particle sizes from 1 to 2 mm for the most effective enzymatic hydrolysis. Zhang et al[152] reported that the best effects increasing the biomass recalcitrance were achieved through mechanical pretreatment with a particle size of 2 mm. Treatment by thermal pressure hydrolysis (230°C, 20-30 bars) leads to the division of organic polymers by hydrolysis into short-chain, easily bioavailable compounds, which raises the biogas yield while considerably reducing the retention time in the digester.[62,153]

5 | CONCLUSIONS

For decades, biological waste, that is, sewage sludge or animal manure, was disposed of directly into soil (as natural manure) or surface waters. However, the notion that biological waste can be recovered and used as raw material is gaining importance. Safe and rational

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waste management is in order, and there is growing awareness of the need of good practice in municipal waste management. Legal regulations require that waste to be stabilized and submitted to mechanical and biological processing, so that the final product could be used as a soil conditioning substance. All these processes generate additional costs, whereas methane fermentation offers dual benefits. In a methane fermentation process, we obtain both eco-friendly biofuel and fertilizer, which are valuable for soil, safe, and free from pathogenic microbes. AD raises many hopes connected with the production of biofuel that will be an alternative to fossil fuels and will be friendlier to the environment. Also, AD generates small amounts of the by-product such as biomass which can be used as valuable fertilizer. The consumption of energy in AD is much smaller than in aerobic processes, which require additional systems to supply oxygen in order to aerate bioreactors. In recent years, there has been a growing interest in the process that, apart from letting us dispose of sewage sludge, can also serve to remove plant residue generated by agriculture or the food processing industry. Moreover, AD contributes to a more widespread recycling of chemicals and nutrients found in organic biomass. Development of technological systems employed to conduct methane fermentation, including progress in molecular biology and genetic engineering methods, facilitates implementation of anaerobic fermentation technologies. Owing to these solutions, the AD process is a subject of increasingly broader research, which helps to determine more optimal parameters of the process and identify technologies which intensify biogas production.

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SUPPORTING INFORMATION

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Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 15.06.2023

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mój wkład polegał na współudziale w opracowaniu metodyki, przeglądu literatury oraz przygotowaniu pierwszej wersji manuskryptu.

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Małgorzata Czatzkowska

Podpis

Załącznik 2

applied sciences

MDPI

Article

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.

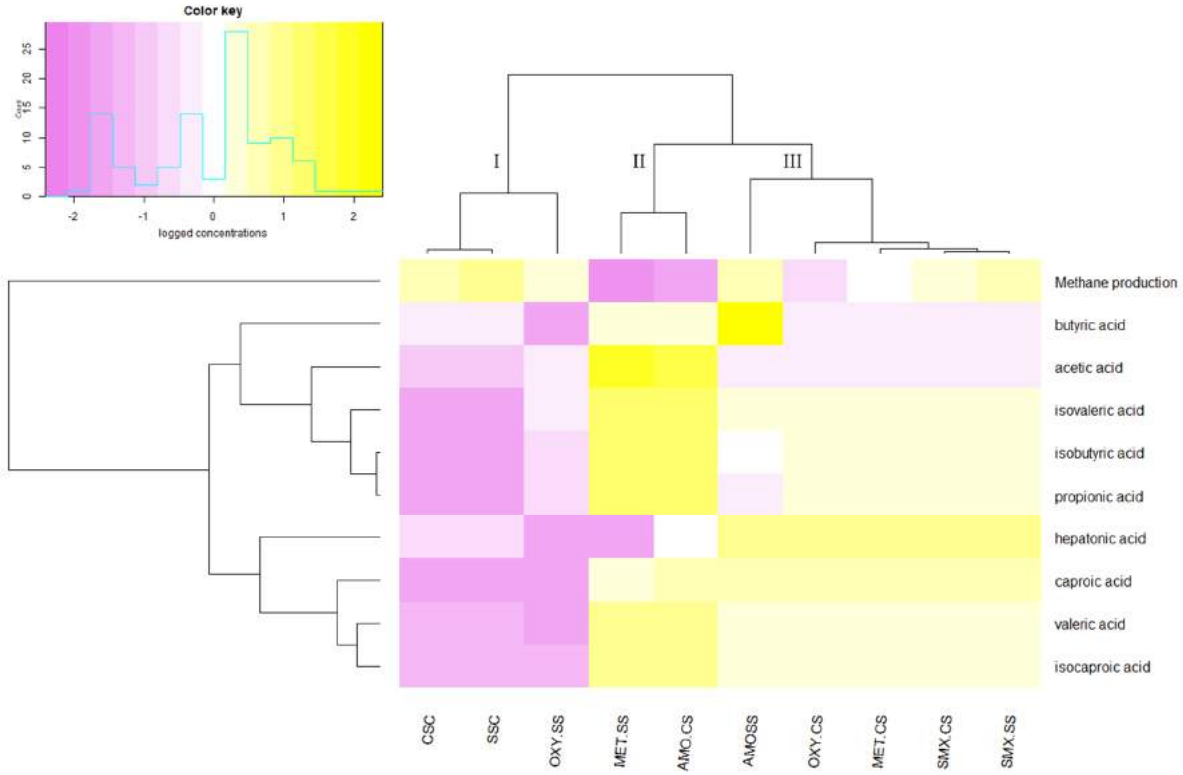


Figure 1. Heatmap with the logged values of methane production (L kg^{-1} VS) and VFAs concentrations (g L^{-1}) in process and control bioreactors with sewage sludge (SS) and cattle slurry (CS). (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry.

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.

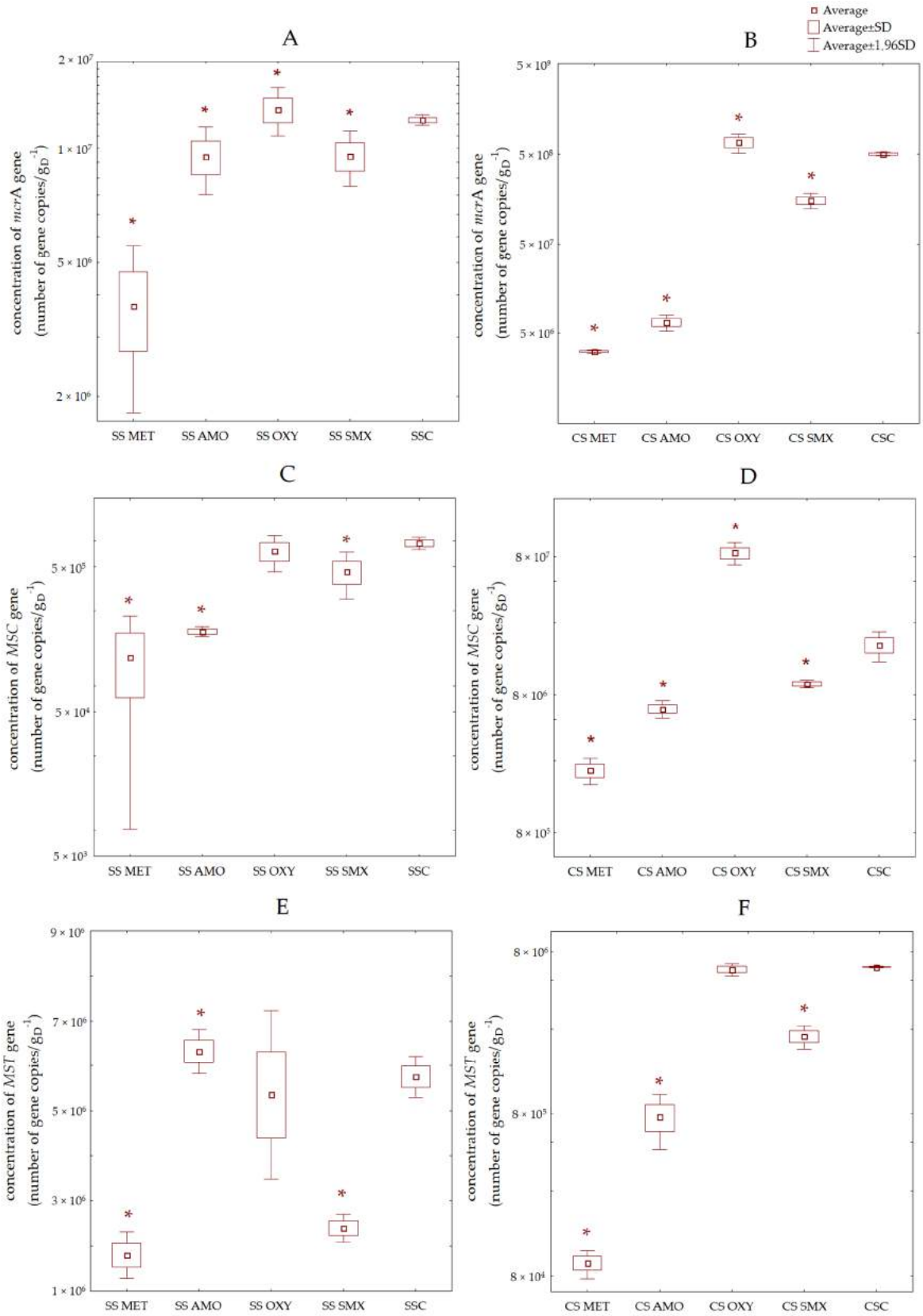


Figure 2. Average concentrations of *mcr*A, *MSC* and *MST* genes in 1 g_D^{-1} of sewage sludge samples (**A**,**C**,**E**, respectively) from the process and control bioreactors and cattle slurry samples (**B**,**D**,**F**, respectively) from the process and control bioreactors. (SSC) control samples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

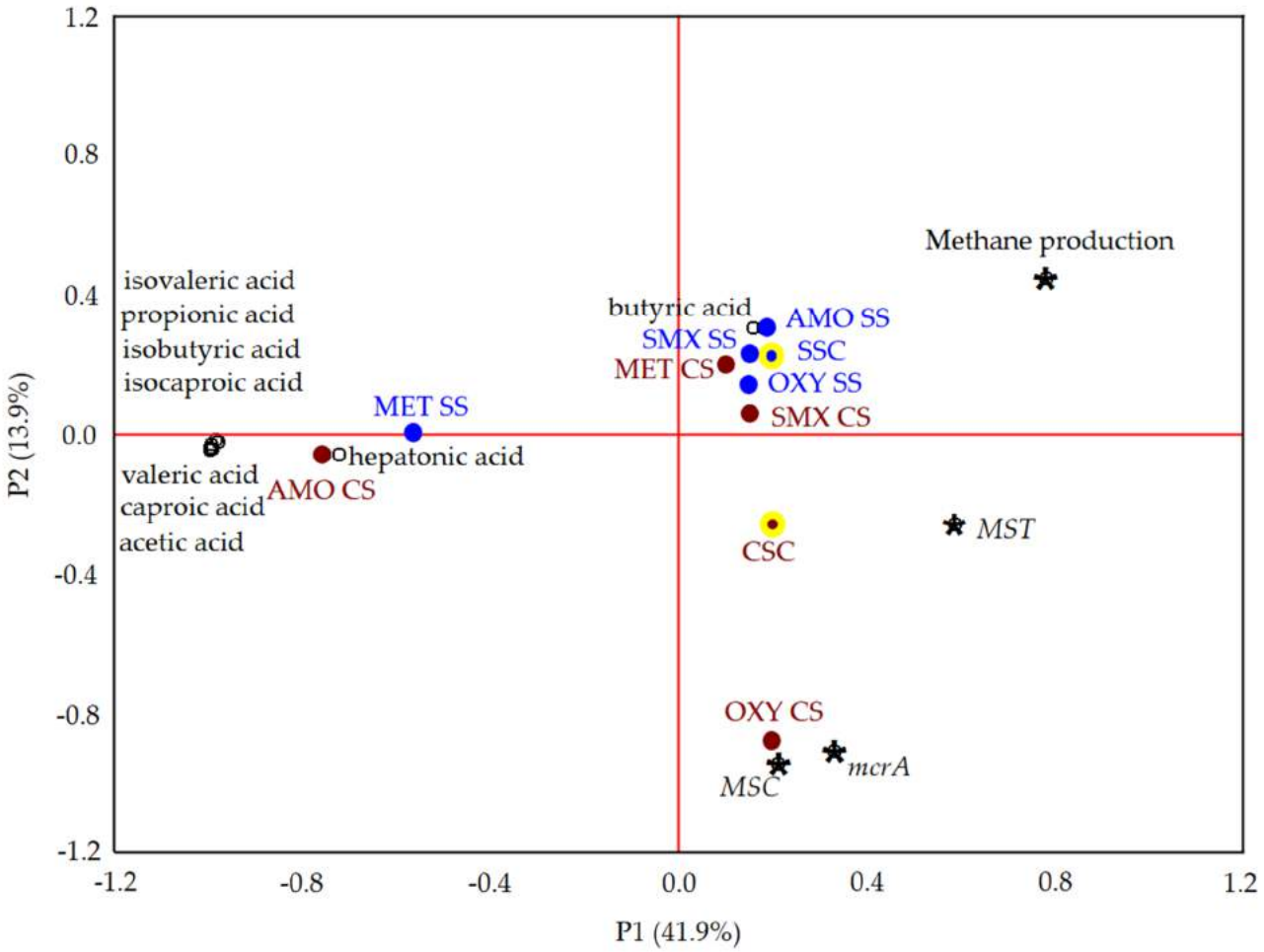


Figure 3. Principal component analysis (PCA) based on the distribution of the analyzed genes characteristic of methanogens and parameters of methane fermentation in bioreactors with sewage sludge (SS) and cattle slurry (CS). (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry.

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].

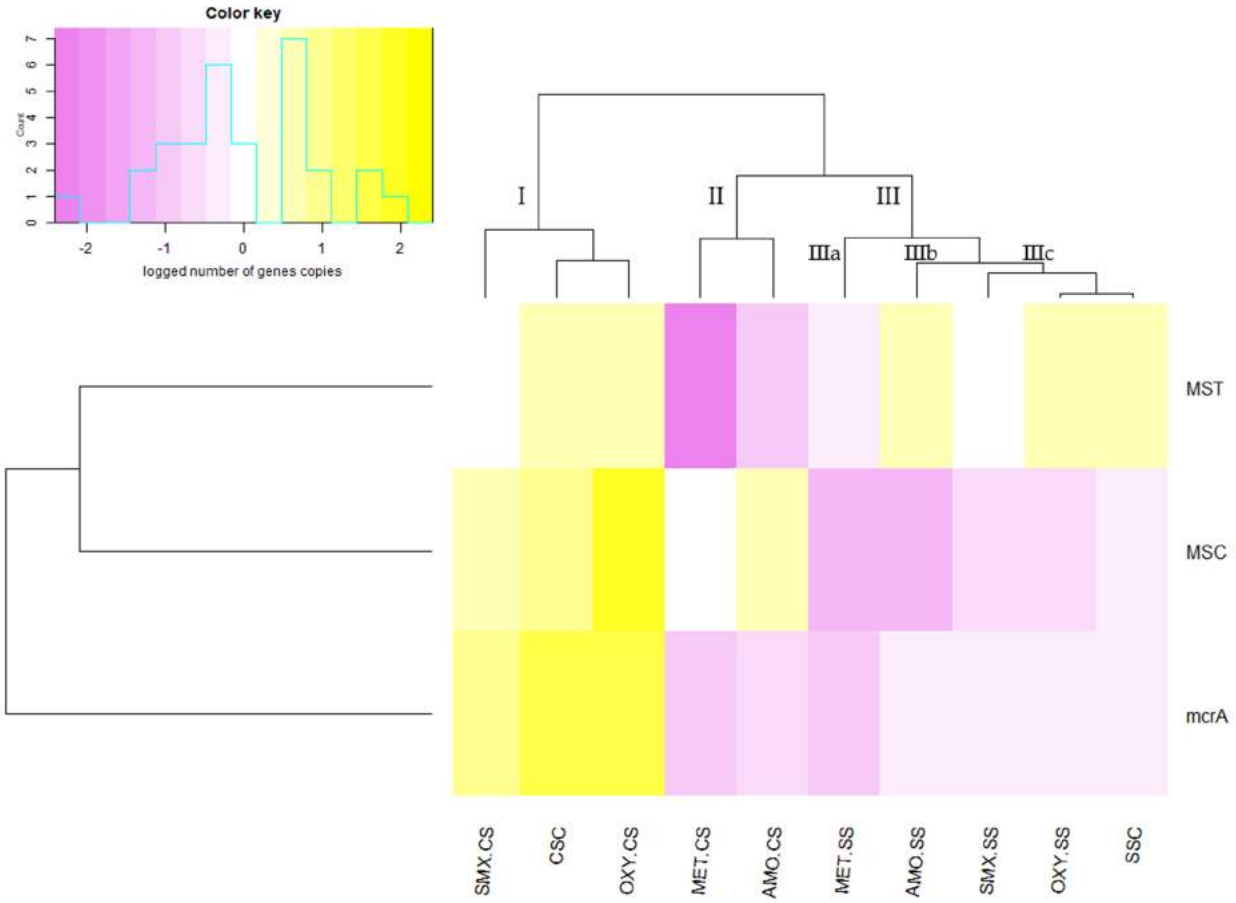


Figure 4. Heatmap with the logged concentrations (number of copies in g_D^{-1}) of *MST*, *MSC* and *mcr*A genes in process and control bioreactors with sewage sludge (SS) and cattle slurry (CS). (SSC) control samples of sewage sludge, (CSC) control samples of cattle slurry.

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

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Table S1. Oligonucleotide primers and PCR reaction profile.

Target Gene	Primer Sequence (5′-3′)	PCR Cycles	Reference
MSC	GAAACCGYGATAAGGGGA	94°C/10s, 60°C/30s, 45 cycles	Yu et al., 2005
	TAGCGARCATCGTTTACG		
MST	TAATCCTYGARGGACCACCA	94°C/10s, 60°C/30s, 45 cycles	Yu et al., 2005
	CCTACGGCACCRACMAC		
*mcr***A**	GGTGGTGTMGGATTCACACARTAYGCWACAGC	95°C/30 s, 55°C/30 s, 72°C/40 s, 45 cycles	Denman et al., 2007
	TTCATTGCRTAGTTWGGRTAGTT		

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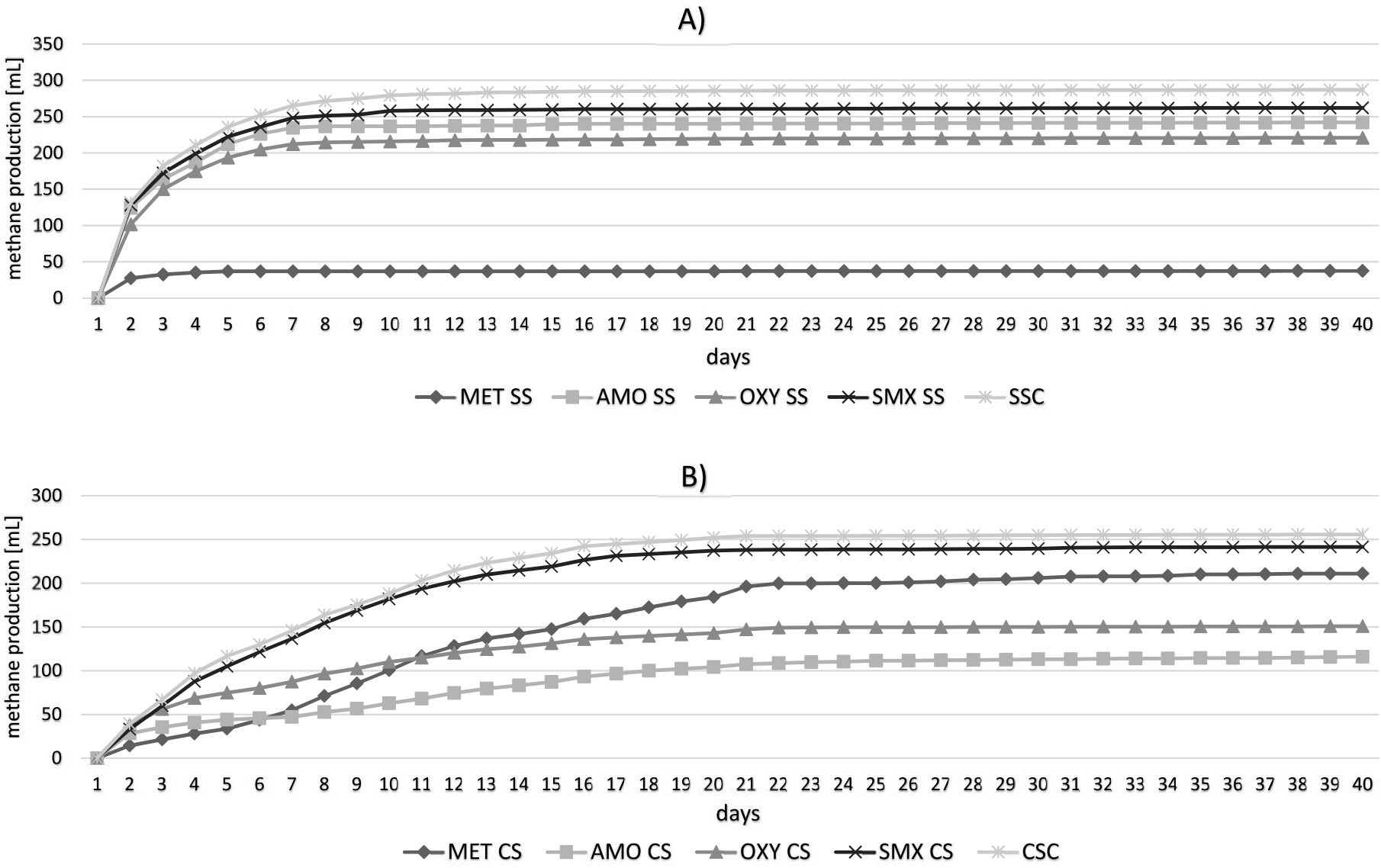


Figure S1. Average values of methane production in bioreactors during anaerobic digestion substrates such as A) sewage sludge (SS) with MET, AMO, OXY, SMX supplementation, and control reactor (SSC), and B) cattle slurry (CS) with MET, AMO, OXY, SMX supplementation, and control reactor (CSC).

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mój wkład polegał na współudziale w opracowaniu metodyki, przygotowaniu pierwszej wersji manuskryptu oraz wykonaniu analiz laboratoryjnych. Byłam również odpowiedzialna za pomoc edytorską przygotowywanego manuskryptu.

Izabela Wolak

Podpis

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mój wkład polegał na współudziale w opracowaniu metodyki. Byłam również odpowiedzialna za pomoc edytorską przygotowywanego manuskryptu.

Małgorzata Czatzkowska

.....

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mój wkład polegał na współudziale w opracowaniu metodyki oraz pozyskaniu środków finansowych. Byłam również odpowiedzialna za pomoc edytorską przygotowywanego manuskryptu.

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mój wkład polegał na współudziale w opracowaniu metodyki. Byłam również odpowiedzialna za pomoc edytorską przygotowywanego manuskryptu.

.....

Podpis

Załącznik 3

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The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure

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Abstract: This study explored the effect of eight antimicrobials on the efficiency of biogas production in the anaerobic digestion (AD) process of cattle manure. The microbiome involved in AD, presence and number of genes *mcr*A, *MSC* and *MST* specific for *Archaea*, and antibiotic resistance genes (ARGs) concentration in digestate ($_D$) were examined. Supplementation of antibiotics to substrate significantly lowered biogas production. Amoxicillin caused a 75% decrease in CH_4 production in comparison with the control samples. Enrofloxacin, tetracycline, oxytetracycline, and chlortetracycline reduced the amount of biogas produced by 36, 39, 45 and 53%, respectively. High-throughput sequencing of 16S *r*RNA results revealed that bacteria dominated the *Archaea* microorganisms in all samples. Moreover, antibiotics led to a decrease in the abundance of the genes *mcr*A, *MSC*, *MST*, and induced an increase in the number of tetracyclines resistance genes. Antibiotics decreased the efficiency of the AD process and lowered the quantity of CH_4 obtained, while stimulating an increase in the number of ARGs in $_D$. This work reveals how antimicrobials affect the cattle manure AD process and changes in microbial biodiversity, number of functional genes and ARGs in the digestate due to drugs exposure. It also, provides useful, practical information about the AD process.

Keywords: mesophilic anaerobic digestion; biogas production; microbiome biodiversity; antibiotic resistance genes; Illumina MiSeq Sequencing

1. Introduction

Anaerobic digestion (AD), which is methane fermentation, applied to the processing of organic waste (e.g., cattle manure (CM)), is a promising production technology generating biogas, the main component which is methane (CH_4) [1]. Cattle manure can be disposed of and recycled through the AD process. However, cattle manure and digestate obtained after AD processing can contain veterinary antibiotics (VAs) and antibiotic transformation products that have not been completely metabolised by animals [2]. Such antibiotics, such as amoxicillin or tetracycline, are metabolised in just 10–20% of the total amount, while others (e.g., sulfamethoxazole) can be metabolised up to 85% [3]. Ahmad et al. [4]

reported that the total volume of antibiotics consumed globally was about 2×10^8 kg a year, of which several thousand kg of VAs are released back into the environment with animal excreta.

Antimicrobial presence in substrates undergoing AD can inhibit the biogas production process and biogas yield as well as the percentage of CH_4 in biogas produced during methane fermentation [5]. Methanogenic microorganisms belonging to the domain of *Archaea* and participating in the AD process are of key importance for biogas production and high CH_4 yield. Methanogens are particularly sensitive to antimicrobials, whose presence in substrate (cattle manure) used in AD can slow down the rate of their growth and decrease their metabolic activity [6]. The risk of the occurrence of drug resistance (DR) and increasing number of antibiotic resistance genes (ARGs) in digestate from cattle manure, an AD by-product, due to the widespread use of antibiotics in veterinary medicine and agriculture, is a significant problem [7]. There is also a thread of the emergence of selection pressure and diffusion of resistant strains of microorganisms as well as their MGEs (mobile genetic elements) in digestate [6].

Over the past decades, the global consumption of antibiotics has been growing constantly. Drugs that belong to the classes of beta-lactams, tetracyclines, sulfonamides, fluoroquinolones and nitroimidazole derivatives are the most popular pharmaceuticals in veterinary medicine [8]. In this research, the aforementioned antibiotics were tested as potential inhibitors of the AD process.

To the best of our knowledge, few researchers have investigated in one experiment the effect of antimicrobials belonging to so many classes of antibiotics on biogas production, biodiversity of microbial consortia occurring in digestate from cattle manure after mesophilic anaerobic digestion process as well as the presence of ARGs, simultaneously. Moreover, only a few authors described the dependence between AD in the presence of antibiotics and the spread of drug resistance. So far, few authors have studied the influence of metronidazole, which directly affects anaerobic microorganisms, on the AD process. Moreover, there is some inconsistency in the literature regarding the effects of antibiotics on the AD process. While some authors report a reduction in CH_4 production at concentrations such as 3.1 mg L^{-1} of the drug [9], other authors have shown no inhibition of AD even at drug concentrations of 125 to 250 mg L^{-1} [10,11]. Hence, there is a need to conduct further experiments, using high doses of antibiotics and metagenomics methods, to determine the changes that occur at the microbiological level and the changes induced by antibiotics in the methanogenic community. Numerous authors [12–14] suggest that it is necessary to investigate the source, fate, and risk of such pollutants, such as antibiotics, and to control them in the AD process, which they can affect directly. Therefore, the main aim of this study was to assess the effect of selected antimicrobial drugs with high doses on (i) the efficiency of AD and biogas production, (ii) occurrence and number of *mcr*A, *MSC*, *MST* genes which are indicators of the presence of methanogenic microorganisms, (iii) the microbiome involved in the methane fermentation processes, and (iv) the presence and number of ARGs. In addition, degradation of antibiotics during biomethanisation was studied. The research provides the basis for understanding the impact of the selected antibiotics on bacterial communities and populations of methanogenic microorganisms that participate in biomethanisation. The article also contains some information useful to help control the AD process.

2. Materials and Methods

2.1. Anaerobic Digestion

The CM was used as substrate, while the anaerobic sludge from the laboratory methane fermentation reactor was used as the inoculum in AD (Table S1). Antibiotics belonging to beta-lactams (AMO—amoxicillin, AMP—ampicillin), tetracyclines (OXY—oxytetracycline, TET—tetracycline, CHLOR—chlortetracycline), fluoroquinolones (ENR—enrofloxacin), sulfamethoxazole (SMX) and nitroimidazole derivatives (MET—metronidazole) have been selected for the study and concentrations of each are present in Table S2.

Beta-lactams and tetracyclines were chosen because of the frequency of use in agriculture and veterinary medicine, while fluoroquinolones and sulfamethoxazole were included in the study because they may affect the growth and activity of microorganisms belonging to the *Archaea* domain. Metronidazole, in turn, inhibits the growth of anaerobic bacteria. The doses of antibiotics were selected based on the data published in the literature [10,15,16] and on the preliminary studies that were conducted to obtain a microbiome response to drugs (unpublished data). This stage of research was conducted in order to select antibiotics that reduce the efficiency of the AD process. Methane fermentation was carried out in nine bioreactors, in eight of which a substrate enriched with one of the selected antibiotics was added. Substrate without antibiotic supplementation was the control (SA—substrate without antibiotic). The whole experiment was loaded in two replicates of each modification of bioreactor feed. The cattle manure retention time in the bioreactors was 39 days. The details related to AD conditions and biogas yield were added to Supplementary Materials (Section S1).

2.2. Analyses of Antibiotic Concentration

Details about sample preparation, analysis of antibiotic concentration and methods used for measuring the concentration of antibiotics in samples have been described in our previous, methodological study [17] and briefly in Supplementary Materials (Section S2).

2.3. Genomic DNA (gDNA) Isolation and Quantitative Analysis by qPCR

Genes specific for *Archaea* (*MSC*, *MST*, *mcr*A), genes encoding resistance to beta-lactams (bla_{TEM}, bla_{OXA}, bla_{SHV}, *cfx*A), tetracyclines (*tet*A, *tet*M, *tet*Q), macrolide, lincosamide and streptogramin (MLS) antibiotics (*erm*F, *lin*A, *mef* A), sulfonamides (*sul*1), fluoroquinolones (*aac(6′)-Ib-cr* and *qep*A) and the integrase gene of class 1 and 2 (*intI*1 and *intI*2) were determined in qPCR reactions (details and conditions presented in Supplementary Materials in Section S3 and in Table S3). To study target genes, three replicate samples of cattle manure digestate were collected after the mesophilic anaerobic digestion process, then gDNA was isolated from each sample and its quantity and quality was measured, after which gDNA samples were pooled and a standardised sample prepared. Absolute abundances (AAs) of each analysed gene were presented as an average of all obtained abundance estimates and presented per gram of analysed digestate from cattle manure ($g_{\ D}^{-1}$). The relative abundances (RAs) of the ARGs were calculated as copy number of ARGs/copy number of 16S rRNA gene.

2.4. Illumina MiSeq Sequencing

To identify the microbiome the high-throughput sequencing of the hypervariable region V3–V4 of 16S gene was performed with an Illumina MiSeq instrument (Seoul, Korea). Primer pairs targeting Bacteria and *Archae*a were selected according to Klindworth et al. [18]. The details have been described in Supplementary Materials (Section S3.3).

2.5. Statistical Analyses

Data were analysed using Statistica 13.1 software (https://www.statsoft.pl/statistica_13/, accessed on 20 October 2020). Tests and diagrams use for analysis have been described in Supplementary Materials (Section S4).

3. Results

Antibiotics present in cattle manure subjected to the AD process may inhibit its efficiency by directly affecting bacteria and methanogens. The microorganisms involved in this process have different metabolic demands, and there is a strict dependence among them. Drugs present in substrates subjected to AD can inhibit DNA replication, RNA transcription, SOS responses, or the production of ATP in microbial organisms [19]. Pharmaceuticals can also interfere with cell division, protein translation, and cell wall synthesis or nucleotide biosynthesis [20]. Wang et al. [21] report that CHLOR in the AD process can completely

inhibit acetoclastic methanogenic *Archaea*. Antibiotics, such as SMX and TET, prevent bacterial growth by binding irreversibly to the 50S and 30S subunits of the bacterial ribosome, respectively [22]. Sulfonamides act as competitive inhibitors of the dihydropteroate synthase enzyme that catalyses the conversion of para-aminobenzoate (PABA) to dihydropteroate (AHHMD), a precursor of folate synthesis. Tetrahydrofolic acid is involved in the synthesis of nucleic acids and, thus, prevent the growth of bacteria [23]. Sulfamethoxazole in the stage of acidogenesis and acetogenesis can destroy cell membranes, thereby improving solubilization, but at the same time increasing the production of VFAs (volatile fatty acids), the accumulation of which may also lead to lower biogas yield or inhibition of the process [24]. In AD, most of the hydrolysis and acidification processes depend on the metabolism of the bacteria, which affects its stability. Bauer et al. [25] observed that increasing the concentration of antibiotics in the process of AD may reduce microbial biodiversity. Amoxicillin had the most significant influence on AD of CM. The CH_4 production from the reactor with amoxicillin was 75% lower than from the SA and the presence of VFAs were noted (Table 1, Figure S1). The rate of biogas production was notably lower in the reactor with amoxicillin than in the other investigated reactors (Figure S1).

Table 1. The results of methane fermentation of cattle manure in the bioreactors with antibiotics supplementation and substrate without antibiotic (SA).

Antibiotic	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	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	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
AMP	138.6 ± 19.6	69.1 ± 3.3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ENR	72.5 ± 14.7	72.9 ± 12.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SMX	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
OXY	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
CHLOR	107.0 ± 54.9	68.7 ± 4.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TET	79.2 ± 23.7	69.4 ± 3.8	6.35 ± 2.54	0.43 ± 0.09	0.48 ± 0.14	0.26 ± 0.07	2.35 ± 1.06	0.68 ± 0.22	0.35 ± 0.08	0.12 ± 0.04	0.03 ± 0.01
SA	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

CH_4—methane, VS—volatile solids, VFAs—volatile fatty acids, MET—metronidazole, AMO—amoxicillin, AMP—ampicillin, ENR—enrofloxacin, SMX—sulfamethoxazole, OXY—oxytetracycline, CHLOR—chlortetracycline, TET—tetracycline, SA—substrate without antibiotic (control), ± indicates standard deviation.

The highest concentration was determined for acetic acid (16.11 ± 2.09 g L^{-1}). However, concentrations of other acids were also higher than in the SA. The higher concentrations of VFAs increased also slightly the FOS/TAC ratio (the ratio of volatile organic acids to alkaline buffer capacity and measure the risk of acidification of a biogas plant) (Table S4). Sun et al. [26] investigated the combined effect of amoxicillin, aureomycin, sulfadimethoxine, and florfenicol on AD of piggery wastewater and observed that AMO was the second most strongly inhibiting antibiotic. Nuengjamnong et al. [27], who measured only the influence of AMO, showed that the presence of AMO inhibited the CH_4 production. Lallai et al. [10] noted a decrease in CH_4 production from pig slurry enriched with amoxicillin trihydrate at a dose of 0.6 and 0.12 mg L^{-1}. The significantly lower CH_4 production was noted in reactors with AMP, OXY, ENRO, CHLOR, and TET. Similarly, Bauer et al. [25] noted that the concentration of ENRO and CHLOR of 0.2 g L^{-1} inhibited CH_4 yields from pig slurry. However, in the present study higher concentrations of VFAs were observed (except the bioreactor with AMO addition) only in the bioreactor with TET. This antibiotic increased mainly acetic acid and iso-valeric acid concentration in the digestate. In the reactor with TET also the lowest rate of biogas production was observed (except the bioreactor with AMO addition) (Figure S1). The indicators of AD (for example FOS/TAC ratio or pH) were not changed in the bioreactors with AMO, OXY, ENRO, CHLOR, and TET. Min et al. [28] showed that a concentration from 0.1 to 0.5 g L^{-1} of OXY only slightly inhibited AD, although a concentration of OXY in the range of 1–2 g L^{-1} decreased the biogas production from 199.6 to 41.9 L kg^{-1} vs. (volatile solids), respectively. These observations were confirmed in the present study, where the OXY concentration was

over 1 g L^{-1}, and the inhibition of CH_4 production was observed. Massé et al. [29] reported that TET reduced CH_4 production by 25% when it was included at the maximum allowable level in the diet of pigs. Arikan et al. [9] reported that cumulative biogas production was 27% lower from anaerobic digesters containing cattle manure treated with 0.2 g kg^{-1} per day OXY for 5 days compared to manure from untreated calves. Wang et al. [15] showed that when the concentration of an antibiotic reached 0.1 g L^{-1}, the rate at which biogas was produced in AD decreased drastically. Having added antibiotics to substrates, these authors noted the accumulation of VFAs in bioreactors, which led to the inhibition of CH_4.

Based on the results of this and other studies it was concluded that drugs significantly reduce the amount of biogas produced. However, the impact of antimicrobials depends on their class, dosage, and the method by which the residues are transferred to bioreactors (i.e., direct supplementation to bioreactor or as a content of manure) [30].

3.1. Fate of Antibiotics During AD

The AMO was not detectable in any fraction of the CM after the AD process—neither in the liquid fraction nor in the solid phase of the samples. Ampicillin was detected only in the liquid phase of the samples, while MET was determined only in the solid phase of the samples (the concentration did not exceed 0.02 μg $g_D{}^{-1}$). Other compounds were detectable in post-treatment samples in both the liquid and solid phases. The results allowed us to estimate the values of drug degradation coefficient (K_d) parameters (according to Equation (1) Section S2.2 in Supplementary Materials), which are known to be a measure of sorption affinity on solid (suspended) particles of a sample. The determined sorption parameters of the tested compounds (Table S5) allowed us to estimate the total compounds concentration in the bioreactor according to Equation (2) (Section S2.2 in Supplementary Materials), (Table S6), which subsequently enabled the calculation of total removal efficiency (Equation (3) (section S2.2 in Supplementary Materials) (Table S7)).

The calculated values of K_d parameters indicated that none of tested antibiotics showed very strong affinity for the solid fraction of the CM (Table S7). The highest values of these coefficients were observed for TET (log K_d = 1.6), although they indicate only moderate affinity for sorption onto the solid (suspended) fractions. Bajkacz et al. [17] observed that the antibiotics, such as tetracyclines and quinolones, were fairly stable under the conditions of anaerobic digestion. Álvarez et al. [30] observed that CHLOR and OXY showed a strong tendency to be adsorbed onto the solid fractions of manure (especially fresh one). Loke et al. [31] reported that OXY binds readily to organic molecules in manure. Kühne et al. [32] reported a TET half-life of 9 days in non-aerated pig manure at ambient temperature. Arikan [33] investigated the fate of CHLOR in calf excrements and found that the concentration of the drug decreased by 75% in a 33 day digestive period, but the concentration of the drug metabolite isochlortetracycline increased 2 fold over the same period. Both of these compounds are able to form strong complexes with divalent cations, which are abundant in pig manure, and demonstrate the capacity to be adsorbed onto proteins and organic matter [31]. However, as a result of biochemical processes occurring during the AD, organic biomass undergoes transformation into biogas and, thus, the solid fraction of the manure remaining after the process significantly changed its physico-chemical properties (including the content of organic compounds). Therefore, the values of K_d coefficients determined in this experiment indicated much greater affinity of the tested compounds for the liquid than the solid phase. In addition, the concentrations of antibiotics used in the investigations were higher than those described by Alvarez et al. [30] and probably the adsorption of these substances could be limited by the available superficial area of the solid fraction of the inoculum and the manure, especially that the hydration of the samples was very high and exceeded 93% (Table S8).

In the experiment, the antibiotics were degradable at varying levels, from 57% (OXY) to 100% (MET, AMO, AMP and SMX). However, even for substances for which the complete degradation was observed, their transformation by-products were present in the solid phase

of the digested CM. Due to the nature of the experiments carried out, the transformation by-products were identified only tentatively, based on the comparison of data from previously published references [2,34] and taking into account collision-induced dissociation mass received in different modes of operation of the mass spectrometer such as: precursor ion, product ion(s) and neutral loss. Some authors [30,35] stated that mainly isomeric transformations at the molecular level may occur at the beginning of the AD. The formation of epimers of TET and CHLOR, that is 4-epi-tetracycline and 4-epi-chlortetracycline, respectively, are mentioned as examples of such transformations [30], but none of these by-products were observed in this study. However, in the case of other antibiotics, such as SMX, AMO, and AMP, the formation of by-products with the same molecular weight (but different retention time) was observed. These products were tentatively identified as 4-amino-N-(5-methyl-1,3-oxazol-2-yl)benzenesulfonamide or 4-amino-N-(5-methyl-1,2-oxazol-3(2H)-ylidene) benzenesulfonamide, diketopiperazine amoxicillin, and diketopiperazine ampicillin. The explicit confirmation of the structures of the tentatively identified transformation by-products requires further research consisting of the comparison of the precursor and product ions as well as the retention times obtained for the tentatively identified by-products with the same parameters of the analytical standards of these substances. However, this may be a certain analytical challenge because the analytical standards of these by-products are not available commercially.

3.2. Impact of AD on Microbial Community

The taxonomic distribution of bacterial diversity as percentages of groups at the phylum level in digestate was visualized with Circos (Figure 1). The analysed digestate mass via the profiling of the gene 16S rRNA, was found to contain 324 OTUs.

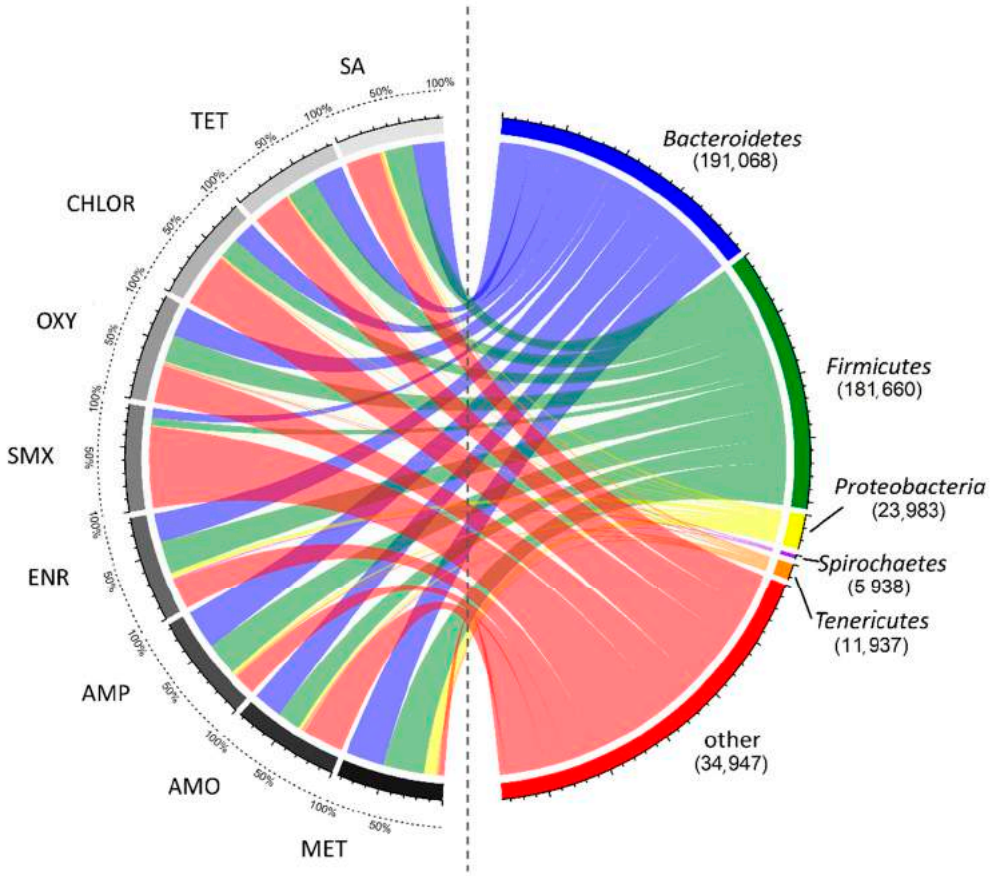


Figure 1. The taxonomic distribution of bacterial diversity as percentage values at the phylum level in digestate. Data visualised in Circos, where on the left is the percentage of microorganisms in the bioreactor and on the right is the sum of sequencing readings for a particular bioreactor.

The high-throughput sequencing analysis revealed considerable changes in the population of microorganisms in digestate depending on the added antibiotic. Among the identified OTUs in the analysed samples of digestate, the most numerous were the microorganisms of the types *Bacteroidetes* and *Firmicutes* which can decompose VFAs, are tolerant to fluctuations of temperature, pH and the availability of oxygen, and are characterised by the potential capability of hydrolysis and hydrogenogenic acidogenesis [36]. As well as degrading proteins, most bacteria of the type *Bacteroidetes* produce lytic enzymes and acetic acid and are widespread in AD of different substrates [37]. Previous studies also showed that the *Firmicutes* to *Bacteroidetes* ratio can serve as an indicator of the stability of AD [38]. In the digestate with added MET or AMP, the *Bacteroidetes* type made up almost 40% of the microbial community, while TS was reduced in the digestate with MET, and CH_4 yield decreased in both digestate samples. In the digestate with AMO, ENRO, OXY or TET, *Bacteroidetes* constituted nearly 30% of all identified microorganisms, while the yield of CH_4 was found to be lower (decreased by three-fold in comparison to SA) and the TS content was decreased relative to the SA variant. The *Bacteroidetes* and *Firmicutes* types made up 22% each of the microbial community in the digestate supplemented with CHLOR. A sample of the digestate including SMX showed the most extensive changes in the microbial community relative to the SA. Numerous genera *Bacteroides* and *Paludibacter* belonging to the type *Bacteroidetes* were found in all bioreactors with the supplementation of antimicrobials (Figure S1). Microorganisms belonging to the *Bacilli* and *Clostridia* classes dominated among the *Firmicutes* type. In most of the bioreactors, numerous reads of the genera *Bacillus*, *Rumellibacillus*, *Enterococcus*, *Turicibacter*, belonging to the *Bacilli* class, were achieved. However, the number of reads per sample for the most of drug-supplemented bioreactors was significantly lower compared to the SA. The types *Bacteroidetes* and *Firmicutes* represented 10% each of the community of microorganisms in the digestate with SMX. It is highly probable that during the process of SMX transformation, the antibiotic degradation products [17] caused a decrease in the abundance of microorganisms, thereby leading to a fall in CH_4 yield due to the insufficient amounts of substrate supplied by bacteria to methanogenic microorganisms.

Microorganisms of the type *Proteobacteria* represented 11.8% of the OTUs in digestate with MET, 5.99% in digestate with ENRO and nearly 4% in samples of digestate with AMO and AMP. Meanwhile, *Proteobacteria* made up slightly over 1% of the microbial community in digestates supplemented with TET drugs. Among the microorganisms belonging to the Proteobacteria type, the *Alpha-*, *Beta-* and *Gammaproteobacteria* classes were dominant. The predominant genus among *Alphaproteobacteria* belonged to the order *Rhizobiales*. The *Oligella* genus belonging to the order *Burkholderiales* dominated among *Betaproteobacteria*. Among the *Gammaproteobacteria*, numerous genera of *Pseudomonas* belonging to the order *Pseudomonadales* were noted. *Proteobacteria* play a very important role in AD by consuming glucose, acetate and propionate (VFAs), which—if accumulated in large amounts—may cause a decrease in pH, which most often leads to some disorder or inhibition of the process [6,39]. Other important types of microorganisms included *Spirochaetes* (more than 1% in samples with AMO, ENRO or OXY), genera *Treponema* and *Tenericutes* (over 2% in digestate with MET, AMO, AMP and 1% in sample with TET), genus *Acholeplasma* (over 1% in samples with MET, AMO, AMP, ENR, OXY, CHLOR and TET). Sequencing revealed a few readings for the *Archaea* domain. The genera *Mathanosarcina* and *Methanosaeta* were identified most frequently (over 1% sequentially in samples with MET, AMP, SMX, OXY, CHLOR, TET and in samples with MET, AMP, ENRO, OXY, CHLOR, TET) which is consistent with the qPCR analysis conducted in this study, targeting the MSC and MST genes, specific for the *Methanosarcinaceae* and *Methanosaetaceae* families (Figure 1 and Figure S2).

Summarising, the microbial community was mainly represented by *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Spirochaetes* and *Tenericutes*, which is consistent with the results of experiments conducted by other researchers [6,40,41]. The identified microorganisms are characterised by hydrolytic capability, and products of hydrolysis serve as substrate

to microorganisms involved in acidogenesis in order to produce VFAs. *Bacteroidetes*, *Firmicutes* and *Proteobacteria* can decompose VFAs and use them as a source of carbon for their metabolic activity. In contrast to SA, most of the analysed digestate samples (except the one with AMO and TET) were not detected to contain VFAs. It is highly probable that microorganisms present in digestate, by using the available substrates as a source of carbon [42,43], diminished the supply of VFAs to acetogenic microorganisms, which in turn, lacking sufficient amounts of substrate, decreased the rate of their production as the substrate for methanogenic microorganisms. This led to the disturbance of the methanogenesis pathway and to the decrease of the efficiency in CH_4 production in all samples of digestate supplemented with antibiotics. The profiling of the 16S rRNA fragment suggests that the identified *Bacteroidetes* and *Firmicutes* can play a significant role in degradation and removal of TS. Based on the research results presented herein, it can be concluded that the types *Bacteroidetes* and *Firmicutes*, in the presence of antibiotics, did not supply adequate quantities of substrates for biogas producing methanogenic microorganisms. Delbès et al. [44] also emphasise that the community of microorganisms engaged in AD can be affected by changes in the environmental conditions, which can lead to reducing the productivity of a reactor or to processing errors, such as excessively lowered pH causing the acidification of the environment and decreased CH_4 yield, a consequence of which, with varying degrees of intensify, was observed in all the digestate samples analysed in this study.

3.3. Impact of AD on the Prevalence of mcrA, MSC and MST Genes

3.3.1. *mcrA* Gene

The absolute abundances of *mcr*A gene copies (in the order of 10^1 to 10^8 copies $g_D{}^{-1}$) can reflect the level of methanogens participating in AD (Table 2) [6,45].

Relative abundances (RAs) of the number of genes are shown in Supplementary Materials (Table S9). In this research, compared to the SA, statistically significant (ANOVA Kruskal–Wallis; $p < 0.05$; $N = 6$) differences in the number of copies of the gene *mcr*A among samples of digestate with added antibiotics were observed (Figure S3). An increase in the number of copies of the gene *mcr*A in the order of 10^6 copies $g_D{}^{-1}$ was observed in the presence of OXY, CHLOR and TET. The *mcr*A gene encoding the subunit of α reductase of methyl-coenzyme M, which catalyses the final stage of methanogenesis, is described as a potential biomarker of the CH_4 yield during methanogenesis [46]. A decrease in the number of copies of the gene *mcr*A in comparison with SA was observed in the digestate samples with MET, AMO, AMP and SMX. An increase in the number of copies of the gene *mcr*A in digestate samples supplemented with tetracycline drugs alongside a decrease in CH_4 yield can indicate that these drugs exerted the selection pressure on methanogenic microorganisms throughout the experiment. As TET is a much more difficult xenobiotic compared to the substrate mixture fed to the bioreactor, it is more likely that the main mechanism for TET removal observed in this study is metabolite formation rather than biodegradation. Tetracyclines inhibited methanogenic microorganisms, but only slightly, as evidenced by a low amount of accumulated VFAs in bioreactors during the AD process [47]. In addition, due to the long-term selection pressure of tetracycline on microorganisms, they developed adaptation and resistance mechanisms, as evidenced by the high number of copies of genes characteristic of methanogens. This interpretation was also fully supported by a metagenomic analysis of the microbial community based on sequencing techniques which revealed numerous readings for the methanogenic *Archaea* (Figure S2).

Table 2. Absolute abundances (AAs) of genes specific for *Archaea*, antibiotic resistance genes, and integrase genes in digestate with antibiotic addition and substrate without antibiotic addition. AAs were calculated as copy number of an antibiotic resistance genes in 1 g digestate ($g_D{}^{-1}$).

	Genes Specific for *Archaea*			**MLS Group**			**Fluoroquiolones**		**Tetracyclines**			**Sulfona-mides**	**Beta-Lactams**			**Integrase Genes**	
	***mcr*A**	***MSC***	***MST***	***erm*F**	***lin*A**	***mef*A**	***qep*A**	***aac6′-Ib-cr***	***tet*A**	***tet*M**	***tet*Q**	***sul*1**	bla_{TEM}	bla_{OXA}	***cfx*A**	***int*I1**	***int*I2**
MET	3.14×10^6	2.14×10^6	9.00×10^4	7.59×10^5	1.03×10^5	1.14×10^7	2.37×10^5	3.41×10^5	6.10×10^7	3.97×10^8	2.56×10^7	1.70×10^7	6.60×10^4	0	5.03×10^5	1.96×10^6	1.16×10^7
AMO	6.63×10^6	5.94×10^6	7.15×10^5	3.71×10^6	9.62×10^5	1.28×10^7	1.58×10^9	2.54×10^6	5.73×10^7	4.05×10^8	3.36×10^8	3.46×10^7	6.57×10^4	8.11×10^5	4.56×10^6	3.91×10^6	1.71×10^7
AMP	3.04×10^7	3.74×10^7	9.35×10^6	2.16×10^7	6.09×10^6	1.27×10^8	3.03×10^9	2.41×10^7	1.76×10^9	8.95×10^9	2.26×10^9	6.18×10^8	1.81×10^5	0	5.41×10^7	8.82×10^7	3.58×10^8
ENR	6.72×10^8	8.02×10^7	5.85×10^6	7.36×10^6	2.44×10^5	3.99×10^6	2.30×10^8	9.59×10^5	1.61×10^8	1.53×10^8	1.15×10^8	1.81×10^7	2.66×10^5	0	7.10×10^6	6.54×10^6	2.16×10^7
SMX	2.10×10^8	1.13×10^7	3.26×10^6	4.33×10^6	4.43×10^5	2.42×10^6	7.00×10^8	1.02×10^6	4.76×10^7	1.29×10^8	4.64×10^7	3.34×10^7	4.12×10^4	0	1.76×10^6	2.35×10^6	1.37×10^7
OXY	1.52×10^8	9.02×10^6	2.25×10^6	2.01×10^6	1.54×10^5	8.50×10^6	1.73×10^9	1.62×10^6	2.10×10^9	1.97×10^9	2.83×10^8	3.95×10^7	1.06×10^4	7.53×10^5	4.90×10^6	2.79×10^6	7.57×10^6
CHLOR	1.11×10^9	4.55×10^7	8.63×10^6	1.41×10^8	4.96×10^5	2.26×10^7	1.31×10^6	4.01×10^6	1.44×10^9	1.82×10^9	1.86×10^8	1.09×10^8	1.01×10^5	0	3.90×10^6	1.43×10^7	3.49×10^7
TET	1.05×10^9	9.75×10^7	2.30×10^7	3.37×10^8	3.24×10^5	2.12×10^7	1.30×10^9	2.13×10^6	2.36×10^9	1.74×10^9	1.30×10^8	7.70×10^7	5.05×10^4	0	7.40×10^6	1.58×10^7	3.75×10^7
SA	5.00×10^8	1.73×10^7	6.03×10^6	2.37×10^8	4.37×10^5	7.49×10^6	1.09×10^9	1.14×10^6	4.11×10^8	2.10×10^8	6.14×10^7	3.41×10^7	6.16×10^4	0	2.54×10^6	3.12×10^6	1.01×10^7

The high drug degradation coefficient (K_d) achieved in this study suggests the presence of a small amount of tetracyclines in digestate throughout the whole experiment. Alvarez et al. [30] report that the half-time of adsorbed tetracyclines in manure is longer than in other environments. Martinez [48] and Wu et al. [49] also observed increased adsorption of tetracycline after the AD process, which was demonstrated to improve the stability of tetracyclines and to minimise their degradation. The results of this study suggest that microorganisms may have used the tetracycline as a source of carbon [50], and that these drugs simultaneously inhibited the expression of the gene *mcr*A. Consequently, the concentration of the gene *mcr*A was high but microorganisms were not active metabolically and produced less CH_4.

3.3.2. *MST* and *MSC* Genes

Presence and number of genes characteristic of the two families of acetoclastic methanogens: *Methanosarcinaceae* (*MSC*) and *Methanosaetaceae* (*MST*), were examined during AD. These methanogens require higher concentrations of acetic acid, which they convert to CH_4; they are characterised by a more rapid rate of substrate conversion than other families of microorganisms [51], and they constitute the majority of *Archaea* in fermentation chambers. In this experiment, statistically significant ($p < 0.05$) differences were determined in numbers of *MSC* and *MST* genes between digestate samples (Figure S3). High concentration of *MSC* and *MST* genes was noted in samples of digestate with AMP, OXY, CHLOR and TET (number of copies of the genes in the order of 10^6 to 10^7 $g_D{}^{-1}$) (Table 2). In the other samples with antibiotics, there was a decrease in the number of copies of the genes *MSC* and *MST* in comparison with SA, possibly indicating the inhibitory effect of MET, AMO, ENR and SMX on the growth and development of the methanogenic organisms described in this paper. Bauer et al. [52] noted small numbers of the *Methanosaetaceae* and *Methanosarcinaceae* in an AD system following the application of antibiotics. Turker et al. [53] also noted reduction in the abundance of *Methanosarcinaceae* in AD of CM with antibiotics. The family *Methanosaetaceae* contains strictly anaerobic microorganisms, extremely sensitive to changes occurring in the environment during AD, especially to the addition of inhibitors like antibiotics, to low pH or to fluctuations in temperature [25].

3.4. Impact of AD Process on the AAs of Analysed ARGs and Integrase Genes, and Their Prevalence in Bioreactors

3.4.1. *tet* Genes

Statistically significant ($p < 0.05$) differences were determined in the number of copies of *tet* genes (in the order of 1.0×10^8 $g_D{}^{-1}$) between samples of digestate with the content of different antibiotics (Figure S4). In most digestate samples, compared to the control, there were numerous ARGs carrying resistance to tetracyclines (Table 2) which agrees with the results provided by other authors [30,49]. In this study, the concentrations of tetracycline antibiotics added to substrates most probably effected selective pressure on the number and dissemination of ARGs in digestate from CM. *tet* genes are widespread in CM and the antibiotics present in AD systems additionally induce the increasing resistance to these antibiotics [54,55], which explains their high abundance in digestate samples observed in this experiment. It is possible that *tet* genes are a stable part of the genomes of animals' physiological microbiota [56] and, therefore, are evacuated from intestines to animal manure, where DR can be spread via HGT. In this study, samples of digestate with MET or SMX were observed to contain a lower number of copies of the *tet*A and *tet*Q genes by one order in comparison to the SA.

3.4.2. *bla* Genes

The AAs of the *bla* genes in $1 g_D{}^{-1}$ varied within the order of 10^4–10^7 of copies of the gene (Table 2). In comparison with SA, statistically significant ($p < 0.05$) differences were observed in the AAs values for the bla_{TEM} gene. The number of copies of the bla_{TEM} gene was higher by one order in 1 g of a sample of digestate with added AMP, ENR or CHLOR

(Figure S5). In addition, an evident increase in the number of copies of the *cfx*A gene by one order in 1 g of digestate in the presence of AMP was observed relative to the SA. Many authors [57,58] describe the bla_{TEM} gene as the one most frequently present among the *bla* genes in environmental samples. The presence of the bla_{OXA} gene was unobserved in most of the analysed digestate samples. Degradation of beta-lactam antibiotics in the AD process could have been the reason why the bla_{OXA} gene was absent. Bacteria show resistance to beta-lactam antibiotics through the acquisition of beta-lactamase genes, responsible for the tolerance to these drugs. Beta-lactamases react on the beta-lactam ring of beta-lactams, which enable the bacteria to acquire resistance to these antibiotics. However, the instability of the beta-lactam ring in beta-lactam antibiotics can cause their rapid degradation in the environment, and therefore prevent the bacteria present in this ambient to gain drug-resistance to these antibiotics [59,60].

3.4.3. *sul*1 Gene

Compared to SA (number of copies of the gene *sul*1 in the order of 10^7 $g_D{}^{-1}$), statistically significant ($p > 0.05$) differences were determined between samples of digestate with the content of different antibiotics in the number of the *sul*1 genes (Figure S6). An increase in the number of the *sul*1 gene by one order (10^8 copies of the gene $g_D{}^{-1}$) was determined in samples of digestate with AMP, TET and CHLOR (Table 2). The formation of transformation products during AD depends on the type of an antibiotic and its concentration added to the substrate in a bioreactor [61]. Complete degradation of SMX was observed in this study, although it is possible that products of the transformation of this compound may appear and remain in digestate mass. Bajkacz et al. [17] observed SMX transformation products in the cattle manure digestate in which the change occurred within the isoxazole ring. The authors noted that epimerisation of antibiotics appears to be the first step in transformation under the anaerobic conditions of the AD process. Similar results were also observed by Spielmeyer et al. [2]. It is highly probable that the supplementation of SMX to substrate caused selection pressure and increased number of ARGs to this drug. On the genetic level, DR results in the presence of ARGs in chromosomes and/or in MGEs, such as integrases, noted in high numbers of the digestate samples analysed in this research [56]. The factors mentioned above, such as selection pressure or presence of the integrase genes, are closely connected with the abundance of ARGs in the AD process, causing mutations of ARBs (antibiotic resistance bacteria) and acquisition of new features, including DR [21,62].

3.4.4. MLS Group Genes

The value of AAs of the *erm*F, *lin*A, *mef*A genes ranged from 10^5 to 10^8 copies of the gene $g_D{}^{-1}$ (Table 2). Relative to SA statistically significant differences were noted in the number of the *mef*A gene between samples of digestate supplemented with antibiotics. In comparison with SA (number of copies in the order of 10^6 $g_D{}^{-1}$), the *mef*A gene appeared in high numbers in digestate with MET, AMO, AMP, CHLOR and TET. In samples of the digestate with AMP, compared to SA, a rise in the number of the *mef*A gene by two orders was noted (Figure S7). In samples of digestate with added MET, AMO, CHLOR or TET, the *mef*A gene was present in quantities in the order of 10^7 copies $g_D{}^{-1}$. Small amounts of metronidazole and considerable amounts of tetracycline drugs were detected in digestate after the AD process. The antibiotics present in digestate may have induced an increase in the DR to these pharmaceutical substances. A significant role in the increase of ARGs in digestate samples may have also been played by the ongoing HGT processes. According to Bouanane-Darenfeld et al. [63] it is also possible that some genera of the methanogenic *Archaea*, including *Methanosaeta*, found in digestate samples are potential hosts of ARGs, responsible for the resistance to MLS antibiotics.

3.4.5. Fluoroquinolones Resistance Genes

In comparison with SA, particularly high and statistically significant (ANOVA Kruskal–Wallis; $p < 0.05$; $N = 6$) differences in the number of the *qep*A genes (1.0×10^9 copies of

the gene g_D^{-1}) were determined for samples of digestate with AMO, AMP and OXY (Table 2) (Figure S8). Fluoroquinolones are compounds that adsorb strongly to organic matter present in all types of faeces [64], owing to which their degradation is much weaker than that of other antibiotics, like beta-lactams. Selvam et al. [55] noted the presence of genes carrying resistance to fluoroquinolones after 56 days of composting pig manure. Qu et al. [65], who analysed poultry droppings, noted numerous presences of genes resistant to fluoroquinolones. In this study, a decrease in the numbers of the *qep*A and *aac 6′-Ib-c*r genes was observed only in the digestate with added MET.

3.4.6. Integrase Genes

The value of AAs of the analysed ARGs in most of the samples of digestate with added antibiotics ranged within 10^6 to 10^8 copies of the gene g_D^{-1} (Table 2, Figure S9). The number of genes in the SA oscillated around 10^6 to 10^7 copies of the gene g_D^{-1}. The high number of copies of the genes *intI*1 and *intI*2 in samples of digestate supplemented with antibiotics and the SA sample can testify to the possibility of spreading ARGs through the HGT process. The abundance of the genes *intI*1 and *intI*2 can therefore serve as an indicator of the HGT processes [66]. In this experiment, the concentrations of *intI*1 and *intI*2 in most samples of digestate supplemented with antibiotics increased by one order in comparison to SA samples (ANOVA Kruskal–Wallis; $p < 0.05$; $N = 6$). A similar number of copies of the tested integrase genes in an AD system may indicate the adaptation of microorganisms to the concentrations of antibiotics applied at the onset of the AD process [6]. The results obtained in this study are consistent with the ones reported by other authors. Gaze et al. [66] showed that the selection pressure exerted by antibiotics can increase the number of copies of the integrase genes. Allen et al. [57] investigated the share of sequences associated with transposition elements and plasmids in the microbiome of farm animals and recorded their higher contribution in animals treated with antibiotics than in the control group. These authors concluded that antibiotics could induce DR as well as the growth and spread of ARGs on a broad scale in the environment. However, it is HGT that remains the main mechanism for the dissemination of ARGs in the environment, which poses an immense threat to animal and human health due to DR, as well as limiting the possibilities of controlling this phenomenon [67].

3.5. Results of Statistical Analyses

The Spearman's correlation analysis applied to investigate ARGs, genes specific for *Archaea* and integrase genes in 1 g of digestate, showed very strong, statistically significant positive correlations between numbers of these genes ($R > 0.8$) (Figure 2).

The *erm*F gene highly correlated with the genes *MST* ($R = 0.8153$, $p < 0.05$), *mcr*A and *MSC* ($R = 0.8158$, $p < 0.05$). High correlation also appeared between the number of *sul*1 and integrase genes, with the correlation with *intI*1 and with *intI*2 reaching $R = 0.8184$ ($p < 0.05$). Previously published studies have also confirmed the positive correlation between ARG and *intI*1 and antibiotic concentrations in livestock and poultry faeces [68]. Moreover, Luo et al. [69] found that *intI*1 was associated with the spread of *sul*1 and *sul*2.

High, statistically significant correlation between genes can suggest that the microorganisms present in digestate may have been drug-resistant, and the selection pressure exerted by antibiotics caused their growth and the spread of DR during the AD process. The genes *intI*1 and *intI*2 showed statistically significant ($p < 0.05$) positive correlations with most of the analysed ARGs (R = od 0.60 do 0.82), which suggests an important role of these genes in HGT. The results of the statistical analysis performed in this study overlap with the data reported by other researchers, who demonstrated a strong and significant correlation between ARGs and the presence of integron [70].

High, statistically significant correlations ($p < 0.05$) were determined between the type *Spirochatetes* and the gene bla_{OXA} ($R = 0.70$) (Figure 3), which implicates the succession of *Spirochaetes*, which can condition changes in ARGs present in digestate obtained from cattle manure [71].

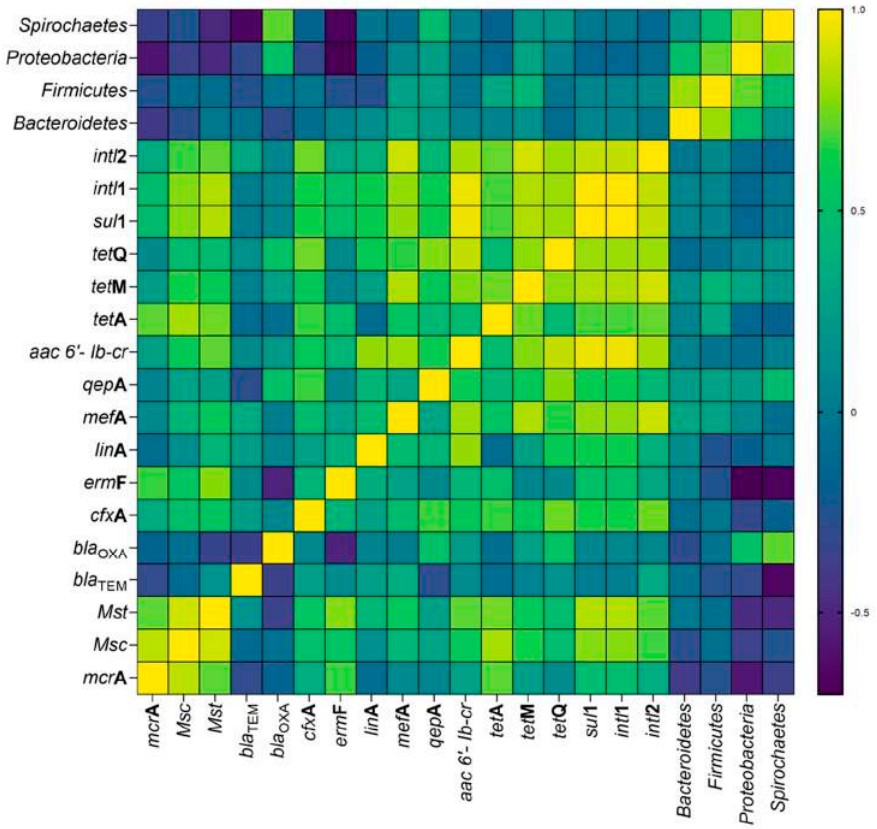


Figure 2. Spearman's rank order correlation analysis between studied genes.

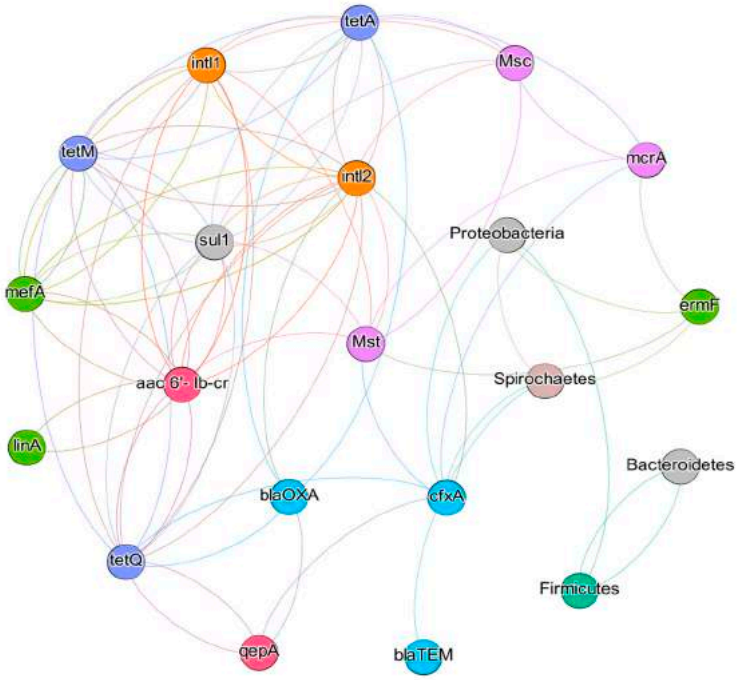


Figure 3. The network analysis showing the co-occurrence patterns between the detected ARGs and microbial taxa (type level). The nodes were coloured according to ARGs types and bacterial taxa. The connection between ARGs and bacterial taxa represents a strong (Spearman's correlation coefficient $R > 0.7$) and significant (p-value < 0.01) correlation. Node size is proportional to the number of connections; edge width is proportional to Spearman's p-value.

Proteobacteria and *Spirochaetes* demonstrated a high, statistically significant ($p < 0.05$) mutual correlation ($R = 0.77$) and correlated negatively with the gene *erm*F ($R = -0.70$), indicating that their hosts were different [41]. The type of bacteria *Firmicutes* highly correlated with the type of bacteria *Bacteroidetes* ($R = 0.70$) and with *Proteobacteria* ($R = 0.72$) (Figure 3). Consistent results of studies carried out by many researchers provide direct evidence that the spread of ARGs can be correlated with residues of antibiotics in the environment, and this information should improve the current understanding of the distribution of ARGs and their connection with the succession of microbial communities.

3.6. Impact of Physicochemical Parameters on the Microbial Community in Bioreactors

Zhang et al. [6] report that fluctuations of pH and accumulation of VFAs in bioreactors can lead to disturbances in the AD process and decrease in the efficiency of methanogenesis. In this experiment, no significant changes in pH or in concentrations of VFAs were noted. It can therefore be concluded that these factors were not responsible for changes in the community of microorganisms or the observed decrease in the yield of CH_4 in most of the analysed digestate samples.

3.7. Future Prospects

This work concluded that antibiotics had the greatest impact on the AD process. The antimicrobials showed high toxicity to microorganisms, which directly reduced the production of biogas. To reduce antibiotic toxicity in digesters treating cattle manure co-fermentation is recommended, i.e., digesting the manure with the addition of, for example, cheese whey [72], which is locally available and easily biodegradable substrate. This method can significantly improve the stability of AD process range compared to the decomposition of a single substrate. The implementation of anaerobic digestion in full-size biogas plants located on large farms can be an environmentally friendly solution ensuring energy security for the farm, allowing for the reduction of transport and management costs. However, the condition for achieving these benefits is rational management of antibiotics and limiting their supply to animals.

4. Conclusions

The research has demonstrated the inhibitory effect of antibiotics on the efficiency of AD and CH_4 production. The AD process in the presence of antibiotics also stimulates an increase in the number of ARGs and their spread in digestate samples. The antibiotics tested in this study were toxic not only to fermentation bacteria but also to methanogens, responsible for CH_4 production. Antibiotics caused a change in the proportions of counts of bacteria relative to *Archaea*. In conclusion, it is necessary to monitor the AD process in biogas plants, the digestate obtained from this process and the environment to which such digestate is introduced. This work will enrich the database supporting the design and commissioning of the full-scale processes.

Supplementary Materials: The following are available online at https://www.mdpi.com/1996-1073/14/4/1125/s1, S1. Anaerobic digestion and Methane fermentation. S2. Analyses of antibiotics concentration. S3. Analyses of genes concentration. S4. Statistical analyses. Table S1: The characteristics of cattle manure used as substrate in AD process and anaerobic sludge used as inoculum. Table S2: The concentrations of antibiotics used in the investigations, per unit of reactor feed volume. Table S3: Primers sequences and parameters use for qPCR analysis. Table S4: The FOS/TAC ratio, concentration of nitrogen and phosphorus in biomass, and pH value in digestate samples from methane fermentation of cattle manure with the addition of selected antibiotics and in bioreactor with substrate without antibiotic supplementation (SA). Table S5: LC-MS/MS parameters for the investigated compounds. Table S6: Total compound concentration at the beginning of the experiment (C0) and in the equilibrium conditions (after the process) (Ct), per unit of reactor volume, $\mu g\ L^{-1}$. Table S7: Concentrations, sorption parameters and removal efficiency of the test substances during the anaerobic digestion. Table S8: Hydration of samples after the anaerobic digestion process. Table S9: Relative abundances (RAs) of genes specific for *Archaea* antibiotic resistance genes, and integrase

genes in digestate with antibiotic addition and substrate without antibiotic addition. The relative abundances (RAs) of the ARGs were calculated as: copy number of ARG/copy number of 16S rRNA. Figure S1: Average values (± standard deviation) of methane production during anaerobic digestion in bioreactors with (A) MET, (B) AMO, (C) AMP, (D) ENR, (E) SMX, (F) OXY, (G) CHLOR, (H) TET supplementation, and (I) control reactor (SA). All the results presented are the means of two independent assays. Figure S2: Detailed taxonomy and community structure of *Archaea* and bacteria (based on the OTUs) up to species in digestate with supplementation of selected antibiotics and in bioreactor with substrate without antibiotic supplementation (SA). Figure S3: Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of genes specific for *Archaea* like (a) *mcr*A gene, (b) *MSC* gene, (c) *MST* gene are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant). Figure S4: Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of *tet* genes like (a) *tet*A, (b) *tet*M, (c) *tet*Q, are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant). Figure S5: Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of *bla* genes like (a) *bla*TEM, (b) *bla*OXA, (c) *cfx*A, are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant). Figure S6: Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of *sul*1 genes are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant). Figure S7: Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of MLS group genes like (a) *erm*F, (b) *lin*A, (c) *mef* A, are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant). Figure S8: Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of fluoroquinolones genes like (a) *qep*A, (b) *aac-(6′)-Ib-cr* are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant). Figure S9: Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of integrase genes like (a) *intI*1, (b) *intI*2 are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant). References [73–84] are cited in the Supplementary Materials.

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The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure

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Supplementary to Materials and Methods

S1. Anaerobic Digestion and Methane Fermentation

Reactors with a volume of 250 mL were filled with 25 g of substrate and 175 g of inoculum for an initial organic loading of 5 g VS L^{-1}. Mixing in the reactors run for 30 seconds each 10 minutes. Rotating speed was 100 rpm. Anaerobic conditions were achieved by continuous flushing of pure nitrogen through the sludge. The Automatic Methane Potential Test System (AMPTS) II (Bioprocess Control, Lund, Sweden) was used to measure the quantity of produced CH_4. The quality of biogas was measured by using a gas chromatograph connected with thermal conductivity detector (GC-TCD) (Agillent 7890 A, Lund, Sweden). In the samples before and after fermentation the volatile fatty acids (VFAs) content, the pH, the FOS/TAC ratio (The FOS/TAC is the ratio of the concentration of volatile fatty acids (FOS) to the basicity (i.e., buffer capacity) (TAC) in the fermentation chamber), the TS, and VS, and the TN and TP content were determined (Table S1). The methane production from bioreactors followed first-order kinetic (Figure S1).

S2. Analyses of Antibiotic Concentration

S2.1. Samples Preparation, Standards and Chemicals

Chemical standards of pharmaceuticals: metronidazole (MET), amoxicillin (AMO), ampicillin (AMP), enrofloxacin (ENR), sulfamethoxazole (SMX), oxytetracycline (OXY), chlortetracycline (CHLOR) and tetracycline (TET) were purchased from Sigma–Aldrich (Saint Louis, MO, USA).

Acetonitrile and water were HPLC grade and were purchased from Merck (DarMstadt, Germany) as well as formic acid. Acetonitrile, methanol, ethyl acetate and ammonium hydroxide solution 25% (all of analytical reagent grade) were obtained from CHEMPUR (Piekary Śląskie, Poland). Acetic acid was provided by POCH S.A. (Gliwice, Poland) and citrate buffer (pH = 4.0) by Merck (Darmstadt, Germany) (both of analytical reagent

grade). Digestate samples were first centrifuged (20 min, 4000 rpm), and the supernatant was then decanted to separate the solid phase from the aqueous phase.

Liquid samples were filtered through a 0.22 μm syringe filter and analysed by LC–MS/MS. If the amounts of pharmaceuticals were above the calibration curve, the samples were diluted before analysis.

Lyophilized and homogenized digestate sample (0.2 g) was accurately weighed and extracted using different mixture of solvents. For extraction of OXY, TET and CHLOR mixture of citrate buffer (pH = 4.0) and acetonitrile (1:1, *v*/*v*). Two steps procedure (2% acetic acid in ethyl acetate and 2% acetic acid in methanol) was applied for extraction of AMO and AMP. Extraction of SMX, MET and ENR was performed in three steps using 2% ammonium hydroxide in methanol, 2% acetic acid in methanol and methanol, respectively. The digestate with 10 mL of solvents (listed above) was successively shaken for 1 h at 750 rpm (Vibramax 100, Heidolph Instruments GmbH & Co., Schwabach, Germany). Then, the extracts were filtrated, supernatants were combined and evaporated. The dry residue was dissolved in 15 mL of 5% methanol in water and then treated by the SPE procedure.

The SPE clean-up (CHROMABOND® SPE-system, Macherey–Nagel, Germany) was performed on an Oasis HLB cartridge (500 mg per 6 mL, Waters, Milford, MA, USA). The cartridge was preconditioned successively with 6 mL methanol and 6 mL water prior to sample load. After sample passage, the cartridge was dried under vacuum for 15 min. The analytes were then eluted with 6 mL of 0.1% acetic acid in methanol. The eluate was evaporated to just dryness. The sample was then reconstituted in 1 mL of 0.1% acetic acid, filtered through a 0.22 μm syringe filter and analysed three times by Liquid Chromatography—Mass Spectrometry (LC–MS/MS).

S2.2. Liquid Chromatography—Mass Spectrometry

Pharmaceutical determination was performed on an Dionex ultra high-performance liquid chromatography 3000 system (Dionex Corporation, Sunnyvale, CA, USA) coupled to a 4500 QTrap triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA).

Chromatographic separation was achieved on a ZORBAX SB-C3 column (150 mm × 3.0 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA). The column temperature was kept at 30 °C and the injected volume was 2 μL. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). For separation of the selected pharmaceuticals, elution started at 10% B, increased to 45% B in 3 min (flow rate 0.8 mL per min), to 90% B in 0.1 min, increased to 100% B in 2 min, back to the initial condition within 0.1 min and held for 3 min (flow rate 1 mL per min). The analyses were performed using the TurboV® (Waltham, MA, USA) ion spray in positive ionization mode (ESI+). The operating conditions for the analysis were the following: ion spray voltage, 4000 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 60 and 50 psi, respectively; probe temperature (TEM), 500 °C. Nitrogen served as the nebulizer and collision gas. Multiple reaction monitoring (MRM) experiments were performed to obtain the maximum sensitivity for the detection of target molecules. The optimization of MS parameters as declustering potential (DP), collision energy (CE) and entrance potential (EP) and collision cell exit potential (CXP) was performed by flow injection analysis (FIA) for each compound (Table S5). Analyst® version 1.4 software (Applied Biosystems/AB Sciex, https://sciex.com/content/SCIEX/na/us/en/products/software/analyst-software.html) was used to control all components of the system and also for data collection and analysis.

S2.3. Calculation of the Solid-Liquid Distribution Coefficient and the Removal Efficiency of the Selected Antibiotics

A measure of the affinity of a given substance for the solid or liquid phase, respectively, can be the parameter known as the solid-liquid distribution coefficient (K_d), which in an equilibrium state may be calculated using the Equation (1) [73]:

$$K_d = \frac{X_p}{S} = \frac{X}{X_{SS} \times S} \quad (1)$$

where:
K_d—solid-liquid distribution coefficient, L g_{SS}^{-1};
X_p—concentration of the compound sorbed onto suspended solids, per amount of dry matter of suspended solid, μg g_{SS}^{-1};
S—concentration of the compound dissolved in the liquid phase, μg L^{-1};
X—concentration of the compound sorbed onto suspended solids, per unit of reactor volume, μg L^{-1};
X_{SS}—suspended solids concentration in the reactor per L, g_{SS} L^{-1}.

Under equilibrium conditions, the total compound concentration in the reactor (C) can be expressed as (2) [73]:

$$C = X + S = S \times (1 + X_{SS} \times K_d) \quad (2)$$

where:
C—total compound concentration in the whole reactor, μg L^{-1}.

The parameters determined by means of Equation (2) may be used to calculate the total removal efficiency (R) of a given compound during the digestion process, according to the Equation (3):

$$R = \frac{C_0 - C_t}{C_0} \times 100 \quad (3)$$

where:
R—total removal efficiency, %;
C_0—initial total compound concentration in the reactor, μg L^{-1};
C_t—total compound concentration in the reactor after the digestion process, μg L^{-1}.

The abovementioned equations were used to calculate the corresponding parameters which are discussed later in the manuscript.

S3. Analyses of Genes Concentration

S3.1. Genomic DNA (gDNA) Isolation

The anaerobic digestion mixture samples (2 g) were transferred to centrifuge tubes with a capacity of 2 mL and centrifuged for 10 min at 8000 rpm. The supernatant was removed and pellet was stored in −20 °C for gDNA isolation. The gDNA was extracted from previously obtained pellets, using a Fast DNA Spin Kit for Soil® (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The total gDNA were eluted with 75 μL of provided DES (DNAse pyrogen free water) solution. Concentration and quality of extracted gDNA were determined with a spectrophotometer (Multiskan Sky, Thermo Scientific™, Waltham, MA, USA) and stored at −20 °C for further analysis.

S3.2. Quantitative Real-Time Polymerase Chain Reactions (qPCR)

A LightCycler® (Roche Diagnostics, Mannheim, Germany) was used to identify genes. All qPCR reactions were performed using 15 μL reaction with the master mix SYBR GREEN (Roche Diagnostics, Mannheim, Germany). The qPCR reaction mixture contained 0.8 μL of DNA template, 0.4 μL of each 10 μM primer, 7.5 μL of SYBR GREEN, and 5.9 μL of RNase-free water. The qPCR analysis was conducted according to the following settings shown in Table S3. The reactions were repeated in triplicate. Melting curves were generated and analysed to detect nonspecific amplification. The fluorescent signal data were processed using LightCycler ® software (version 1.5.0, https://lifescience.roche.com/en_de/products/light-cycler14301-480-software-version-15.html).

S3.3. Illumina MiSeq Sequencing

To identify the microbiome the high-throughput sequencing of the hypervariable region V3–V4 of 16S gene was performed with the Illumina MiSeq instrument (Seoul, Korea) (Table S3), using MiSeq Reagent v3 Kit (Macrogen, Seoul, Korea) with 2 × 250 bp paired-end reads and primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG-NGGC WGCAG) and 785R (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG-GACTACHVGGGTATCTAATCC) designed by Klindworth et al.,[74]. The quality control was performed using both FastQC software version 0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Qualitative Insights Into Microbial Ecology (QIIME2) version 2019.4 (https://qiime2.org/). Adapter trimming and quality filtration were carried out by using both Trimmomatic version 0.38 (http://www.usadellab.org/cms/?page=trimmomatic) [75] and QIIME2 and denoising, merging reads, and chimera removal were done by using DADA2 tool [76] in QIIME2 environment. The sampling depth was set to 17,200 reads per sample. The operational taxonomic units (OTUs) were classified basing on the predefined 99% OTUs Greengenes database version 13.8. A representative sequence of each OTU has been deposited in European Nucleotide Archive (ENA) database under the accession numbers PRJEB40682 (EMBL–EBI service, https://www.ebi.ac.uk/).

S4. Statistical Analyses

Data were analysed using Statistica 13.1 software (https://www.statsoft.pl/statistica_13/). Spearman's rank correlation coefficient was calculated to determine the correlations between the analysed genes. The distribution of variables was compared by the Kruskal–Wallis test. Network analysis based on the Spearman's Correlation analysis between ARGs and the bacterial community composition (based on OTUs) was determined using the Gephi 0.9.2 (https://gephi.org/) platform. Circos diagrams were generated using the online software Circos©2004-2016 (http://circos.ca/). Heatmap was created in GraphPad Prism 8.4 (https://www.graphpad.com/scientific-software/prism/).

Table S1. The characteristics of cattle manure used as substrate in AD process and anaerobic sludge used as inoculum.

	TS [a] g_D^{-1} (mg)	**VS [b] g_D^{-1} (mg)**	**pH**	**TP [c] g_{TS}^{-1} (mg)**	**TN [d] g_{TS}^{-1} (mg)**
Substrate	150.0 ± [e] 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] VS—volatile solids; [c] TP—total phosphorus; [d] TN—total nitrogen; [e] ±—standard deviation.

Table S2. The concentrations of antibiotics used in the investigations, per unit of reactor feed volume.

Class of Antibiotics	**Antibiotics**	**Concentration of Antibiotics (μg mL^{-1})**
Nitroimidazole derivatives	Metronidazole (MET)	512
Beta-lactams	Amoxicillin (AMO)	1024
	Ampicillin (AMP)	1024
Fluoroquinolones	Enrofloxacin (ENR)	1024
Sulfonamides	Sulfamethoxazole (SMX)	512
Tetracyclines	Oxytetracycline (OXY)	1024
	Chlortetracycline (CHLOR)	1024
	Tetracycline (TET)	1024
-	Substrate without antibiotic addition (SA)	-

Table S3. Primers sequences and parameters use for qPCR analysis.

Target Gene	Primer Sequence (5′-3′)	Annealing Temperature (°C)	Amplicon Size (bp)	References
*bla*TEM	AGTGCTGCCATAACCATGAGTG	58	431	[77] (Kim et al., 2009)
	CTGACTCCCCGTCGTGTAGATA			
*bla*OXA	ATTATCTACAGCAGCGCCAGTG		296	
	TGCATCCACGTCTTTGGTG			
*cfx*A	TGACTGGCCCTGAATAATCT	55	312	
	ACAAAAGATAGCGCAAATCC			
*erm*F	TAGATATTGGGGCAGGCAAG	58	126	[78] (Eitel et al., 2013)
	GGAAATTGCGGAACTGCAAA			
*lin*A	CTGGGGAGTGGATGTCTTGT	60	230	
	AGTTGGCTTGTTTGGAAGTG			
*mef*A	ATACCCCAGCACTCAATTCG	56	346	
	CAATCACAGCACCCAATACG			
*qep*A	CCAGCTCGGCAACTTGATAC	58	570	[79] (Li et al., 2012)
	ATGCTCGCCTTCCAGAAAA			
aac(6′)-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	52	482	[80](Park et al., 2006)
	CTCGAATGCCTGGCGTGTT			
*tet*A	GCTACATCCTGCTTGCCTTC	53	211	
	GCATAGATCGCCGTGAAGAG			
*tet*M	GTGGACAAAGGTACAACGAG	55	406	[81] (Nawaz et al., 2006)
	CGGTAAAGTTCGTCACACAC			
*tet*Q	TTATACTTCCTCCGGCATCG	55	904	[82] (Ng et al., 2001)
	ATCGGTTCGAGAATGTCCAC			
*sul*1	CGCACCGGAAACATCGCTGCAC	52	163	
	TGAAGTTCCGCCGCAAGGCTCG			
16S *r*RNA	AGAGTTTGATCATGGCTCAG	60	904	[83] (Nadkarni et al., 2002)
	GGTACCTTGTTACGACTT			
*intI*1	CCTCCCGCACGATGATC	54	280	[84] (Goldstein et al., 2001)
	TCCACGCATCGTCAGGC			
*intI*2	TTATTGCTGGGATTAGGC	54	233	
	ACGGCTACCCTCTGTTATC			
MSC	GAAACCGYGATAAGGGGA	60	408	[85] (Luton et al., 2002)
	TAGCGARCATCGTTTACG			
MST	TAATCCTYGARGGACCACCA	60	164	
	CCTACGGCACCRACMAC			
*mcr*A	GGTGGTGTMGGATTCACACARTAYGCWACAGC	95	467	
	TTCATTGCRTAGTTWGGRTAGTT			
V3-V4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	72	185	[74] (Klindworth et al., 2013)
	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC			

Table S4. The FOS/TAC ratio, concentration of nitrogen and phosphorus in biomass, and pH value in digestate samples from methane fermentation of cattle manure with the addition of selected antibiotics and in bioreactor with substrate without antibiotic supplementation (SA).

	FOS [a]/TAC [b] ratio	Nitrogen (mg g_{TS}^{-1})	Phosphorus (mg g_{TS}^{-1})	TS [c] (mg TS g^{-1})	VS [d] (mg VS g^{-1})	pH
MET	0.17 ± 0.02	2.6 ± 0.2	0.7 ± 0.3	46.5 ± 5.3	31.3 ± 7.4	8.7 ± 0.1
AMO	0.26 ± 0.00	3.0 ± 0.3	0.3 ± 0.4	62.4 ± 9.2	45.5 ± 6.3	8.6 ± 0.1
AMP	0.16 ± 0.02	2.6 ± 0.1	0.2 ± 0.5	51.1 ± 5.6	35.3 ± 8.3	8.7 ± 0.1
ENR	0.16 ± 0.02	2.7 ± 0.2	0.8 ± 0.5	31.0 ± 9.7	16.9 ± 8.0	8.7 ± 0.0
SMX	0.17 ± 0.03	2.3 ± 0.1	0.7 ± 0.3	45.8 ± 7.2	30.6 ± 4.9	8.7 ± 0.1
OXY	0.17 ± 0.02	2.6 ± 0.2	0.2 ± 0.5	43.2 ± 4.3	28.9 ± 6.2	8.7 ± 0.0
CHLOR	0.16 ± 0.02	2.4 ± 0.3	0.1 ± 0.2	47.7 ± 7.1	32.3 ± 6.9	8.7 ± 0.0
TET	0.18 ± 0.03	2.6 ± 0.4	0.2 ± 0.4	47.7 ± 4.8	32.2 ± 5.5	8.7 ± 0.0
SA	0.17 ± 0.02	2.7 ± 0.1	0.2 ± 0.3	48.4 ± 3.2	31.8 ± 5.2	8.7 ± 0.0

[a] FOS—volatile organic acids; [b] TAC—buffer capacity; [c] TS—total solids; [d] VS—volatile solids; ± indicates standard deviation.

Table S5. LC-MS/MS parameters for the investigated compounds.

Analyte	tR [a] (min)	Q1 [b] (m/z)	Q3 [c] (m/z)	DP [d] (V)	EP [e] (V)	CE [f] (V)	CXP [g] (V)	LOD [h] (ng g^{-1})	LOQ [i] (ng g^{-1})
MET	2.34	172.1	128.1	76	7	21	8	1.23	3.70
			82.1			33	6		
AMO	1.59	365.9	349.1	56	7	13	10	6.57	19.7
			114.1			31	8		
AMP	2.52	350.0	106.1	81	7	31	6	2.63	7.90
			160.2			19	14		
ENR	3.11	360.8	316.2	101	7	29	8	10.0	30.0
			245.2			27	10		
SMX	3.70	253.9	108.1	96	7	37	8	8.37	25.1
			92.1			43	6		
OXY	2.67	461.1	426.0	106	7	29	20	6.10	18.3
			443.0			17	14		
CHLOR	3.28	479.1	444.1	111	7	29	20	6.57	19.7
			462.0			23	18		
TET	2.89	445.1	410.2	106	7	27	12	5.97	17.9
			427.2			19	18		

[a] tR—retention time; [b] Q1—precursor ion; [c] Q3—fragment ion; [d] DP—declustering potential; [e] EP—entrance potential; [f] CE—collision energy; [g] CXP—cell exit potential; [h] LOD—limit of detection; [i] LOQ—limit of quantification.

Table S6. Total compound concentration at the beginning of the experiment (C_0) and in the equilibrium conditions (after the process) (C_t), per unit of reactor volume, μg L^{-1}.

Compound(s)	C_0 (μg L^{-1})	C_t (μg L^{-1})
MET	512,000	2.1 *
AMO	1,024,000	7.0 *
AMP	1,024,000	8.6 *
ENR	1,024,000	176,950.0
SMX	512,000	28.0
OXY	1,024,000	443,058.4
CHLOR	1,024,000	19,734.0
TET	1,024,000	370,339.0

* Calculated according to the limit of detection (LOD).

Table S7. Concentrations, sorption parameters, and removal efficiency of the test substances during the anaerobic digestion.

Compound	Xp (μg g^{-1})	S (μg L^{-1})	log Kd	R (%)
MET	0.02 ± 0.001	<LOD	n.a.	≈100
AMO	<LOD	<LOD	n.a.	100
AMP	<LOD	14.3	n.a.	≈100
ENR	4350.0 ± 254.56	42,100.0 ± 1272.79	2.0	83
SMX	0.24 ± 0.08	25.6	1.1	≈100
OXY	962.0 ± 43.08	401,500.0 ± 3535.53	0.4	57
CHLOR	163.5 ± 7.78	11,935.0 ± 2920.35	1.1	98
TET	5070.0 ± 1725.34	128,500.0 ± 13,435.03	1.6	64

Xp—concentration of the compound sorbed onto suspended solids; S—concentration of the compound dissolved in the liquid phase; LOD—limit of detection; log Kd—log of the calculated value of Kd (expressed in L kg^{-1}); n.a.—not available; ± indicates standard deviation.

Table S8. Hydration of samples after the anaerobic digestion process.

Compound(s)	Hydration (%)
MET	95.4
AMO	93.8
AMP	94.9
ENR	96.9
SMX	95.4
OXY	95.7
CHLOR	95.2
TET	95.2

Table S9. Relative abundances (RAs) of genes specific for *Archaea* antibiotic resistance genes, and integrase genes in digestate with antibiotic addition and substrate without antibiotic addition. The relative abundances (RAs) of the ARGs were calculated as: copy number of ARG/copy number of 16S rRNA.

	Genes Specific for *Archaea*			MLS Group		Fluoroquinolones			Tetracyclines		Sulfonamides			Beta-Lactams		Integrase Genes	
	*mcr*A	*MSC*	*MST*	*erm*F	*lin*A	*mef*A	*qep*A	*aac6′-Ib-cr*	*tet*A	*tet*M	*tet*Q	*sul*1	*bla*$_{TEM}$	*bla*$_{OXA}$	*cfx*A	*int*I1	*int*I2
MET	1.10×10^{-3}	7.51×10^{-4}	3.17×10^{-5}	2.67×10^{-4}	3.61×10^{-5}	4.00×10^{-3}	8.33×10^{-5}	1.20×10^{-4}	2.14×10^{-2}	1.40×10^{-1}	9.02×10^{-3}	5.97×10^{-3}	2.32×10^{-5}	0	1.77×10^{-4}	6.89×10^{-4}	4.08×10^{-3}
AMO	2.29×10^{-4}	2.05×10^{-4}	2.46×10^{-5}	1.28×10^{-4}	3.31×10^{-5}	4.42×10^{-4}	5.45×10^{-2}	8.74×10^{-5}	1.98×10^{-3}	1.40×10^{-2}	1.16×10^{-2}	1.19×10^{-3}	2.26×10^{-6}	2.79×10^{-5}	1.57×10^{-4}	1.35×10^{-4}	5.88×10^{-4}
AMP	8.47×10^{-5}	1.04×10^{-4}	2.61×10^{-5}	6.03×10^{-5}	1.70×10^{-5}	3.55×10^{-4}	8.45×10^{-3}	6.74×10^{-5}	4.92×10^{-3}	2.50×10^{-2}	6.29×10^{-3}	1.72×10^{-3}	5.05×10^{-7}	0	1.51×10^{-4}	2.46×10^{-4}	9.98×10^{-4}
ENR	3.10×10^{-2}	3.70×10^{-3}	2.69×10^{-4}	9.24×10^{-5}	7.10×10^{-6}	3.92×10^{-4}	7.99×10^{-2}	7.48×10^{-5}	9.67×10^{-2}	9.08×10^{-2}	1.30×10^{-2}	1.82×10^{-3}	4.88×10^{-7}	3.47×10^{-5}	2.26×10^{-4}	3.02×10^{-4}	9.94×10^{-4}
SMX	2.19×10^{-2}	1.18×10^{-3}	3.41×10^{-4}	7.70×10^{-4}	2.55×10^{-5}	4.17×10^{-4}	2.40×10^{-2}	1.00×10^{-4}	1.69×10^{-2}	1.60×10^{-2}	1.20×10^{-2}	1.89×10^{-3}	2.78×10^{-5}	0	7.42×10^{-4}	2.46×10^{-4}	1.43×10^{-3}
OXY	1.17×10^{-2}	6.92×10^{-4}	1.73×10^{-4}	3.32×10^{-4}	3.40×10^{-5}	1.86×10^{-4}	5.38×10^{-2}	7.86×10^{-5}	3.65×10^{-3}	9.93×10^{-3}	3.56×10^{-3}	2.56×10^{-3}	3.16×10^{-6}	0	1.35×10^{-4}	2.14×10^{-4}	5.81×10^{-4}
CHLOR	2.87×10^{-2}	2.66×10^{-3}	6.28×10^{-4}	3.84×10^{-3}	1.35×10^{-5}	6.17×10^{-4}	3.58×10^{-5}	1.09×10^{-4}	3.93×10^{-2}	4.96×10^{-2}	5.06×10^{-3}	2.96×10^{-3}	2.76×10^{-6}	0	1.06×10^{-4}	4.32×10^{-4}	1.02×10^{-3}
TET	2.57×10^{-2}	1.05×10^{-3}	1.99×10^{-4}	7.78×10^{-3}	7.48×10^{-6}	4.88×10^{-4}	3.01×10^{-2}	4.92×10^{-5}	5.44×10^{-2}	4.02×10^{-2}	3.00×10^{-3}	1.78×10^{-3}	1.17×10^{-6}	0	1.71×10^{-4}	3.30×10^{-4}	8.05×10^{-4}
SA	2.89×10^{-2}	9.98×10^{-4}	3.48×10^{-4}	1.37×10^{-2}	2.52×10^{-5}	4.33×10^{-4}	6.32×10^{-2}	6.56×10^{-5}	2.37×10^{-2}	1.21×10^{-2}	3.55×10^{-3}	1.97×10^{-3}	3.56×10^{-6}	0	1.47×10^{-4}	1.80×10^{-4}	5.85×10^{-4}

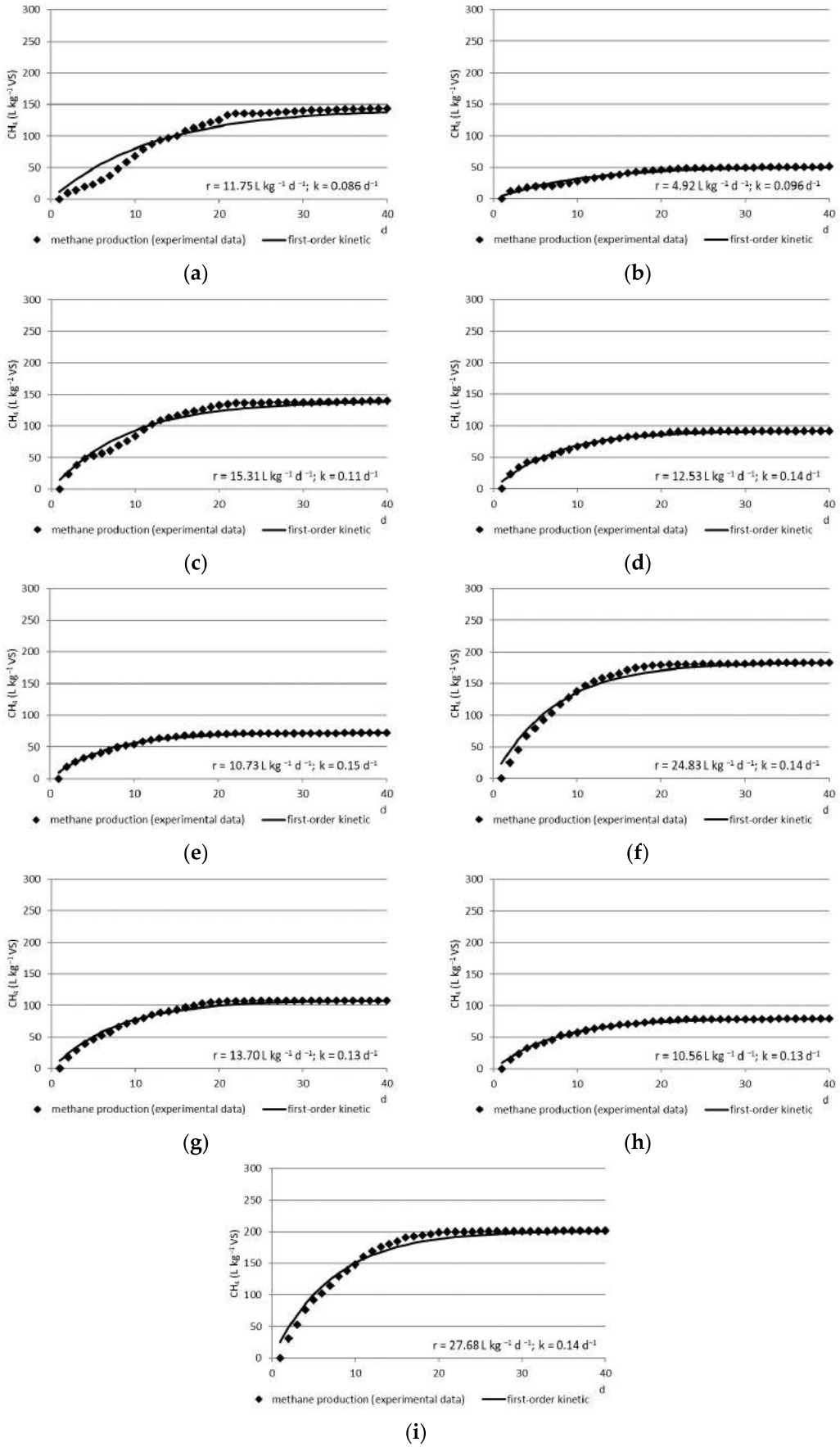


Figure S1. Average values of specific methane production during anaerobic digestion in bioreactors with (**a**) MET, (**b**) AMO, (**c**) AMP, (**d**) OXY, (**e**) ENR, (**f**) SMX, (**g**) CHLOR, (**h**) TET supplementation, and (**i**) control reactor (SA).

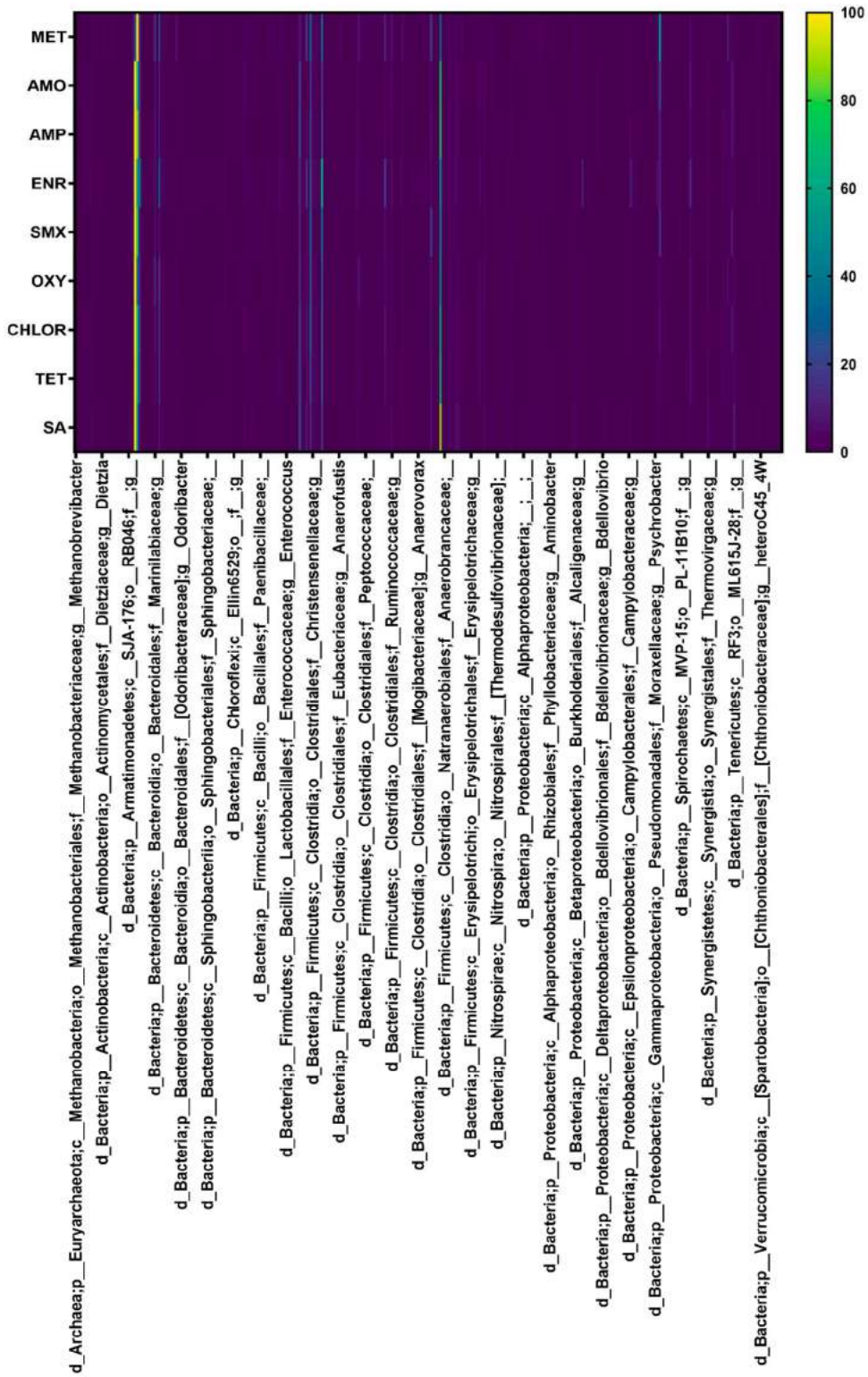
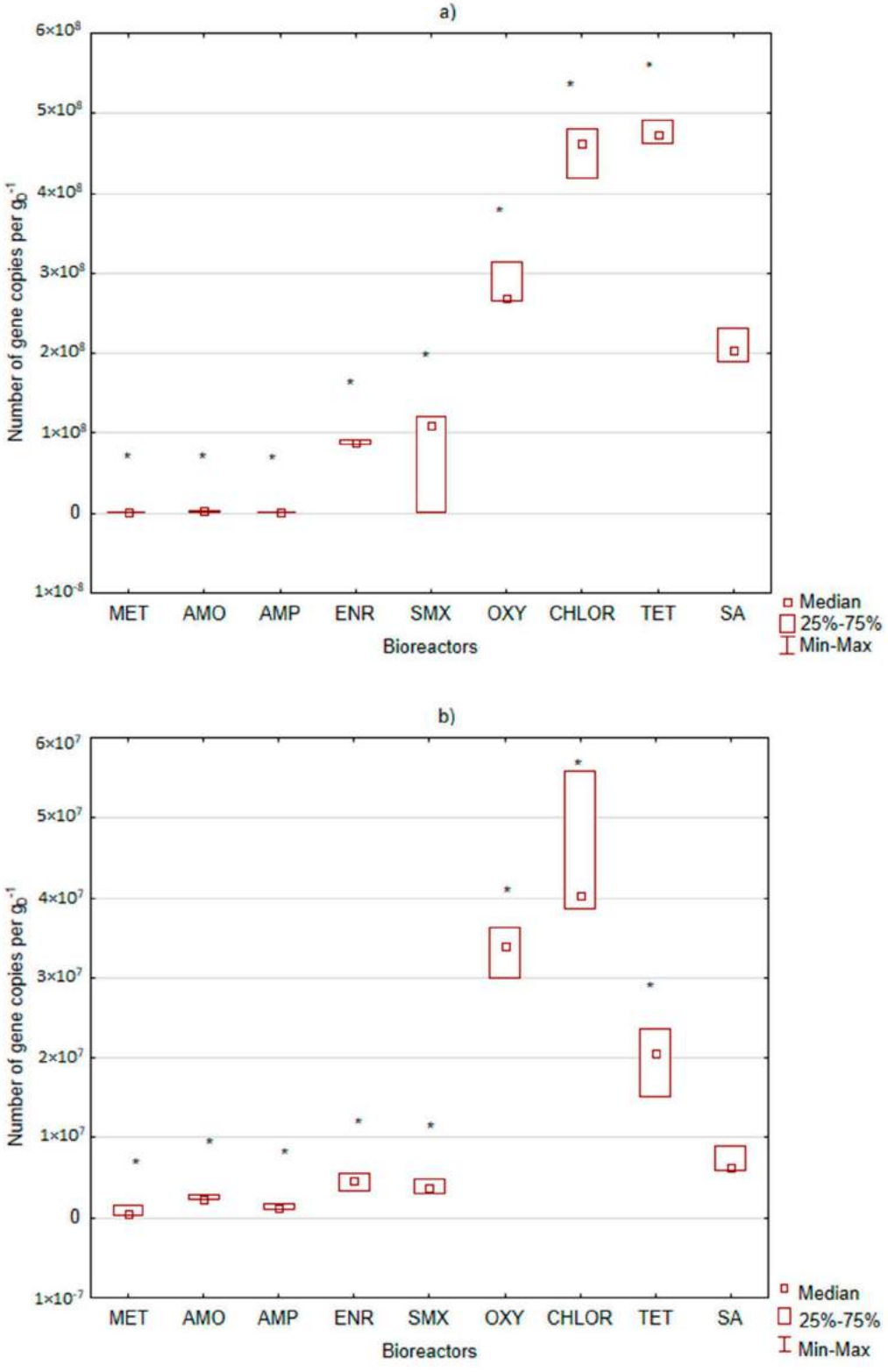


Figure S2. Detailed taxonomy and community structure of *Archaea* and bacteria (based on the OTUs) up to species in digestate with supplementation of selected antibiotics and in bioreactor with substrate without antibiotic supplementation (SA).


a)
6×10^8
5×10^8
4×10^8
3×10^8
2×10^8
1×10^8
0
1×10^-8
Number of gene copies per g_D^-1
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max
b)
6×10^7
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
1×10^-7
Number of gene copies per g_D^-1
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max

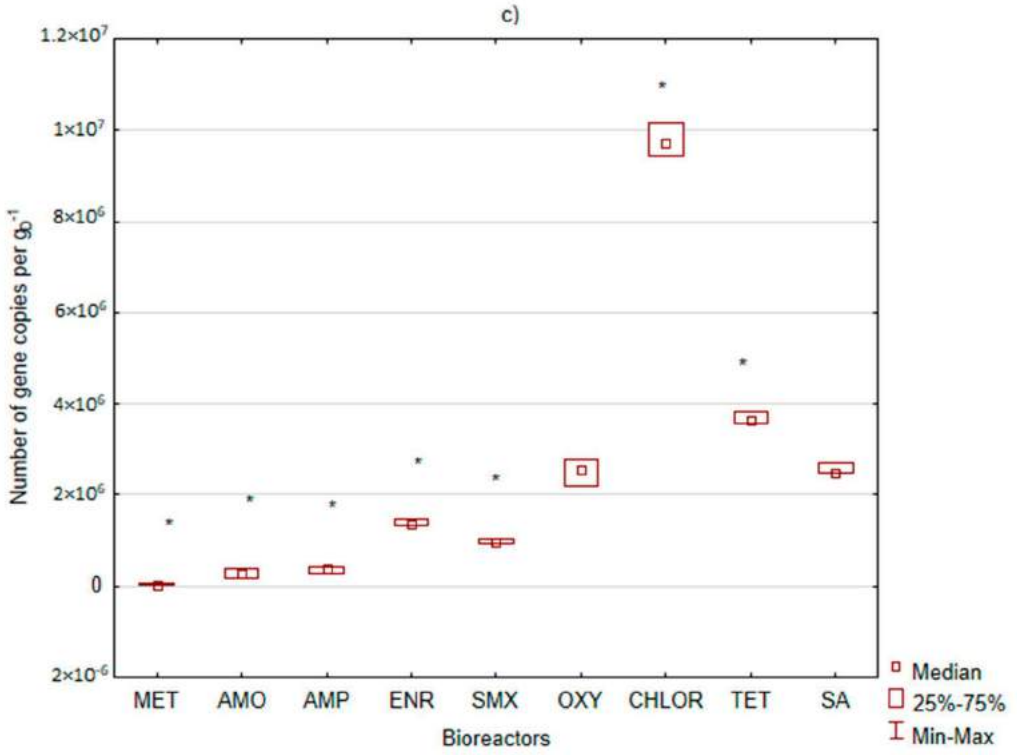
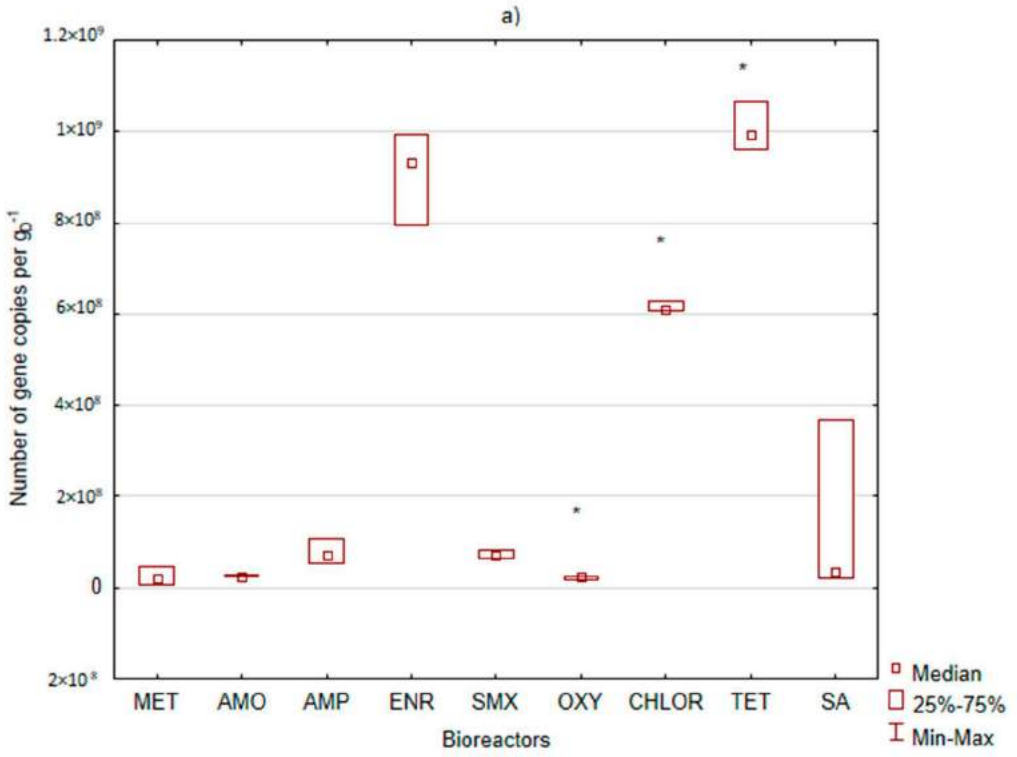


Figure S3. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of genes specific for *Archaea* like (**a**) *mcr*A gene, (**b**) *MSC* gene, (**c**) *MST* gene are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant).



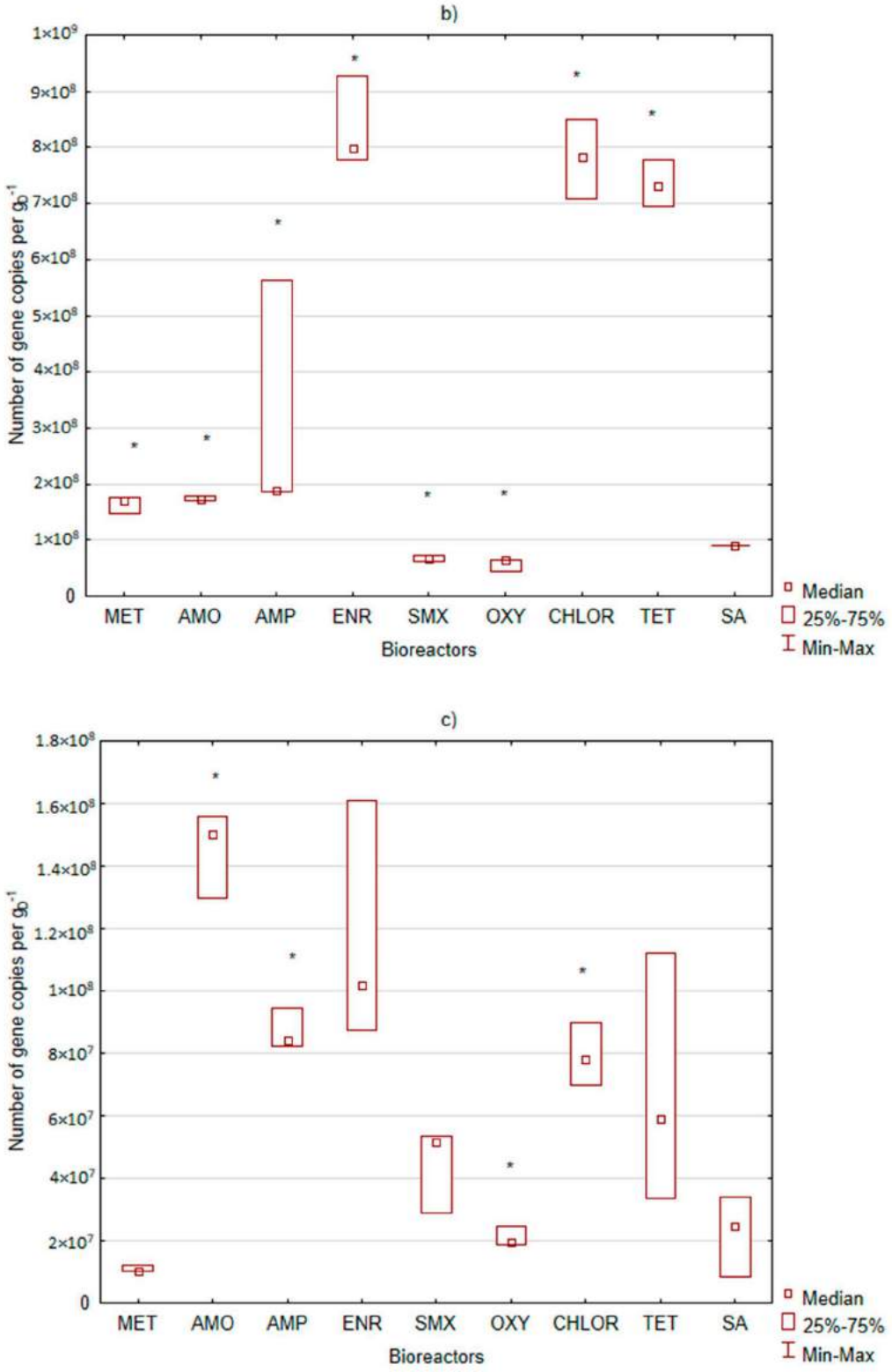
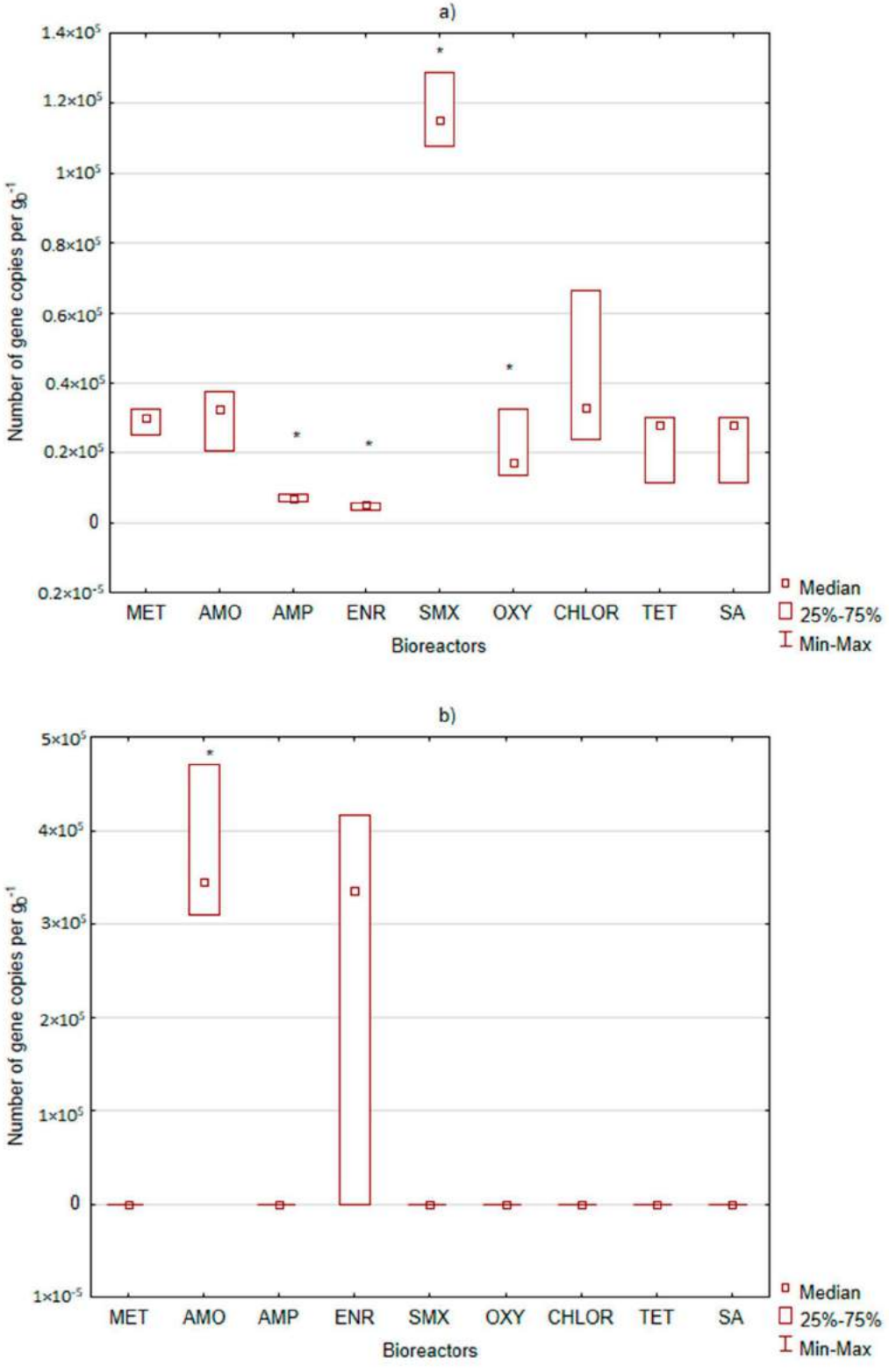


Figure S4. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of *tet* genes like (**a**) *tet*A, (**b**) *tet*M, (**c**) *tet*Q are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant).


a)
1.4×10⁵
1.2×10⁵
1×10⁵
0.8×10⁵
0.6×10⁵
0.4×10⁵
0.2×10⁵
0
0.2×10⁻⁵
Number of gene copies per g⁻¹
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max
b)
5×10⁵
4×10⁵
3×10⁵
2×10⁵
1×10⁵
0
1×10⁻⁵
Number of gene copies per g⁻¹
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max

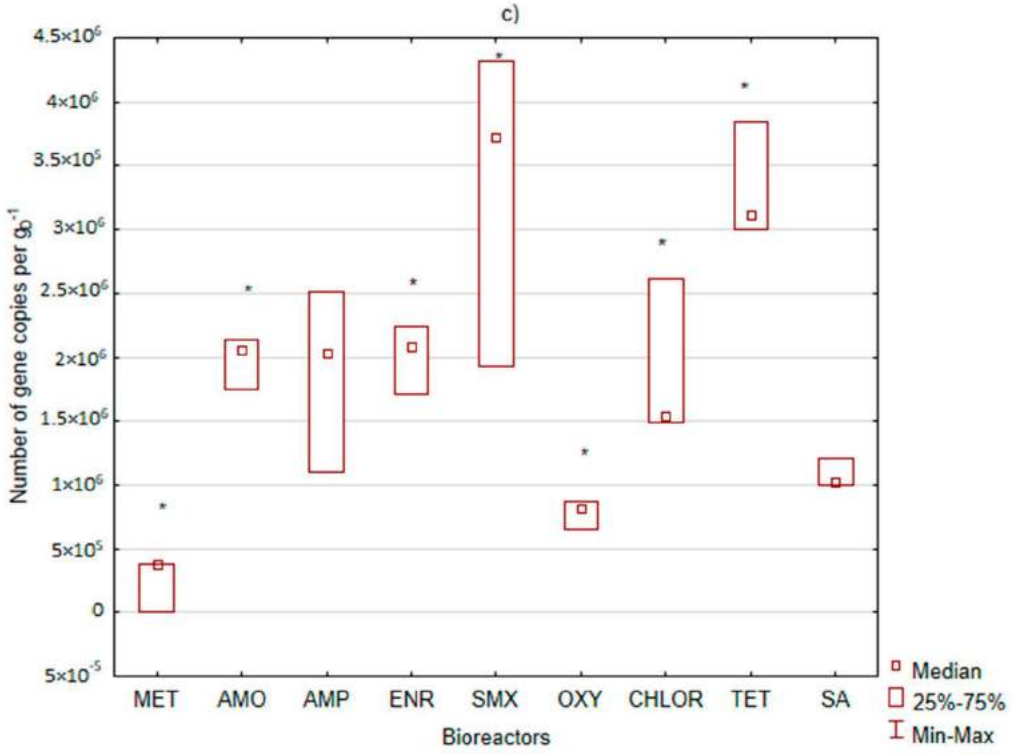


Figure S5. Frame-mustache charts showing the results of the Kruskal-Wallis test (ANOVA). Statistically significant changes in the concentration of *bla* genes like (**a**) *bla*TEM, (**b**) *bla*OXA, (**c**) *cfx*A are marked with an asterisk (*) (p values <0.05 were considered statistically significant).

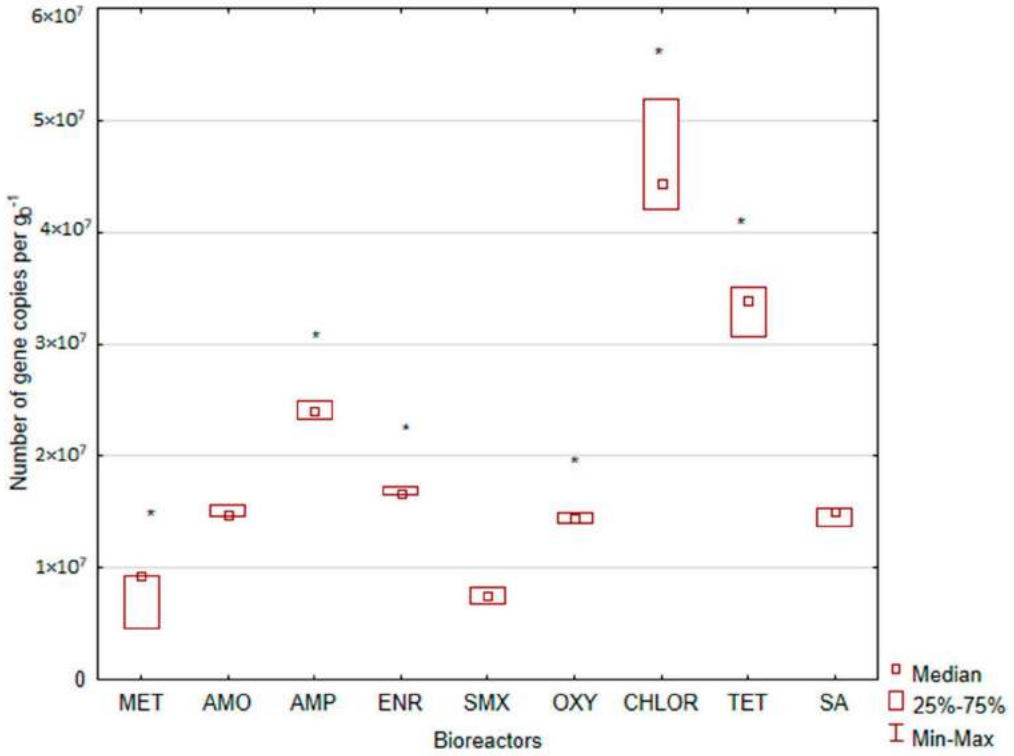
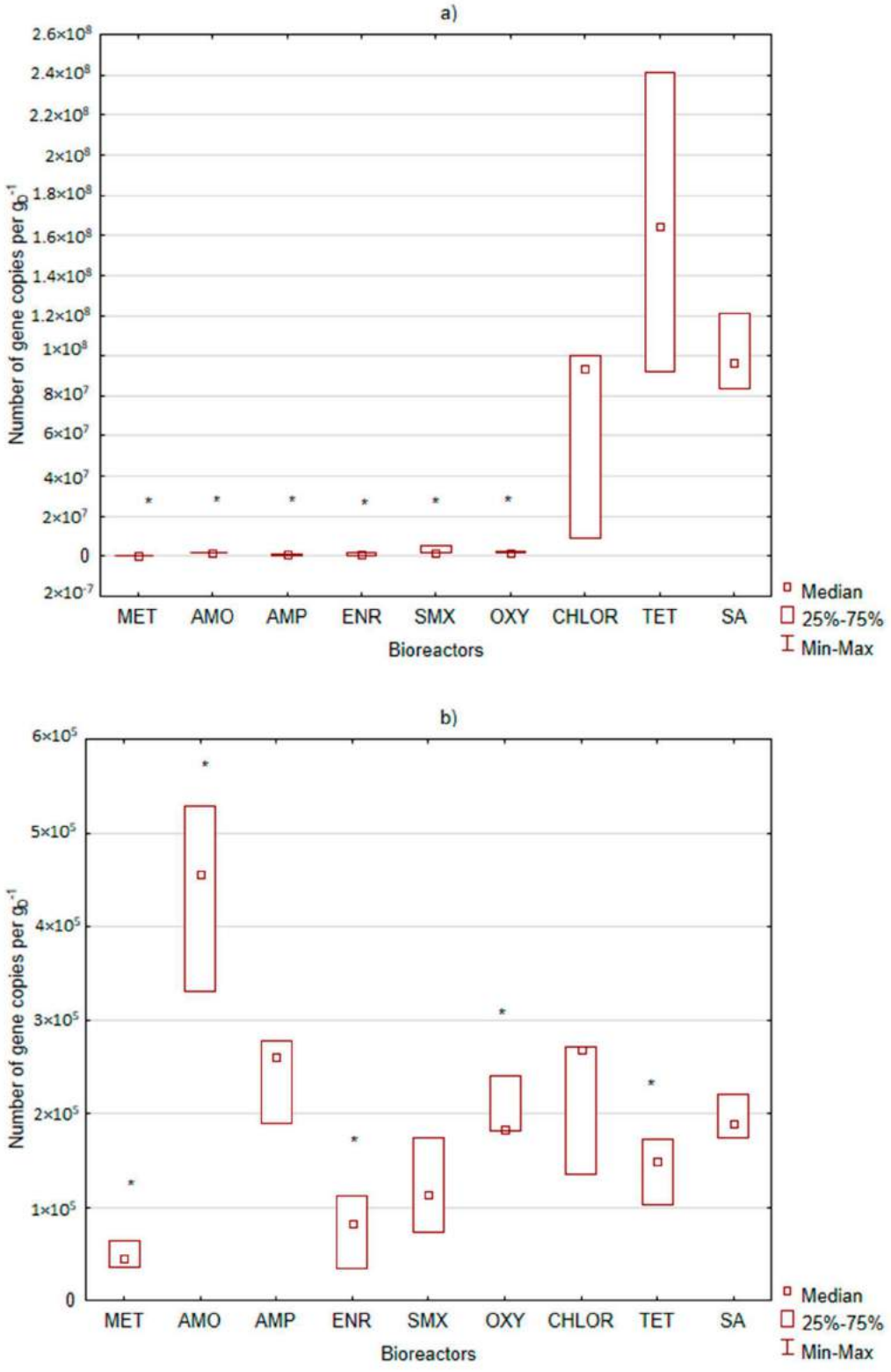


Figure S6. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of *sul*1 genes are marked with an asterisk (*) (*p*-values < 0.05 were considered statistically significant).


a)
Number of gene copies per g$_D^{-1}$
2.6×10^8
2.4×10^8
2.2×10^8
2×10^8
1.8×10^8
1.6×10^8
1.4×10^8
1.2×10^8
1×10^8
8×10^7
6×10^7
4×10^7
2×10^7
0
2×10^{-7}
MET AMO AMP ENR SMX OXY CHLOR TET SA
Bioreactors
Median
25%-75%
Min-Max
b)
Number of gene copies per g$_D^{-1}$
6×10^5
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
MET AMO AMP ENR SMX OXY CHLOR TET SA
Bioreactors
Median
25%-75%
Min-Max

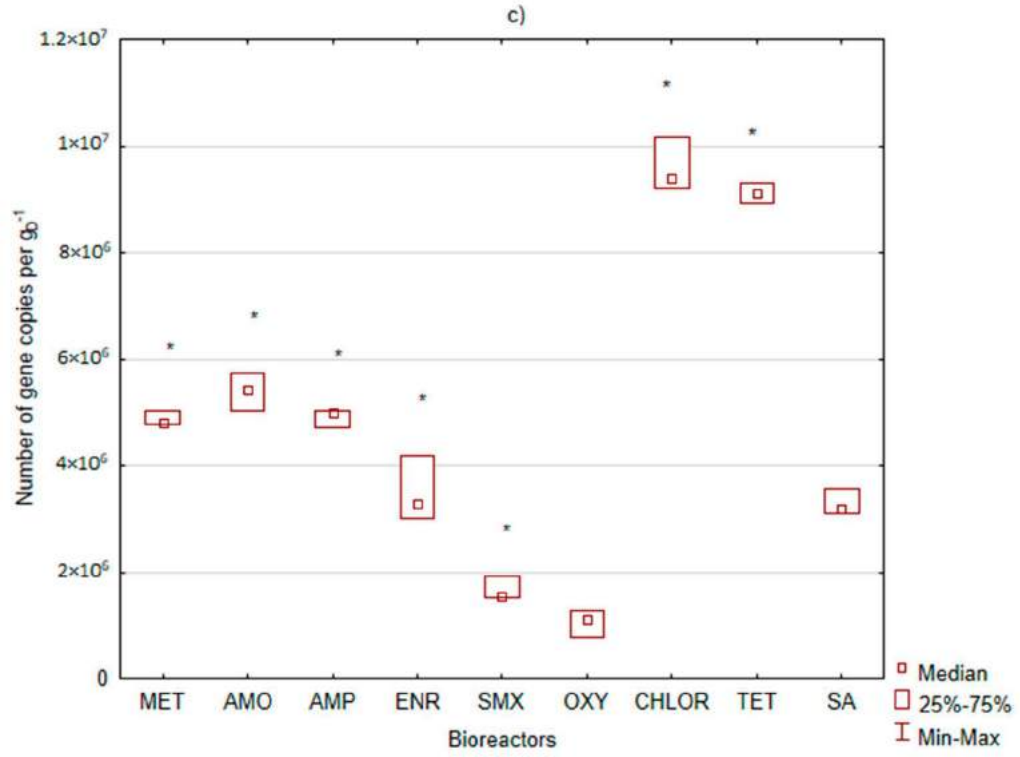
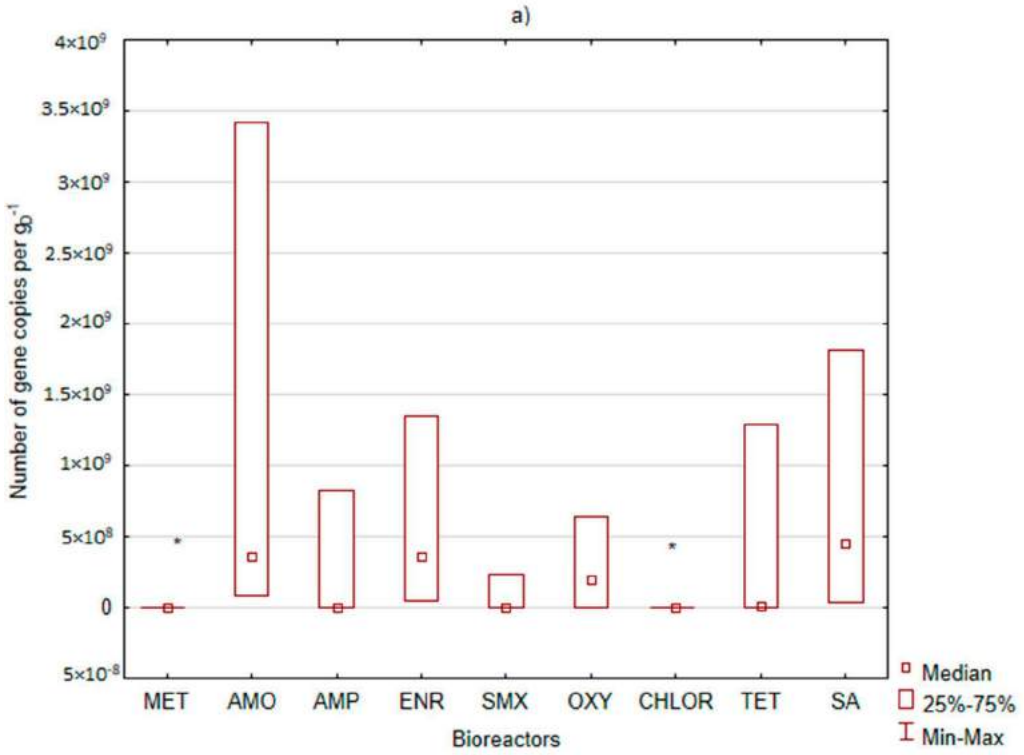


Figure S7. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of MLS group genes like (**a**) *erm*F, (**b**) *lin*A, (**c**) *mef*A are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant).



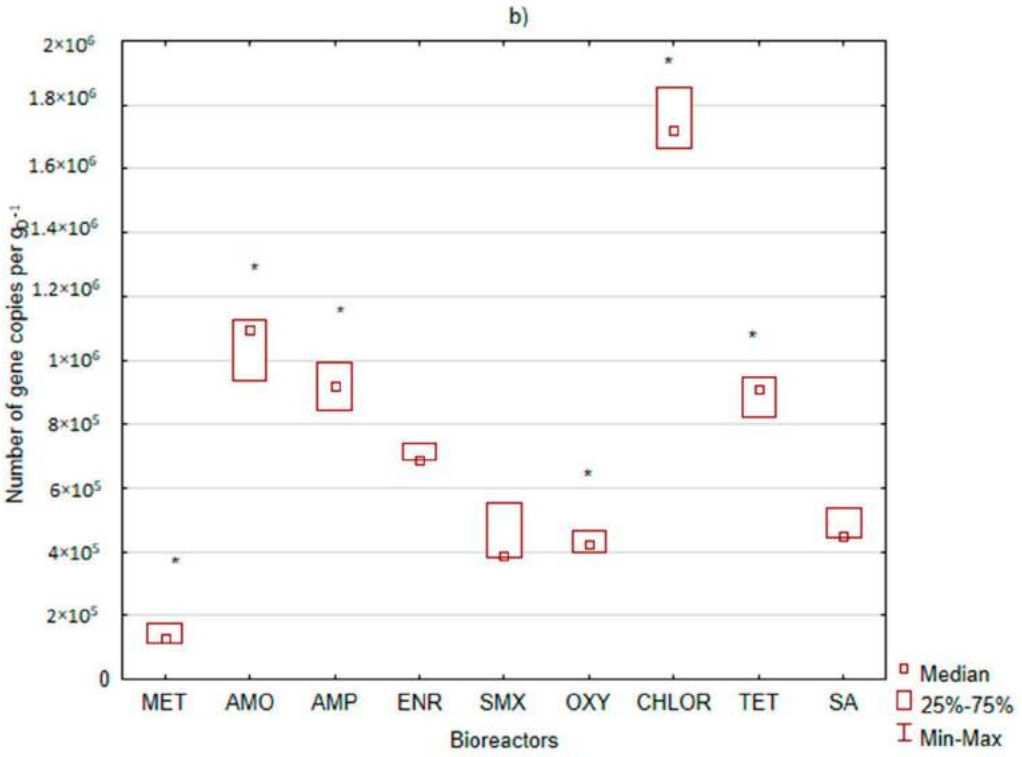
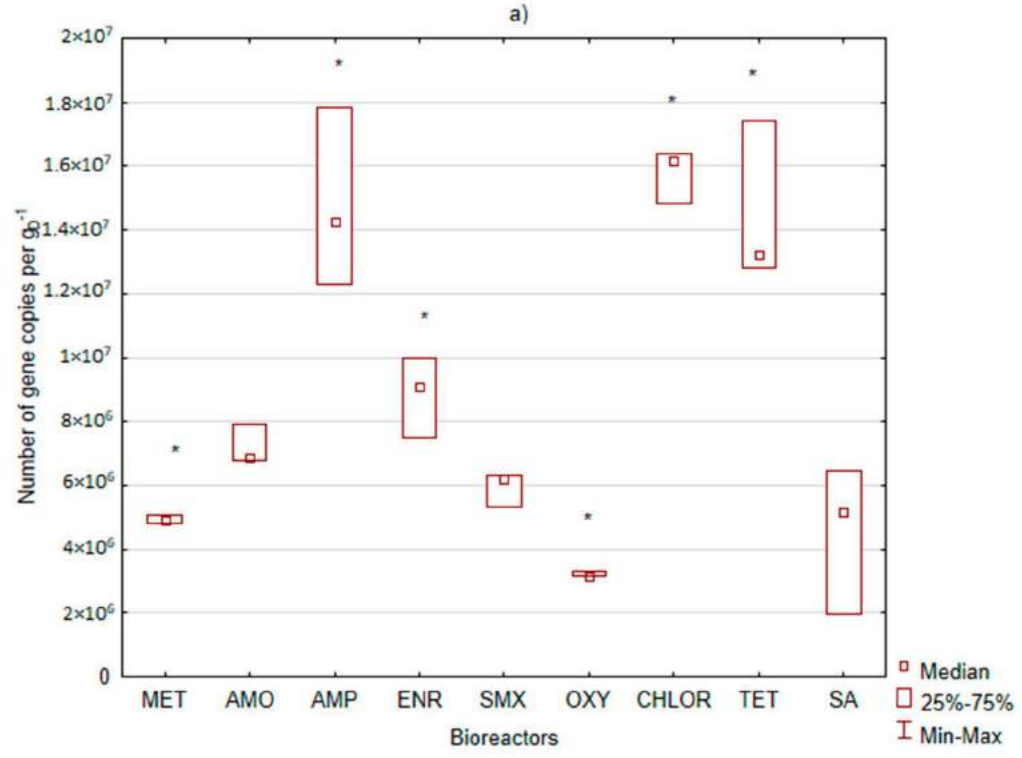


Figure S8. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of fluoroquinolones genes like (**a**) *qep*A and (**b**) *aac-(6′)-Ib-cr* are marked with an asterisk (*) (*p*-values < 0.05 were considered statistically significant).



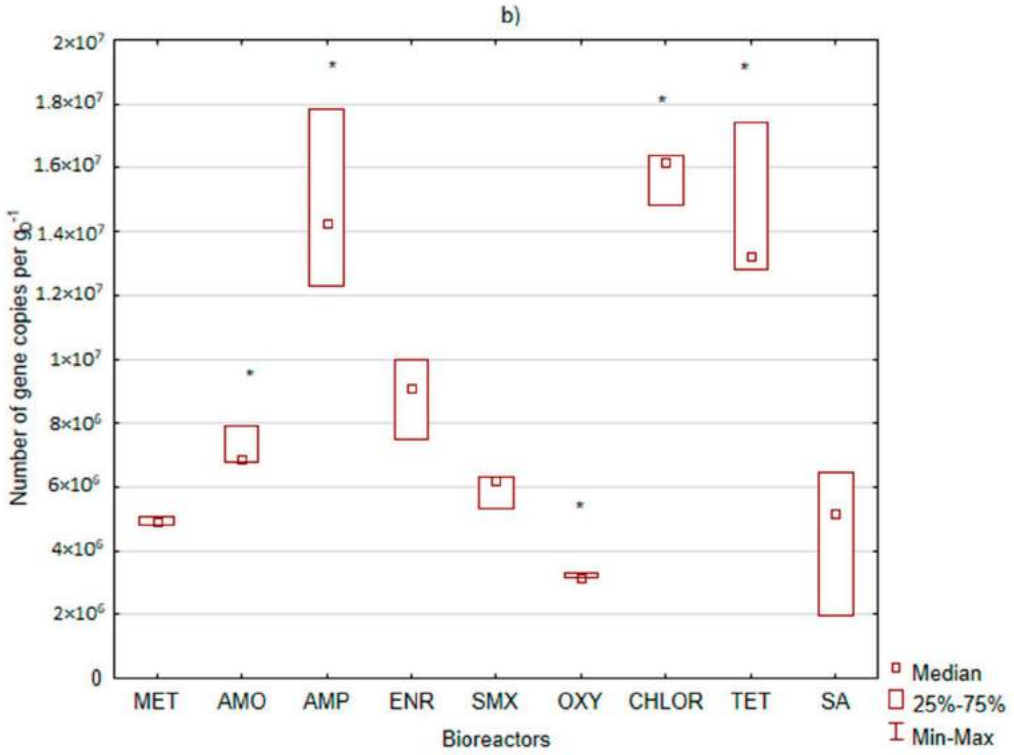


Figure S9. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of integrase genes like (**a**) *int*I1, (**b**) *int*I2 are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant).

mgr Izabela Wolak

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Wydział Geoinżynierii

Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 15.06.2023

OŚWIADCZENIE

Oświadczam, że w pracy:

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(4):1125. https://doi.org/10.3390/en14041125

mój wkład polegał na współudziale w opracowaniu metodyki, przygotowaniu pierwszej wersji manuskryptu oraz wykonaniu analiz laboratoryjnych.

Podpis

Prof. dr hab. inż. Monika Harnisz

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mój wkład polegał na współudziale w opracowaniu metodyki i eksperymentu oraz zaplanowaniu analiz laboratoryjnych. Byłam również odpowiedzialna za pozyskanie środków finansowych i pomoc edytorską przygotowywanego manuskryptu.

Monika Harnisz

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mgr inż. Małgorzata Czatzkowska

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Olsztyn,

OŚWIADCZENIE

Oświadczam, że w pracy:

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mój wkład polegał na współudziale w opracowaniu metodyki. Byłem również odpowiedzialny za wykonanie analiz bioinformatycznych i pomoc edytorską przygotowywanego manuskryptu.

Łukasz Paukszto

Podpis

Załącznik 4

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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,*]

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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

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).

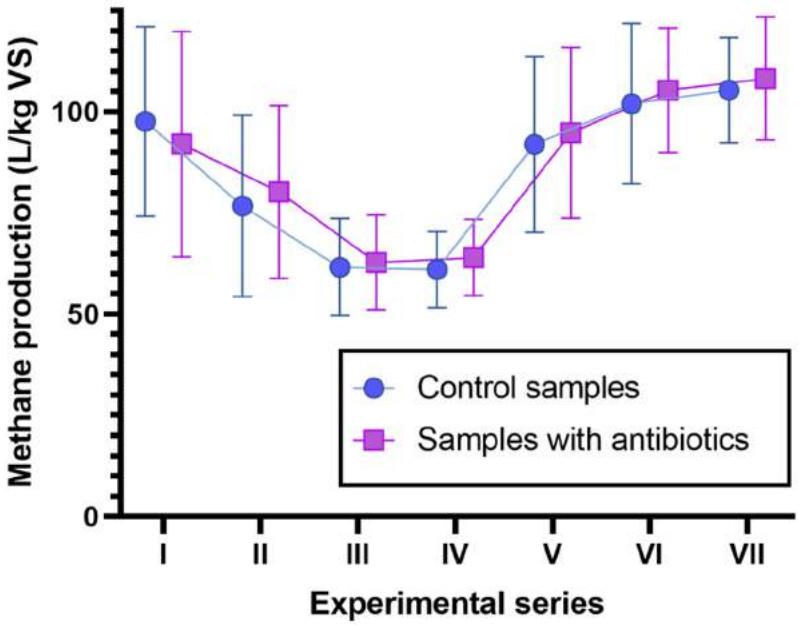


Figure 1. Methane production in seven experimental series (L/kg VS). The presented data are the average values in each experimental series with triplicate measurements and two replicates. Error bars represent standard deviation (SD).

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.

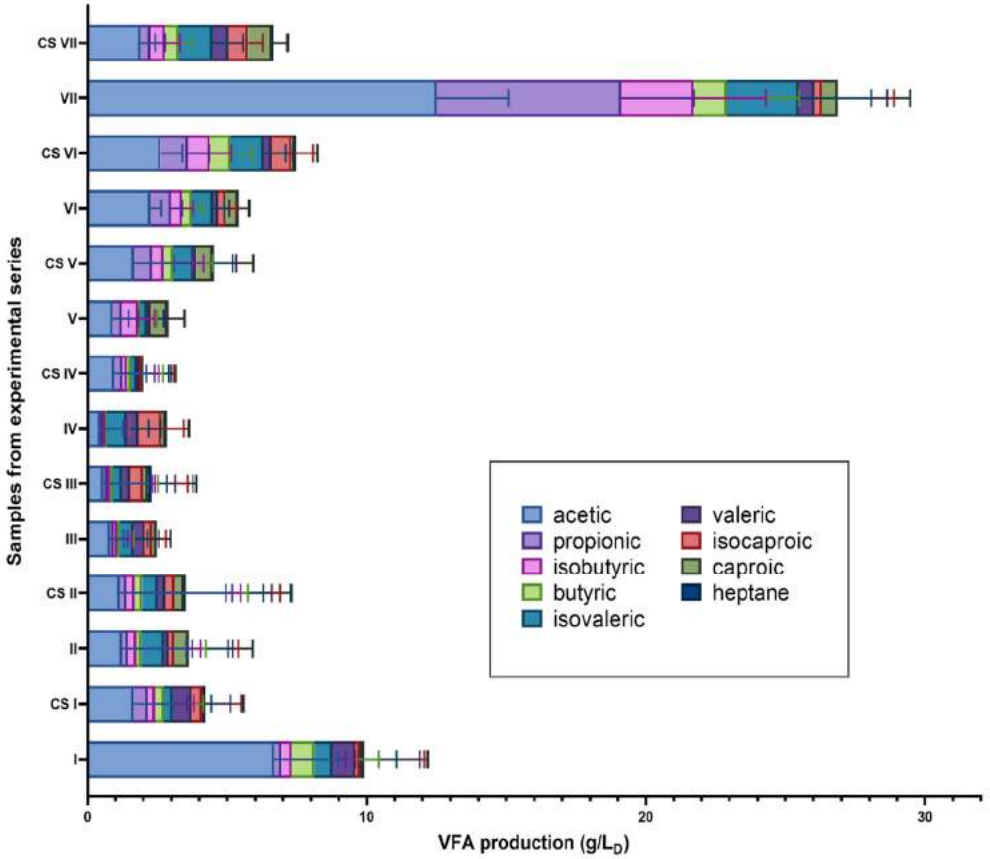


Figure 2. Average concentrations of VFAs (g/L $_D$ ± SD) in the experimental samples collected from series I–VII. Control samples without AMO, ENRO and MET are marked as CS and are labeled with the number of the corresponding experimental series (I–VII).

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].

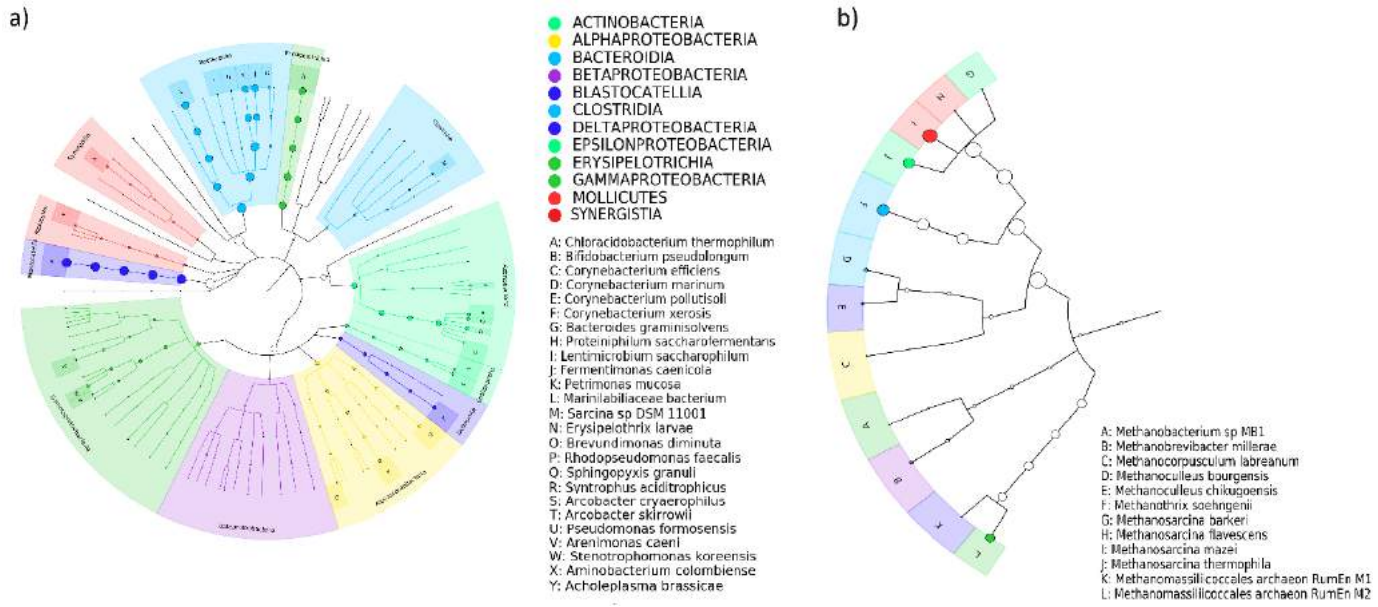


Figure 3. Circos presenting the (**a**) most abundant bacterial consortia in digestate samples and (**b**) most abundant *Archaea* consortia in digestate samples. The OTU values of the presented species exceeded 1 ppm.

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].

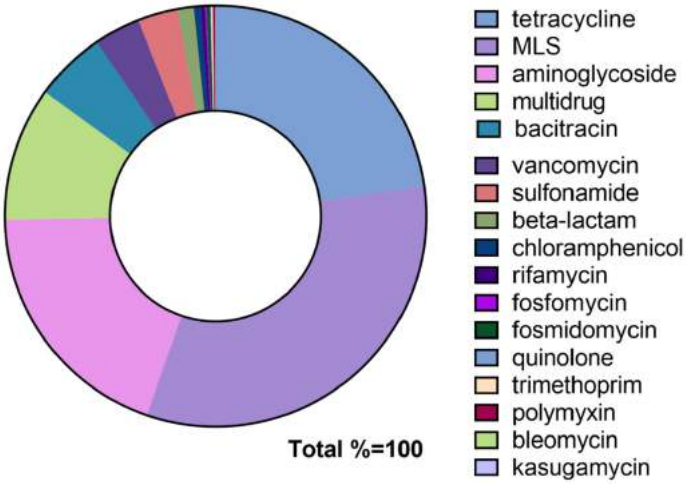


Figure 4. Percentage (%) distribution of different ARG types in digestate samples. MLS—macrolide–lincosamide–streptogramin antibiotics.

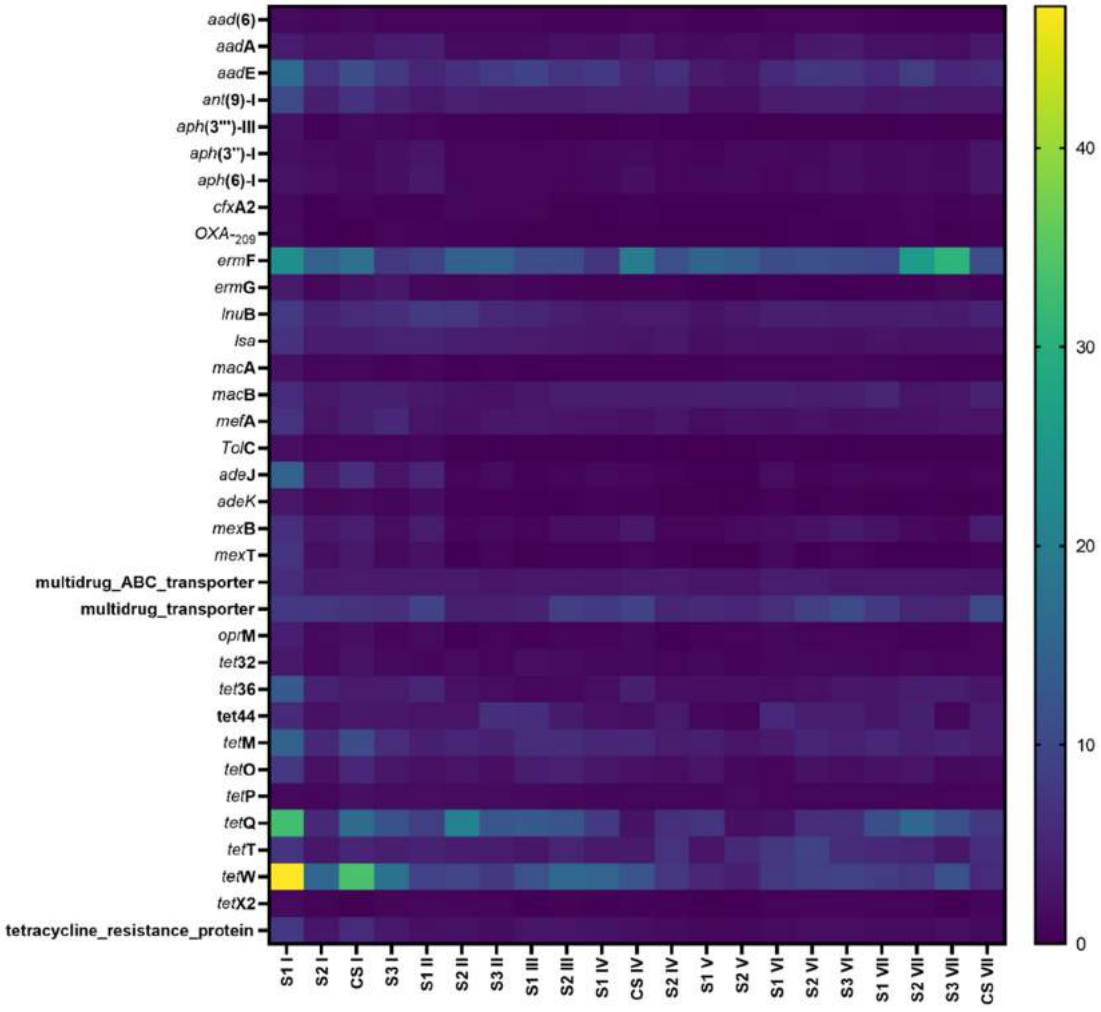


Figure 5. Heatmap presenting the abundance (ppm) of ARG subtypes in the experimental samples (S1, S2, S3) collected from seven experimental series (I–VII) and in control samples (CS).

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.

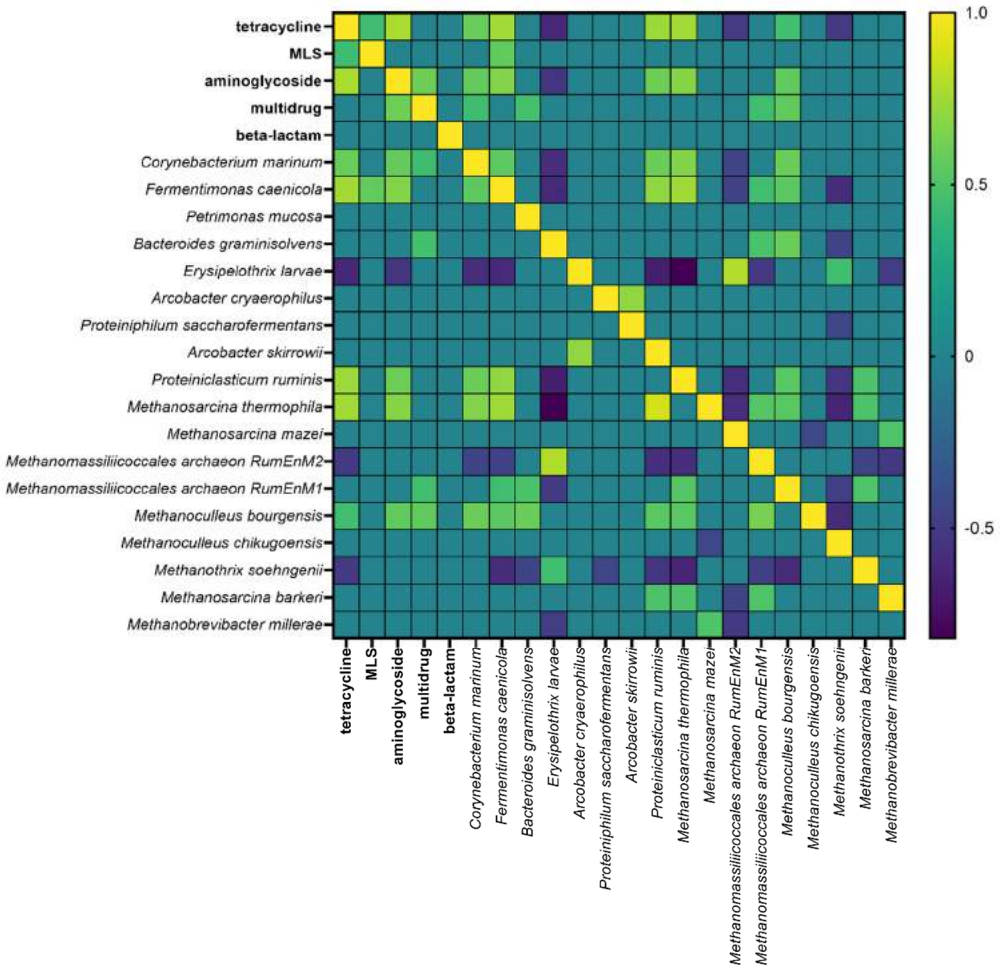


Figure 6. Spearman's rank order correlation coefficients for the relationships between microbial abundance and the abundance of different ARG types.

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).

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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).

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Metagenomic analysis of the long-term synergistic effects of antibiotics on the anaerobic digestion of cattle manure

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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).

Experimental series	Day	CH_4 production (L/kg VS[a])	
		Control samples	Samples with AMO, ENRO and MET
I	1	75.0	194.5
	2	83.0	189.1
	3	89.7	150.3
	4	84.3	128.3
	5	116.1	130.5
	6	97.7	110.8
	7	111.3	131.1
	8	120.0	133.0
	9	116.7	114.8
	10	122.9	134.8
	11	129.8	116.0
	12	130.8	103.3
	13	110.9	95.5
	14	119.6	102.1
	15	112.9	92.4
	16	90.7	92.5
	17	92.7	113.6

	18	93.3	95.9
	19	127.6	113.0
	20	101.9	94.9
	21	93.0	88.3
	22	134.5	116.4
	23	131.2	118.1
	24	112.0	102.5
	25	116.3	110.6
	26	107.6	92.5
	27	92.2	111.9
	28	99.5	99.1
	29	102.9	97.3
	30	79.7	84.7
	31	109.6	102.7
	32	86.7	83.8
	33	113.1	106.2
	34	134.1	118.1
	35	121.8	115.5
	36	109.7	138.4
	37	118.0	114.0
	38	106.0	106.2
	39	87.9	85.5
	40	60.4	63.9
	41	47.3	58.4
	42	43.7	62.4
	43	69.2	126.5
	44	73.8	118.3
	45	63.2	67.5
	46	86.5	89.1
	47	73.6	84.8
	48	47.5	58.5
	49	36.6	47.8
	50	69.8	75.9
	51	66.7	77.5
	52	68.6	68.2
	53	72.8	77.2
	54	78.1	72.0
	55	76.1	70.4
	56	76.2	70.5
	57	97.1	78.1
	58	92.7	79.4
	59	94.9	83.0
	60	88.8	82.3
	61	66.7	65.9
	62	64.7	63.6

	63	67.1	69.3
	64	74.9	104.2
	65	80.6	91.8
	66	71.5	68.2
	67	81.1	78.6
	68	78.4	77.3
	69	83.8	80.4
	70	105.5	95.6
	71	92.0	97.4
	72	99.1	84.7
	73	94.5	84.1
	74	74.5	66.5
	75	92.6	89.5
	76	99.3	94.8
	77	105.2	99.4
	78	106.8	104.7
	79	107.3	111.0
	80	106.5	99.7
	81	94.7	104.7
	82	100.7	90.2
	83	96.2	87.2
	84	96.5	89.0
	85	100.7	97.9
	86	102.3	112.0
	87	99.5	121.2
	88	95.1	96.9
II	89	82.9	89.3
	90	88.2	97.0
	91	78.3	95.3
	92	90.6	107.2
	93	97.5	106.1
	94	118.7	98.9
	95	94.4	112.1
	96	108.7	103.2
	97	77.3	81.6
	98	83.4	93.3
	99	79.4	87.8
	100	76.3	84.1
	101	77.4	87.0
	102	80.0	90.5
	103	82.8	89.3
	104	76.3	79.6
	105	71.2	74.0
	106	72.6	74.6
	107	75.7	76.6

	108	75.5	80.7
	109	73.5	80.4
	110	50.5	56.7
	111	44.2	51.5
	112	44.7	58.5
	113	47.7	59.1
	114	56.8	68.2
	115	59.8	67.6
	116	63.9	68.6
	117	64.0	67.6
	118	46.4	47.2
	119	59.0	61.1
	120	62.3	65.2
	121	59.9	74.1
	122	11.8	12.8
	123	50.0	50.9
	124	60.6	58.5
	125	56.7	54.0
	126	47.9	49.3
	127	57.9	62.9
	128	41.0	46.9
	129	35.6	44.5
	130	59.2	65.4
III	131	58.6	62.9
	132	69.9	69.2
	133	72.0	69.0
	134	68.5	71.6
	135	50.0	50.0
	136	44.6	53.5
	137	72.8	63.8
	138	75.0	78.1
	139	66.3	67.6
	140	62.2	57.3
	141	71.3	61.0
	142	56.1	47.4
	143	74.1	68.4
	144	66.7	65.2
	145	64.6	65.8
	146	74.3	69.6
	147	69.2	69.7
	148	78.7	76.3
	149	75.3	78.5
	150	71.3	74.1
	151	68.3	78.5
	152	76.2	78.2

	153	71.2	74.8
	154	60.6	62.6
	155	70.5	70.6
	156	77.7	78.9
	157	69.0	66.3
	158	75.0	77.0
	159	65.4	67.4
	160	53.8	53.7
	161	63.4	62.0
	162	54.2	56.5
	163	60.2	63.0
	164	57.4	77.3
	165	58.0	60.7
	166	29.6	42.9
	167	46.9	49.4
	168	54.5	54.0
	169	40.3	41.3
	170	61.4	63.7
	171	61.4	62.2
	172	57.4	67.5
	173	55.4	55.4
	174	55.3	58.0
	175	48.6	48.3
	176	57.3	56.8
	177	61.5	69.4
	178	60.5	59.8
	179	63.1	62.0
	180	62.5	63.9
	181	10.3	10.0
	182	60.1	63.0
	183	64.5	65.1
	184	50.6	53.6
	185	63.5	62.5
	186	60.5	63.4
	187	63.2	71.9
	188	57.0	62.0
	189	56.4	58.6
	190	49.0	51.4
	191	62.8	61.3
IV	192	49.1	47.6
	193	65.9	68.6
	194	73.8	80.1
	195	69.1	59.6
	196	63.3	60.5
	197	56.5	54.6

	198	63.2	60.1
	199	58.8	56.1
	200	57.1	60.9
	201	65.5	67.6
	202	58.2	58.7
	203	60.6	66.5
	204	53.0	61.6
	205	47.2	53.0
	206	64.8	71.3
	207	59.5	63.8
	208	53.0	59.6
	209	54.3	63.2
	210	56.1	62.9
	211	53.7	56.0
	212	46.3	53.0
	213	53.9	57.9
	214	68.6	69.3
	215	53.0	60.2
	216	80.3	60.7
	217	42.4	42.5
	218	50.0	53.6
	219	64.2	67.1
	220	46.6	51.9
	221	51.5	59.8
	222	65.6	74.2
	223	59.8	62.3
	224	72.9	77.6
	225	69.0	67.1
	226	58.6	63.3
	227	65.0	66.4
	228	60.7	64.1
	229	62.9	68.0
	230	62.7	70.9
	231	81.7	76.8
	232	73.1	68.1
	233	71.0	69.1
	234	50.2	51.1
	235	64.3	65.1
	236	69.2	71.1
	237	77.9	72.2
	238	68.4	69.9
	239	67.1	79.5
	240	73.4	90.2
	241	52.1	60.4
	242	45.5	55.6

	243	79.1	91.8
V	244	65.0	72.6
	245	64.6	69.2
	246	60.5	63.7
	247	51.0	52.1
	248	42.9	45.4
	249	36.4	39.6
	250	34.9	38.2
	251	47.5	55.0
	252	98.1	96.3
	253	97.2	98.8
	254	93.4	98.2
	255	86.7	89.4
	256	84.1	92.0
	257	108.7	104.8
	258	116.3	128.6
	259	99.2	105.9
	260	70.7	71.0
	261	95.1	92.2
	262	93.4	79.8
	263	117.2	110.2
	264	113.6	108.3
	265	84.2	80.3
	266	66.0	61.7
	267	104.0	108.2
	268	98.4	98.8
	269	96.2	98.8
	270	108.1	103.7
	271	123.4	132.2
	272	101.1	103.3
	273	102.1	98.5
	274	113.3	97.6
	275	110.3	108.2
	276	82.5	93.4
	277	97.6	98.0
	278	109.2	101.7
	279	114.9	105.3
	280	95.9	103.1
	281	95.1	93.8
	282	104.4	103.9
	283	65.7	85.4
	284	49.8	64.1
	285	90.0	85.8
	286	80.0	91.9
	287	114.0	106.9

	288	97.5	104.3
	289	83.5	104.8
	290	79.4	99.9
	291	103.6	106.1
	292	118.7	131.1
	293	92.7	127.2
	294	109.1	121.8
	295	96.8	98.4
	296	99.4	98.8
	297	98.2	100.6
	298	79.9	81.6
	299	107.2	110.0
	300	116.2	118.6
	301	116.7	118.9
	302	98.7	102.5
	303	94.7	99.1
	304	103.6	102.8
	305	116.3	114.3
	306	101.3	111.7
	307	76.9	88.2
	308	86.7	100.0
	309	74.6	88.3
	310	82.4	96.7
	311	95.3	119.4
	312	95.4	110.4
	313	108.9	119.1
	314	89.4	104.5
	315	96.3	115.2
	316	113.7	114.9
	317	74.0	94.2
	318	73.0	69.6
VI	319	83.1	97.7
	320	84.6	102.9
	321	78.5	78.4
	322	87.1	98.2
	323	76.8	88.6
	324	90.3	88.9
	325	99.1	112.1
	326	100.0	95.1
	327	93.2	106.7
	328	86.3	97.8
	329	81.6	87.9
	330	87.7	100.6
	331	85.3	93.9
	332	106.1	114.2

	333	86.2	106.4
	334	102.7	110.0
	335	91.3	99.1
	336	94.6	96.7
	337	101.2	107.0
	338	108.1	104.8
	339	83.5	83.3
	340	113.4	108.2
	341	112.6	114.0
	342	130.6	112.6
	343	130.2	134.6
	344	143.6	111.5
	345	118.2	98.5
	346	124.7	75.7
	347	117.4	104.5
	348	99.4	87.0
	349	120.4	107.6
	350	145.6	107.0
	351	120.7	129.7
	352	79.6	93.8
	353	87.9	99.0
	354	100.6	105.6
	355	147.8	126.4
	356	120.4	118.3
	357	127.0	122.9
	358	122.1	123.7
	359	125.8	132.3
	360	136.2	141.4
	361	115.2	138.8
VII	362	148.2	155.2
	363	151.5	159.4
	364	113.8	136.4
	365	110.1	158.4
	366	83.3	100.6
	367	112.6	87.9
	368	95.1	97.8
	369	99.1	93.9
	370	98.5	96.7
	371	114.0	106.7
	372	108.2	106.4
	373	112.1	111.5
	374	119.4	134.6
	375	110.4	119.1
	376	110.0	114.2
	377	104.8	107.0

	378	88.2	96.7
	379	88.3	104.5
	380	115.2	114.9
	381	94.2	100.0
	382	69.6	97.7
	383	98.2	102.9
	384	88.6	88.9
	385	101.0	101.4
	386	103.6	107.5
	387	116.6	119.1
	388	111.8	114.5
	389	100.6	105.3
	390	94.8	97.4
	391	100.9	98.8
	392	100.9	101.2
	393	94.5	96.8
	394	104.9	105.1
	395	102.9	103.3
	396	114.1	107.7
	397	118.7	112.3
	398	120.7	121.2
	399	112.2	114.3
	400	105.3	107.4
	401	112.0	114.4
	402	112.1	114.5
	403	106.6	108.5
	404	100.9	105.3
	405	98.6	96.9
	406	94.3	90.4
	407	96.4	99.1
	408	107.5	103.6
	409	98.9	96.9
	410	96.8	94.7
	411	114.1	105.8
	412	103.2	103.2
	413	107.7	110.3
	414	111.7	112.5
	415	102.9	98.9
	416	94.7	96.9
	417	100.7	103.2

[a]- volatile solids

Table S2. Dates and duration of seven experimental anaerobic digestion (AD) series.

Experimental series	Date
I	20 September 2018 – 28 October 2018
II	29 October 2018 – 27 December 2019
III	28 December 2019 – 21 February 2020
IV	22 February 2020 – 19 April 2020
V	20 April 2020 – 20 June 2020
VI	21 June 2020 – 16 September 2020
VII	17 September 2020 – 11 October 2020

Table S3. Correlations between microbial counts[a] and concentrations of antibiotic resistance genes (ARGs).

Erysipelothrix larvae	*Bacteroides graminisolvens*	*Petrimonas mucosa*	*Fermentimonas caenicola*	*Corynebacterium marinum*	multidrug	aminoglycoside	MLS	tetracycline	
-0.62	0.00	0.00	0.75	0.58	0.00	0.77	0.44	0.00	tetracycline
0.00	0.00	0.00	0.56	0.00	0.00	0.00	0.00	0.44	MLS[b]
-0.54	0.00	0.00	0.66	0.57	0.60	0.00	0.00	0.77	aminoglycoside
0.00	0.46	0.00	0.00	0.44	0.00	0.60	0.00	0.00	multidrug
-0.58	0.00	0.00	0.55	0.00	0.44	0.57	0.00	0.58	*Corynebacterium marinum*
-0.61	0.00	0.00	0.00	0.55	0.00	0.66	0.56	0.75	*Fermentimonas caenicola*
0.00	0.00	0.00	0.00	0.00	0.46	0.00	0.00	0.00	*Bacteroides graminisolvens*
0.00	0.00	0.00	-0.61	-0.58	0.00	-0.54	0.00	-0.62	*Erysipelothrix larvae*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Arcobacter cryaerophilus*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Proteiniphilum saccharofermentans*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Arcobacter skirrowii*
-0.67	0.00	0.00	0.70	0.59	0.00	0.60	0.00	0.73	*Proteiniclasticum ruminis*
-0.82	0.00	0.00	0.74	0.66	0.00	0.67	0.00	0.75	*Methanosarcina thermophila*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Methanosarcina mazei*
0.79	0.00	0.00	-0.47	-0.45	0.00	0.00	0.00	-0.51	*Methanomassiliicoccales archaeon RumEnM2*
-0.51	0.48	0.00	0.45	0.00	0.45	0.00	0.00	0.00	*Methanomassiliicoccales archaeon RumEnM1*
0.00	0.59	0.00	0.54	0.59	0.56	0.56	0.00	0.45	*Methanoculleus bourgensis*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Methanoculleus chikugoensis*
0.45	-0.45	0.00	-0.59	0.00	0.00	0.00	0.00	-0.51	*Methanothrix soehngenii*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Methanosarcina barkeri*

Methanosarcina barkeri	*Methanothrix soehngenii*	*Methanoculleus chikugoensis*	*Methanoculleus bourgensis*	*Methanomassili icoccales*	*Methanomassili icoccales*	*Methanosarcina mazei*	*Methanosarcina thermophila*	*Proteiniclasticu m ruminis*	*Arcobacter skirrowii*	*Proteiniphilum saccharofermen*	*Arcobacter cryaerophilus*
0.00	-0.51	0.00	0.45	0.00	-0.51	0.00	0.75	0.73	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.56	0.00	0.00	0.00	0.67	0.60	0.00	0.00	0.00
0.00	0.00	0.00	0.56	0.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.59	0.00	-0.45	0.00	0.66	0.59	0.00	0.00	0.00
0.00	-0.59	0.00	0.54	0.45	-0.47	0.00	0.74	0.70	0.00	0.00	0.00
0.00	-0.45	0.00	0.59	0.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.45	0.00	0.00	-0.51	0.79	0.00	-0.82	-0.67	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.70	0.00	0.00
0.00	-0.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.70
0.49	-0.54	0.00	0.53	0.00	-0.57	0.00	0.88	0.00	0.00	0.00	0.00
0.49	-0.64	0.00	0.53	0.52	-0.58	0.00	0.00	0.88	0.00	0.00	0.00
0.00	0.00	-0.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
-0.45	0.00	0.00	0.00	0.00	0.00	0.00	-0.58	-0.57	0.00	0.00	0.00
0.50	-0.48	0.00	0.63	0.00	0.00	0.00	0.52	0.00	0.00	0.00	0.00
0.00	-0.60	0.00	0.00	0.63	0.00	0.00	0.53	0.53	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	-0.43	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	-0.60	-0.48	0.00	0.00	-0.64	-0.54	0.00	-0.44	0.00
0.00	0.00	0.00	0.00	0.50	-0.45	0.00	0.49	0.49	0.00	0.00	0.00

Methanobrevibacter millerae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.50	0.00	0.00	0.00	0.00	0.00	0.50	-0.52	0.00	0.00	0.00	0.00	0.00

[a]- microbial counts and ARG concentrations based on the percentage of OTUs, [b] MLS – macrolide-lincosamide-streptogramin antibiotics

mgr Izabela Wolak

Katedra Inżynierii Ochrony Wód

i Mikrobiologii Środowiskowej

Wydział Geoinżynierii

Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 15.06.2023

OŚWIADCZENIE

Oświadczam, że w pracy:

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(5):1920. https://doi.org/10.3390/en15051920

mój wkład polegał na współudziale w opracowaniu metodyki, przygotowaniu pierwszej wersji manuskryptu oraz wykonaniu analiz laboratoryjnych.

Izabela Wolak

Podpis

mgr inż. Małgorzata Czatzkowska

Katedra Inżynierii Ochrony Wód

i Mikrobiologii Środowiskowej

Wydział Geoinżynierii

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mój wkład polegał na współudziale w opracowaniu metodyki. Byłam również odpowiedzialna za wykonanie analiz laboratoryjnych oraz statystycznych i pomoc edytorską przygotowywanego manuskryptu.

Małgorzata Czatzkowska

.....

Podpis

Prof. dr hab. inż. Monika Harnisz

Katedra Inżynierii Ochrony Wód

i Mikrobiologii Środowiskowej

Wydział Geoinżynierii

Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 15.06.2023

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mój wkład polegał na współudziale w opracowaniu metodyki i eksperymentu oraz zaplanowaniu analiz laboratoryjnych. Byłam również odpowiedzialna za pozyskanie środków finansowych i pomoc edytorską przygotowywanego manuskryptu.

Monika Harnisz

Podpis

dr Łukasz Paukszto

15.04.2023

Katedra Botaniki

i Ochrony Przyrody

Wydział Biologii i Biotechnologii

Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn,

OŚWIADCZENIE

Oświadczam, że w pracy:

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Podpis

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Uniwersytet Warmińsko-Mazurski w Olsztynie

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Podpis

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Article

Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment

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Abstract: Antimicrobials and antibiotic resistance genes (ARGs) in substrates processed during anaerobic digestion in agricultural biogas plants (BPs) can reach the digestate ($_D$), which is used as fertilizer. Antimicrobials and ARGs can be transferred to agricultural land, which increases their concentrations in the environment. The concentrations of 13 antibiotics in digestate samples from biogas plants (BPs) were investigated in this study. The abundance of ARGs encoding resistance to beta-lactams, tetracyclines, sulfonamides, fluoroquinolones, macrolide-lincosamide-streptogramin antibiotics, and the integrase genes were determined in the analyzed samples. The presence of cadmium, lead, nickel, chromium, zinc, and mercury was also examined. Antimicrobials were not eliminated during anaerobic digestion. Their concentrations differed in digestates obtained from different substrates and in liquid and solid fractions (ranging from 62.8 ng/g clarithromycin in the solid fraction of sewage sludge digestate to 1555.9 ng/L doxycycline in the liquid fraction of cattle manure digestate). Digestates obtained from plant-based substrates were characterized by high concentrations of ARGs (ranging from 5.73×10^2 copies/g_D *cfx*A to 2.98×10^9 copies/g_D *sul*1). The samples also contained mercury (0.5 mg/kg dry mass (dm)) and zinc (830 mg/kg dm). The results confirmed that digestate is a reservoir of ARGs (5.73×10^2 to 8.89×10^{10} copies/g_D) and heavy metals (HMs). In addition, high concentrations of integrase genes (10^5 to 10^7 copies/g_D) in the samples indicate that mobile genetic elements may be involved in the spread of antibiotic resistance. The study suggested that the risk of soil contamination with antibiotics, HMs, and ARGs is high in farms where digestate is used as fertilizer.

Keywords: ARGs; heavy metals; sewage sludge; slaughterhouse waste; cattle manure; maize silage

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1. Introduction

Due to growing levels of waste generation around the world and a higher demand for alternative and environmentally friendly energy, anaerobic digestion (AD) is becoming an increasingly popular method of stabilizing various types of organic matter substrates [1]. The popularity of installations such as biogas plants (BPs) is on the rise in Europe, including in Poland, and the number of BPs continues to increase. A total of 132,000 engineering biogas projects have been implemented around the world, including 17,783 in Europe. Biogas is upgraded to biomethane in 700 plants worldwide, 540 of which are located in Europe [2,3]. In Poland, the first BP was commissioned for use in 2005, and there were 128 agricultural BPs at the end of 2021 [4].

Most BPs use manure [5], sewage sludge [6], slaughterhouse waste [7], meat and bone meal [8], ensilaged biomass such as maize silage [9] and sugar beet silage [10], potato pulp [11], and confectionery press cake [12] as substrates for AD. These substrates are

processed into nutrient-rich digestate. Digestate contains organic and mineral compounds, nitrogen, macronutrients, and micronutrients, and it is colonized by digestate-specific microbiota composed of diverse microbial communities which participate in AD [13]. For many years, digestate has been used mainly as agricultural fertilizer around the world [14]. Digestate is a rich source of nutrients that promote plant growth, but it also contains antibiotics and their metabolites, antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), and heavy metals (HMs). Therefore, there is a high risk that these micropollutants will be transmitted to the soil when digestate is used as organic fertilizer [15].

Most antimicrobials, ARB, ARGs, and HMs present in digestate originate from substrates that are used in the AD process. Sewage sludge, the byproduct of wastewater processing in wastewater treatment plants (WWTPs), is also used as feedstock in BPs, and it contains large quantities of drugs, ARB, ARGs, and HMs [13] because most pharmaceuticals are not fully metabolized in the human body and are evacuated to WWTPs with wastewater. Moreover, HMs also reach WWTPs with industrial wastewater and manure, and they contribute to environmental pollution and can participate in the transmission of ARGs. Heavy metals such as cadmium (Cd), mercury (Hg), nickel (Ni), chromium (Cr), and lead (Pb) are characterized by high biostability and a long half-life in the environment, and they can also exert selection pressure on microorganisms [16]. Sewage sludge produced during wastewater treatment is used as a substrate for AD in agricultural BPs, often in combination with other agricultural feedstocks.

In digestate, pharmaceuticals and HMs can also originate from manure and agricultural wastes that are rich sources of mineral substances and microorganisms and are most widely used as substrates in AD. According to the literature, global annual antibiotic consumption in livestock production has been estimated at 172 mg/kg in pigs, 148 mg/kg in chickens, and 45 mg/kg in cattle [14]. In animals treated with antibiotics, 30% to even 90% of antimicrobials and their metabolites are retained in urine and feces, and thousands of tons of drugs are evacuated to the environment with manure [14,15]. In China, the world's largest meat producer, 45,000 tons of antibiotics were excreted with animal feces in 2013 [14].

Drugs that are introduced to the environment with digestate-based fertilizers can be highly toxic, and they can cause selection pressure which increases the concentrations of ARGs in soil and contributes to antibiotic resistance (AR) [9]. In addition, no distinction between human and veterinary medicinal products is made in Europe; therefore, the same antimicrobials or drugs belonging to the same class are used in human and veterinary medicine. For example, fluoroquinolones and sulfonamides are classified as veterinary drugs in China, but they account for 2.2% and 9.2%, respectively, of all antimicrobials sold in Europe, and are used in both human and veterinary medicine. Beta-lactams and tetracyclines are also widely used in human and veterinary medicine in Europe [17–19]. Macrolides [20], lincosamides [21], and dihydrofolate reductase inhibitors (trimethoprim) [22] have also been identified in agri-food products. Due to the wide availability of pharmaceutical products on the market, pathogens that are resistant to a single drug can develop resistance to an entire class of related antimicrobials [23,24].

The rate at which chemotherapeutics and HMs are degraded in soil is determined by temperature, soil texture, organic carbon content, and the resistance profile of soil-dwelling microorganisms [14]. Pharmaceuticals accumulated in soil can be carried by surface runoffs to water bodies which are used in livestock production [15], and they can be ingested by animals in successive stages of the food chain. These drugs can then be transmitted to humans via contaminated animal-based products [16]. Fresh produce can be a source of clinically significant drug residues, ARB, and ARGs for consumers [25]. Antibiotic-resistant bacteria pose a significant risk of infection, and the therapeutic efficacy of antimicrobials is being compromised by growing levels of AR in humans and animals.

Antibiotics and HMs present in the environment can contribute to the spread of ARGs and ARB in soil, and they can trigger the emergence of new ARGs or increase

their concentrations in a given environment [16]. Antimicrobial resistance genes can also spread rapidly between microbial communities via horizontal gene transfer (HGT) because microorganisms present in substrates subjected to AD and digestate can harbor mobile genetic elements (MGEs) such as integrons [17]. For this reason, the concentrations and spread of antibiotics, HMs, and ARGs in digestate produced by agricultural BPs should be monitored to prevent or limit the transmission of ARB and ARGs in the environment, and to minimize selection pressure on environmental microorganisms.

The digestate can be considered a rich source of valuable minerals, but at the same time, unfortunately, it can also be a reservoir of antibiotics, ARGs and heavy metals for plants and microorganisms. There is a large research gap in the literature related to this topic, because there are still only few data on the content of ARGs, HMs, and pharmaceuticals and their persistence in the fermentation mass based on various substrates, which during soil fertilization with digestate can significantly affect their content in soil and edible vegetables. So far, there are no studies available with the content of so many antibiotic groups, HMs, and ARGs in digestate based on different substrates and the possible environmental implications for the soil–plant–animal–human system related to their transfer to the soil along with digestate used as fertilizer. Therefore, in this preliminary study, digestate samples from agricultural BPs stocked with various substrates, including sewage sludge, animal manure, and green waste, were analyzed for the presence of beta-lactam (amoxicillin, ampicillin), tetracycline (tetracycline, oxytetracycline, doxycycline, chlortetracycline), sulfonamide (sulfamethoxazole, sulfonamide), fluoroquinolone (enrofloxacin, ciprofloxacin), macrolide (clarithromycin), lincosamide (clindamycin) antibiotics, and dihydrofolate reductase inhibitors (trimethoprim). Digestate samples were also examined for the presence of HMs such as Cd, Pb, Ni, Cr, zinc (Zn), and Hg, as well as genes encoding resistance to beta-lactams (bla_{TEM}, bla_{OXA}, *cfx*A), tetracyclines (*tet*A, *tet*M), sulfonamides (*sul*1, *sul*2), fluoroquinolones (*aac 6′-Ib-cr*, *qep*A), and macrolide-lincosamide-streptogramin (MLS) class antibiotics (*erm*F). The prevalence of class 1 and 2 integrase genes (*intI*1 and *intI*2) was also determined in digestate samples. The environmental implications of uncontrolled transmission of drugs and ARGs to agricultural land were assessed.

2. Materials and Methods

2.1. Characteristics of Biogas Plants and Digestate Samples

Three fully operational agricultural BPs in Poland were selected for the study and labeled as BP1, BP2, and BP3. The selected BPs process various types of substrates whose characteristics and composition are presented in Table 1.

Table 1. Characteristics and composition of the examined digestate samples.

Sample	Composition of Substrates	pH	Dry Mass (%)	FOS/ TAC Ratio	NH_4+ g/L	TN g/L	TP g/L
BP1S	Sewage sludge	8.40	14.9	0.15	1.10	6.50	2.80
BP1W		7.62	16.00	0.17	1.16	6.50	2.46
BP2S	Cattle manure, maize silage	6.70	3.01	0.19	1.20	na	3.00
BP2W		6.88	3.00	0.14	1.43	na	3.65
BP3S	Maize silage, slaughterhouse waste, potato pulp, confectionery press cake	7.83	4.80	0.18	2.40	na	na
BP3W		7.90	4.30	0.14	1.60	na	na

na—not available.

Digestate samples collected from each BP were labeled with the number of the corresponding BP and the sampling season (S—spring, W—winter): BP1S, BP1W, BP2S, BP2W, BP3S, and BP3W.

The presence and concentration of drugs and HMs, as well as the presence and abundance of ARGs, were examined depending on the substrate composition of digestate samples. Digestates were sampled in winter and spring, which differ in the availability of various substrates and weather conditions (such as temperature) and are characterized by increased

disease incidence and, consequently, antibiotic consumption. Digestate samples were collected directly from the outlets of full-scale bioreactors operating under mesophilic conditions. Digestate produced by all three BPs is used as organic fertilizer in agricultural production.

2.2. Determination of Antibiotics in Digestate Samples

The analytical procedure for identifying antibiotics in the preliminary study was based on the methods that had been used in our previous research [26–28]. Digestate samples were analyzed for the presence of the following antibiotics according to the procedure presented in Table S1: amoxicillin—AMO, ampicillin—AMP, tetracycline—TC, oxytetracycline—OXY, doxycycline—DOX, chlortetracycline—CHLOR, sulfamethoxazole—SMX, sulfonamide—SFD, enrofloxacin—ENF, ciprofloxacin—CIP, clarithromycin—CLR, clindamycin—CLD, and trimethoprim—TRI.

The limit of quantification (LOQ) was set at 0.1 ng/L of fresh liquid digestate fraction and 0.1 ng/g of fresh solid digestate fraction.

2.3. Determination of HMs in Digestate Samples

The presence and concentrations of six HMs, i.e., Cd, Pb, Ni, Cr, Zn, and Hg in digestate samples were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). The analyses of digestate samples were conducted in an accredited laboratory of the Institute for Ecology of Industrial Areas (IETU), Katowice, Poland [29,30].

2.4. gDNA Isolation

Digestate samples (2 g) were placed in 2 mL Eppendorf tubes and centrifuged at 8000 rpm for 10 min. The supernatant was discarded after centrifugation. Genomic DNA (gDNA) was isolated from the pellet in triplicate, according to the instructions provided by the manufacturer of the Fast DNA Spin Kit for Soil® (MP Biomedicals™, Carlsbad, CA, USA). The concentration and quality of the isolated gDNA were evaluated with the Multiskan Sky spectrophotometer (Thermo Scientific™, Waltham, MA, USA). Additionally, gDNA was isolated in triplicate from each sample, and the isolates were pooled to obtain a representative gDNA sample for each digestate sample. The samples were freeze-stored (−20 °C) until analysis.

2.5. Quantification of ARGs and Integrase Genes

The presence and abundance of genes encoding resistance to beta-lactams (bla_{TEM}, bla_{OXA}, *cfx*A), tetracyclines (*tet*A, *tet*M), sulfonamides (*sul*1, *sul*2), fluoroquinolones (*aac 6′-Ib-cr*, *qep*A), and macrolide-lincosamide-streptogramin (MLS) class antibiotics (*erm*F) were analyzed in digestate samples. The prevalence of class 1 and 2 integrase genes (*intI*1 and *intI*2) was also determined. Genes conferring resistance to the above classes of antibiotics were analyzed because these antimicrobials are widely used in agriculture and in human and veterinary medicine. The presence and abundance of integrase genes were examined to assess the degree to which MGEs participate in the spread of AR. Antibiotic resistance genes were quantified in the quantitative Polymerase Chain Reaction (qPCR) in the LightCycler® system (Roche Diagnostics, Mannheim, Germany). The SYBR Green dye (Roche Diagnostics, Mannheim, Germany) was used in the reaction mixture with a total volume of 15 µL. The qPCR mix contained 0.8 µL of the DNA matrix, 0.4 µL of the corresponding primer with a concentration of 10 µM, 7.5 µL of SYBR Green, and 5.9 µL of RNase-free water. Genes were amplified in triplicate, and amplification parameters are presented in Table S2 (Supplementary Materials). Melt curves were generated and analyzed for non-specific amplification. Fluorescence excitation spectra were analyzed with the use of LightCycler® v. software (version 1.5.0). The number of gene copies was expressed in terms of absolute abundance (AA) in 1 g of digestate (ARG copy number/g_D).

2.6. Statistical Analysis

Significant differences between ARG and HM concentrations in samples were determined in the Kruskal–Wallis non-parametric test (Statistica v. 13.3). The correlations between ARG abundance, antibiotic concentrations, and HM concentrations in samples were determined in the Principal Component Analysis (PCA). Principal Component Analysis was applied using as variables all data from samplings BP1S, BP1W, BP2S, BP2W, BP3S, and BP3W regarding the concentration of 13 antibiotics such as AMO, AMP, TC, OXY, DOX, CHLOR, SMX, SFD, ENF, CIP, CLR, CLD, and TRI; ARGs concentration, such as *bla*TEM, *bla*OXA, *cfx*A, *tet*A, *tet*M, *sul*1, *sul*2, *aac 6′-Ib-cr*, *qep*A, *erm*F; and the integrase genes concentration (*intI*1 and *intI*2) and HMs concentration such as Cd, Pb, Ni, Cr, Zn, and Hg. Moreover, non-parametric Spearman's correlation analysis was performed to indicate the effect of antibiotic concentration on the ARGs and integrase genes studied. The results were regarded as statistically significant at $p < 0.05$. The results of statistical analyses were visualized with the use of GraphPad Prism 8.4 software (v. 9.5.0).

3. Results and Discussion

3.1. Presence and Concentrations of Antibiotics in Digestate Samples

The study revealed that 8 out of the 13 tested antibiotics were present in digestate samples. The presence of pharmaceuticals was determined by the composition of the examined digestates. Digestate samples differed significantly in antibiotic concentrations (Figure 1; Table S3, Supplementary Materials) (Kruskal-Wallis, ANOVA, $p < 0.05$). None of the examined digestate samples contained SMX, OXY, CHLOR, AMO, or AMP.

The abundance of antibiotics was highest in samples BP1S and BP1W from the BP stocked with sewage sludge. The concentrations of drugs belonging to each antibiotic class also differed considerably between the samples of sewage sludge digestate. Moreover, liquid and solid fractions of digestate also differed in terms of the presence and concentrations of the analyzed drugs. Antibiotics were identified more often and with similar frequency in the solid fraction. The presence of drugs in sewage sludge digestate could be attributed to substrate contamination before AD. Antibiotics are widely prescribed around the world, and they are not fully metabolized in the body, which is why high concentrations of pharmaceuticals are present in sewage sludge. Moreover, according to the literature, the incidence of acute respiratory infections varies on a seasonal basis [31], which is why drug consumption and transmission to WWTPs with wastewater is intensified in selected seasons of the year [32]. Therefore, significant quantities of antimicrobials that are not biodegraded in the wastewater treatment process can be carried to sewage sludge, which is used as a substrate in bioreactors, which explains their presence in the examined digestate samples. It should also be noted that pharmaceuticals including antibiotics are often overused and used without medical guidance, which can significantly increase their concentrations in sewage sludge [33,34].

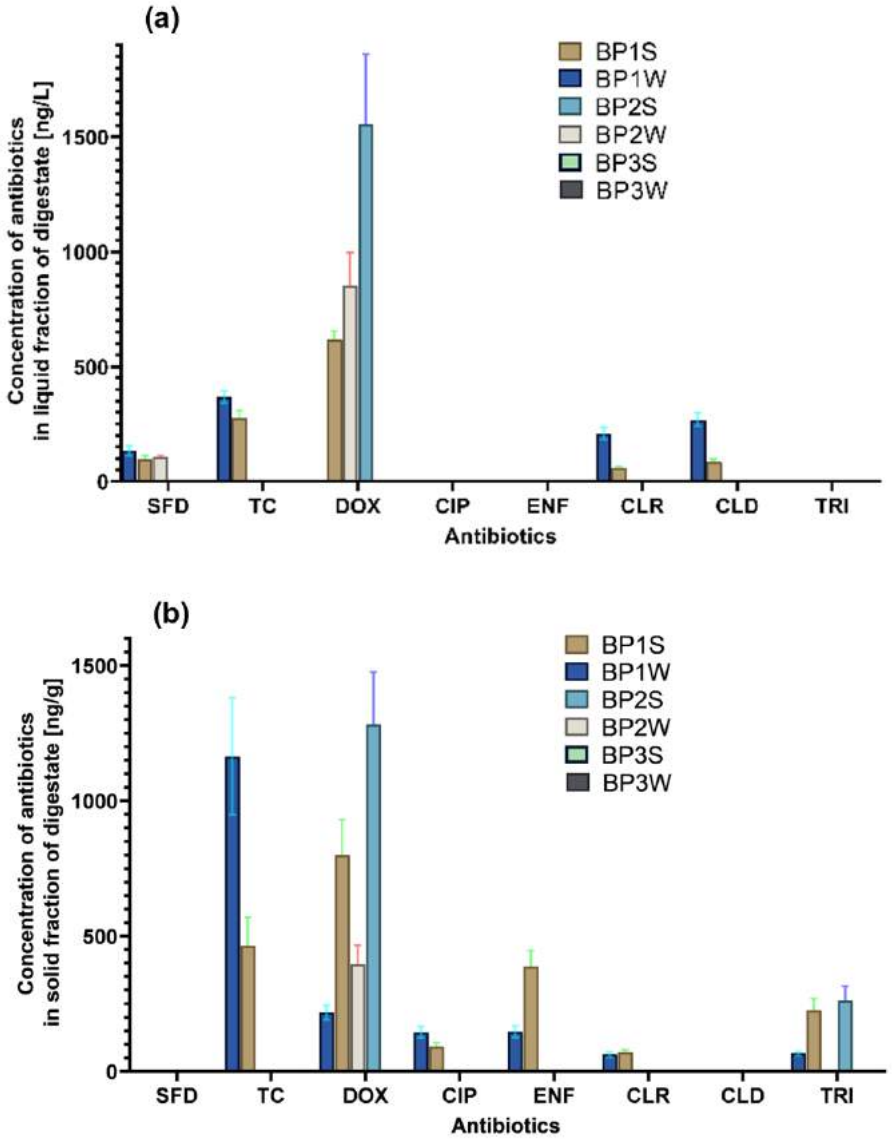


Figure 1. Concentrations of antibiotics in (**a**) liquid and (**b**) solid fractions of digestate. Error bars denote standard deviation (SD).

3.1.1. Tetracyclines

The analyzed digestate samples contained TC and DOX antibiotics from the tetracycline class. Tetracycline was identified in samples BP1W and BP1S, in both liquid and solid fractions of the examined matrix. However, TC concentrations differed significantly (Kruskal–Wallis, ANOVA, $p < 0.05$) between liquid and solid fractions. In sample BP1W, TC concentration was determined at 1164.4 ng/g in the solid fraction and 368.7 ng/L in the liquid fraction. In sample BP1S, TC concentration was determined at 464.8 ng/g in the solid fraction and 275.6 ng/L in the liquid fraction.

Significant differences in DOX concentrations were observed between digestate samples, depending on their substrate composition (Kruskal–Wallis, ANOVA, $p < 0.05$). Doxycycline was identified in the samples collected from two out of the three analyzed BPs (samples BP1W, BP1S, BP2W, and BP2S). Samples BP1W and BP1S were obtained from a BP processing sewage sludge, whereas samples BP2W and BP2S were collected in a bioreactor stocked with cattle manure and maize silage. Doxycycline was identified in nearly all of the above samples, in both solid and liquid fractions. Only the liquid fraction of sample BP1W was free of DOX. In the solid fractions of samples BP1W, BP1S, BP2W and BP2S, DOX concentration ranged from 218.1 ng/g to even 1282.5 ng/g. In the liquid fractions

of samples BP1S, BP2W and BP2S, DOX concentration was determined in the range of 620.4 ng/L to 1555.9 ng/L. In the studied digestate samples, DOX was present in higher concentrations than the remaining antibiotics. Doxycycline levels were influenced by the substrate composition of digestate samples, but not by digestate fraction (solid or liquid).

Similar results were reported by other authors [35] who identified DOX in digestate samples and found that the half-life of DOX in digestate can be as long as 91 days. In another experiment [36], the concentration of TC reached up to 36 µg/L in both liquid and solid digestate fractions. The results of this study and the findings of other authors indicate that tetracycline class drugs are not fully removed in the AD process, and that repeated soil fertilization with digestate containing tetracyclines can lead to their accumulation in soil and can directly affect the soil microbiome and resistome [37].

3.1.2. Sulfonamides

Significant differences in the presence and concentrations of SFD were noted in solid and liquid fractions of digestate samples (Kruskal–Wallis, ANOVA, $p < 0.05$). Sulfonamides were identified in liquid fractions of samples BP1W, BP1S, and BP2W (Kruskal–Wallis, ANOVA, $p < 0.05$). Samples BP1W and BP1S were obtained from a BP processing sewage sludge, whereas the sample BP2W was collected from a bioreactor stocked with cattle manure. Sulfonamide concentrations ranged from 98.2 ng/L to 136.5 ng/L. According to other authors, SFD are effectively biodegraded during AD, but their removal rate can be affected by the conditions inside the bioreactor, such as the presence of iron, sulfates, and methane-producing microorganisms [16,17]. The biodegradation of SFD during AD can be attributed to the presence of $–NH_2$ and $–CH_3$ electron donating groups in the drugs' molecular structures. The biotransformation of SFD during AD can lead to the reduction in the electron-withdrawing sulfonyl group [16] or the iron- and sulfate-catalyzed cleavage of the isoxazole ring [17]. Interestingly, in the present study, SFD were identified in the liquid, but not in the solid phase of digestate. According to many authors, SFD are biodegraded more rapidly in the liquid fraction, but this class of antibiotics could have been more effectively eliminated from the solid fraction due to the accumulation of chemical compounds that promote their biodegradation, whereas in the liquid fraction, these compounds were diluted, which could have affected their biodegradation pathway [38]. The SFD biodegradation pathway could have also been influenced by the presence of iron and sulfates in digestate, which is why this drug was not identified in half of the examined digestate samples.

Similar results were reported in other studies [20]. Numerous authors identified SFD in digestate obtained under laboratory conditions [18] and in digestate from industrial bioreactors [19]. In the present study, SFD concentrations were highest in sewage sludge and cattle manure digestates. These observations can probably be attributed to direct consumption of SFD by patients and increased use of SFD in livestock production. Sulfonamides are broad-spectrum antibiotics that are widely used in veterinary medicine, and they can significantly contribute to the spread of AR in the environment due to their high mobility and low susceptibility to biodegradation [39]. For example, pharmaceuticals introduced to soil with digestate can directly affect microbial communities, reduce bacterial groups sensitive to chemotherapeutics, and contribute to AR. Drugs can also exert a negative impact on crops by promoting the spread of ARGs in edible plant parts [28] and by inhibiting plant growth [40].

3.1.3. Fluoroquinolones

Ciprofloxacin was identified in samples BP1W and BP1S collected from a BP stocked with sewage sludge. Ciprofloxacin concentration was significantly higher in the solid fraction of sample BP1W (145.3 ng/g) than sample BP1S (92.4 ng/g) (Kruskal–Wallis, ANOVA, $p < 0.05$). This drug was not identified in the liquid fraction. Enrofloxacin was detected only in the solid fraction, and similarly to CIP, it was identified in samples BP1W

and BP1S (digested sewage sludge) at concentrations of 147.1 and 387.9 ng/g, respectively (Kruskal–Wallis, ANOVA, $p < 0.05$).

In an Austrian study, antibiotics were identified in digestate samples, and digestate-fertilized soils were contaminated with ENF and CIP [41]. According to the literature, fluoroquinolones are easily adsorbed on various types of organic substrates [42] during AD. These observations indicate that ENF and CIP pose a considerable risk of ecotoxicity when introduced to soil with digestate. These drugs can exert selection pressure on soil-dwelling microorganisms and contribute to the spread of ARGs and AR in the soil microbiome [43].

3.1.4. Macrolides and Lincosamides

In sewage sludge digestate (BP1W), CLR and CLD concentrations were significantly higher (more than three times) in the liquid fraction (208.9 and 268.4 ng/L, respectively) than in the liquid fraction (56.97 ng/L and 88.6 ng/L, respectively) of sewage sludge digestate (BP1S) (Kruskal–Wallis, ANOVA, $p < 0.05$).

Other studies [44–46] revealed that CLR and CLD were adsorbed by sewage sludge, which indicates that the use of sewage sludge as fertilizer, even after AD, without further processing can be a source of soil and groundwater contamination with antibiotics and ARGs. This is an important observation in the context of European and Polish directives which regulate only the content of nutrients such as nitrogen in organic fertilizers, but not the content of antibiotics which reach the soil with fertilizers from processed agricultural waste [47]. The above increases the risk that significant quantities of pharmaceuticals will be introduced the environment in an uncontrolled manner with digestate-based fertilizers.

3.1.5. Trimethoprim

Trimethoprim was identified in the solid fraction of samples BP1W and BP1S at concentrations of 66.8 ng/g and 226.1 ng/g, and in the solid fraction of sample BP2S (cattle manure and maize silage digestate) at 261.9 ng/g (Kruskal–Wallis, ANOVA, $p < 0.05$). Trimethoprim contains two aromatic rings, a double aminated pyrimidine ring and phenyl-trimethyl ether. According to the literature, this drug is effectively biodegraded (in more than 80%) in both liquid and solid fractions of organic matter [48]. Anaerobic biodegradation of TRI can be catalyzed by the cleavage of phenyl methyl ether or the substitution of the pyrimidine ring, depending on AD conditions inside the bioreactor [49]. Trimethoprim contains only functional electron donating groups, such as $–NH_2$, –OH, $–CH_3$ and $–OCH_3$, in its molecular structure, which increases its susceptibility to biodegradation. Moreover, the drug has different biodegradation pathways under anaerobic conditions, depending on the availability of electron acceptors, both inorganic (such as NO_3^-, SO_4^{2-}, Fe^{3+}, and Mn^{4+}) and organic (such as sulfoxide and dimethyl sulfide) [50]. The presence of TRI in both sewage sludge and manure digestates suggests that this drug had been administered to human and veterinary patients in seasons with increased disease prevalence. In human medicine, TRI is a first-line antibiotic in the treatment and prevention of urinary tract infections, and it is used as a prophylactic in veterinary medicine and livestock production. When administered frequently, this drug can be transferred to sewage sludge and cattle manure which are used as feedstocks in agricultural BPs. The above could explain the presence of TRI in the analyzed digestate samples. Moreover, according to the literature, TRI has a long half-life in organic biomass [51–53], which contributes to its persistence in digestate.

The concentrations of the analyzed antimicrobials were worryingly high in the studied samples, and they could induce irreversible changes in the environment by promoting the spread of AR and compromising the therapeutic efficacy of pharmaceuticals in human and veterinary medicine. According to the literature, predicted no-effect concentration (PNEC) values for resistance selection range from 8 ng/L to 64 µg/L [17], whereas in this study, drug concentrations were several or more than ten times higher. This observation gives significant cause for concern because the analyzed matrix—digestate—is often used as organic fertilizer, and therefore introduced to the natural environment.

The composition of individual digestate samples has a significant impact on the availability and degradation of antibiotics. For example, sulfadiazine and ciprofloxacin are widely used antibiotics in both human and veterinary medicine, and their residues are among the most frequently detected pharmaceuticals in the environment [6]. Sulfadiazine can be used in combination with trimethoprim in the treatment and prevention of respiratory infections and mastitis in cattle. These drugs are not fully metabolized in animals; they are excreted in feces and urine and subjected to AD in plants that process cattle slurry. Sewage sludge also increases the availability of antibiotics in the environment [8]. Wastewater treated in WWTPs contains large quantities of drugs, and contaminants such as pharmaceuticals are not fully removed during wastewater treatment, and are transferred to water bodies together with the treated wastewater [9]. In addition, the resulting sewage sludge is used as fertilizer in fields, which further increases the pool of drugs that are released into the environment. Cattle manure, sewage sludge, and digestates are widely used as fertilizers in agriculture. These potential sources of ARB, ARGs, and antibiotics exert a significant impact on agricultural ecosystems and, ultimately, on consumers of fresh vegetables or meat [5]. Digestates based on cattle slurry or sewage sludge are introduced to the soil, which is why plant substrates should also be analyzed for the presence of drugs absorbed from the applied fertilizer [1].

Furthermore, according to many authors [6,48], drugs differ in half-life and degree of biodegradation in various digestate fractions. Some drugs are biodegraded more rapidly in the solid fraction due to the accumulation of chemical compounds that favor their decomposition, whereas the compounds present in the liquid fraction are often dispersed and diluted, which may also affect their biodegradation rate. However, depending on the drug class, selected pharmaceuticals are absorbed more rapidly by the solid fraction of organic matter, where they persist for longer periods of time [16].

3.2. Distribution of ARGs and Integrase Genes in Digestate Samples

As demonstrated in Figure 2, 11 ARGs conferring resistance to five antibiotic classes, as well as class 1 and 2 integrase genes, which promote the spread of AR via HGT driven by MGEs, were identified in digestate samples. Antibiotic resistance genes were detected in all digestate samples (Figure 2; Table S4, Supplementary Materials) at concentrations of 1.16×10^3 to 2.25×10^6 copies/g of digestate. The statistical analysis based on the Kruskal–Wallis non-parametric test did not reveal significant differences in ARG copy numbers between the analyzed samples (Kruskal–Wallis, ANOVA, $p < 0.05$) (data not shown). The abundance of ARGs was highest in sewage sludge digestate (samples BP1S and BP1W) and digestate derived from a mixture of cattle manure and maize silage (samples BP2S and BP2W).

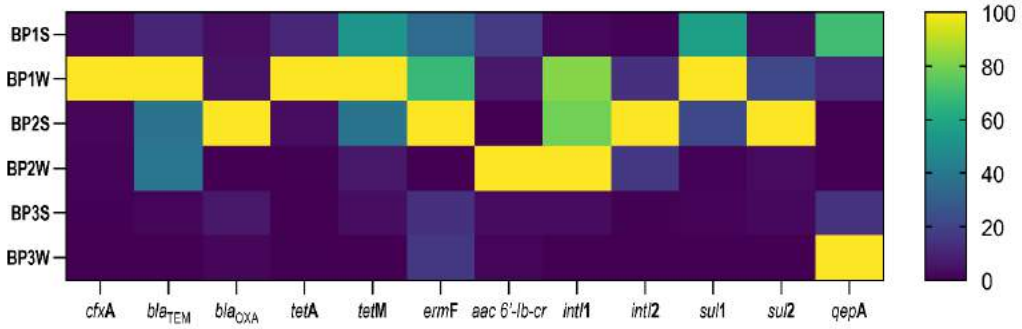


Figure 2. Normalized heatmap (%) presenting the absolute abundance (AA) of antibiotic resistance and integrase genes in digestate samples (number of copies/g of digestate).

According to Koniuszewska et al., [6] antimicrobial substances influence the prevalence of the evaluated ARGs. The presence of antibiotics in sewage sludge significantly increased (up to two orders of magnitude) the concentrations of these genes in 1 g_D compared with the

control [16]. An increase in ARG concentrations in the digestate exposed to antimicrobials, relative to the control, could indicate that microorganisms have acquired new ARGs or have gradually adapted to the new environment [6]. According to the literature [6,16], *intI*1 and *intI*2 play an important role in the transfer of ARGs during AD. The gene *intI*1 is the most ubiquitous integrase gene in the environment which, together with sulfonamide resistance genes, has recently been recognized as an indicator of contamination with ARB, ARGs, and other anthropogenic pollutants. Integrase genes are implicated in the HGT mechanism; therefore, integron-associated genes should be included in environmental AR analyses [16]. The results of other authors are consistent with the results obtained in this study (Figure S1, Supplementary Materials), in which numerous statistically significant positive correlations between the concentration of drugs and the number of individual ARGs and integrase genes were noted.

3.2.1. *tet*A and *tet*M Genes

The abundance of *tet*A and *tet*M genes encoding resistance to tetracyclines was high in the analyzed digestate samples. The abundance of the *tet*M gene ranged from 10^5 to 10^6 copies/g_D, whereas the abundance of the *tet*A gene was determined at 10^4 copies/g_D in most samples. The *tet*A gene was more prevalent only in sewage sludge digestate (from 10^5 copies/g_D in sample BP1S to 10^6 copies/g_D in sample BP1W).

The *tet* genes are ubiquitous in both Gram-negative and Gram-positive bacteria, and most bacterial *tet* genes are associated with mobile plasmids, transposons, conjugative transposons, and integrons. These genes confer tetracycline resistance via an active efflux of tetracycline (*tet*A) and by preventing bacterial ribosomes from binding to the drug (*tet*M) [54,55]. The above could explain the high concentrations of *tet* genes in the analyzed digestate samples. The high abundance of ARGs suggests that digestate-based fertilizers could pose a threat to human health. Moreover, digestate offers a supportive environment for the development of AR because it is characterized by high microbial diversity and a high content of organic carbon, nitrogen, phosphorus, and other nutrients that promote bacterial growth, even in the presence of multiple antibiotics [15].

3.2.2. *sul*1 and *sul*2 Genes

The gene *sul*1 was the most abundant gene in all digestate samples, and its concentration ranged from 2.45×10^9 to 8.89×10^{10} copies in 1 g_D. The abundance of the *sul*2 gene was also high, but its concentration ranged from 6.25×10^4 to 7.20×10^5 in 1 g_D and was even five orders of magnitude lower in comparison with *sul*1.

Other authors also reported high concentrations of *sul*1 in cattle manure digestate [56] and *sul*2 in manure-fertilized soil [28]. Sulfonamides were the first antimicrobials to be used systemically and, according to the literature, hospital wastewater appears to be the main source of the *sul*1 gene [57]. However, sulfonamides are also widely used in the treatment and prevention of diarrhea in veterinary medicine. Sulfonamides inhibit dihydropteroate synthase in the bacterial folic acid biosynthesis pathway, and *sul*1 and *sul*2 genes confer resistance by encoding forms of dihydropteroate synthase that are not sensitive to the drug [58]. These genes can be located on transposons and mobile plasmids which confer multi-drug resistance in numerous hosts [59], which suggests that digestate-based fertilizers can promote the spread of AR in the environment.

3.2.3. *aac 6′-Ib-cr* and *qep*A Genes

The abundance of the *qep*A gene ranged from 10^4 to 10^5 copies/g_D in digestate samples. The concentration of the *aac-(6′)-Ib-cr* resistance gene was one order of magnitude lower, at 10^3 copies/g_D in most samples. The concentration of the *aac-(6′)-Ib-cr* gene was one order of magnitude higher (10^4 copies/g_D) only in sample BP1W, relative to the remaining samples. The *aac-(6′)-Ib-cr* gene encodes an enzyme which inactivates ciprofloxacin and norfloxacin by N-acetylation [60]. The *qep*A gene encodes efflux pump proteins of the major facilitator superfamily, which transport drugs to extracellular space. In the present study,

the high abundance of *qep*A in digestate samples can be attributed to its non-specificity. The efflux pump encoded by *qep*A can decrease the toxic accumulation of antimicrobials inside cells, thus contributing to microbial growth during AD and promoting the transfer of ARGs between bacterial cells [61].

Other authors also reported high abundance of the *aac-(6′)-Ib-cr* gene in digestate [62], wastewater [63], and river water [64], as well as the high abundance of the *qep*A gene in sewage sludge [57]. The presence of this gene in environmental samples can be explained by the fact that it is introduced to the soil and the environment with digestate-based fertilizers. In the current study, high CIP concentrations in digestate samples suggest that this drug can increase the abundance of *aac(6′)-Ib-cr* and *qep*A genes in soil fertilized with digestate.

3.2.4. *erm*F Genes

The *erm*F gene was abundant in all digestate samples, regardless of their origin, and its concentration ranged from 10^7 to 10^8 copies/g_D. The *erm*F gene was most abundant (10^8 copies/g_D) in samples of cattle manure digestate. These results suggest that digestate is a hot spot of ARGs. In the literature, strains of *Enterococcus* resistant to erythromycin were identified in ready-to-eat meat products, which implies that these genes can be transmitted to the gut microbiota of livestock and animal-based foods [65].

3.2.5. *cfx*A, bla_{TEM} and bla_{OXA} Genes

The gene *cfx*A was the most abundant beta-lactam-resistance gene, and its concentration reached 3.33×10^5 copies/g of digestate in sample BP1W (sewage sludge digestate).

All beta-lactam-resistance genes were also identified in digestate samples from BP3, which processes maize silage, slaughterhouse waste, potato pulp, and confectionary press cake (samples BP3S and BP3W), but their concentrations were one order of magnitude lower on average (10^3 to 10^4 copies/g_D) than in the remaining samples (10^5 copies/g_D). Genes encoding resistance to beta-lactams are ubiquitous in the environment. The analyzed digestate samples were free of beta-lactams that could exert selection pressure, but they were characterized by high concentrations of beta-lactam-resistance genes that can be transported the soil environment with digestate, accumulate in plants and, consequently, reach the animals' gut microbiota [28,66].

3.2.6. *intI*1 and *intI*2 Genes

Integrons participate in the spread of ARGs via HGT mediated by MGEs, and the *intI*1 gene is regarded as an indicator of HGT [67]. In the present study, both integrase genes (*intI*1 and *intI*2) were abundant in all digestate samples (regardless of their substrate composition), and their concentrations ranged from 4.37×10^5 to 5.84×10^7 copies/g_D. These results could indicate that MGEs are involved in the spread of AR.

Class 1 and 2 integrons (*intI*1, *intI*2) were the first integrons to be strongly associated with MGEs. In other studies, sewage sludge and manure digestates frequently harbored *intI*1 [68,69] and *intI*2 genes [70]. There is evidence to suggest that digestate-based fertilizers containing integrase genes contribute to the transmission of ARGs [71] and accelerate the spread of AR with the involvement of MGEs [72].

In summary, the results of this study indicate that digestate contaminated with ARGs can lead to the development of antibiotic resistance in soil bacteria. Antibiotic resistance genes are transported to the soil environment by digestate and are accumulated by host bacteria in soil. They can spread via HGT or novel mutations in soil bacteria [73]. Moreover, ARGs can spread even if antibiotics have been completely or partially degraded [74]. This is due to the fact that other environmental contaminants [75], such as HMs [76], can also co-select for resistance genes and contribute to the persistence of ARGs in the environment [77].

3.3. Heavy Metals in Digestate Samples

The presence and concentrations of the analyzed HMs differed significantly (ANOVA, Kruskal–Wallis, $p < 0.05$) in digestate samples, depending on their origin. Heavy metals were identified in all samples, but not every digestate sample contained all of the analyzed HMs. Heavy metals such as Cd, Ni, Cr, Zn, and Hg were present in all samples. Lead was detected in sewage sludge digestate and digested mixtures of cattle manure and maize silage, but not in digestate originating from green waste. Sewage sludge digestate and digested mixtures of cattle manure and maize silage were characterized by similar HM levels (Table 2). Zinc was the most abundant HM, and its concentration ranged from 595 mg/kg $_{dm}$ in sample BP2W (digested cattle manure and maize silage) to 830 mg/kg $_{dm}$ in sample BP1S.

Table 2. Content of heavy metals (HMs) in digestate samples.

	Digestate Samples					
HMs (mg/kg$_{dm}$)	**BP1S**	**BP1W**	**BP2S**	**BP2W**	**BP3S**	**BP3W**
Cd	1.60	0.90	1.60	0.90	0.02	0.03
Pb	18.00	7.13	20.00	7.90	0.00	0.00
Ni	17.00	11.00	19.00	12.00	0.37	0.32
Cr	44.00	28.00	40.00	26.00	0.2	0.25
Zn	830.00	645.00	790.00	595.00	10.40	11.90
Hg	0.51	0.4144	0.49	0.3112	0.0097	0.0037

HMs—heavy metals; dm—dry mass; Cd—cadmium; Pb—lead; Ni—nickel; Cr—chromium; Zn—zinc; Hg—mercury.

Relatively high Zn concentrations in digested cattle manure and maize silage can probably be attributed to growth-promoting feed additives with increased levels of Cu and Zn. Zinc induces changes in the gut microbiome and inhibits anaerobic fermentation in the intestines, which decreases the availability of nutrients for fermenting bacteria and suppresses the growth of gut pathogens. Zinc is poorly absorbed in the intestines; it is excreted with feces and transferred to BPs that digest manure. Zinc and other HMs can accumulate in soils that are frequently fertilized with digestate [78]. In other studies, high concentrations of HMs were also reported in manure [28], agricultural soil [79], sewage sludge [80], and aquatic environments [81], which suggests that in addition to antibiotics, toxic HMs such as Hg, Cu, and Zn can exert strong selection pressure and increase ARG levels in the environment [28,78].

Heavy metal pollution from the livestock sector could promote the spread of AR by co-selection. Therefore, bacterial communities colonizing agricultural soils are strongly exposed to a combination of HMs and antibiotics. Exposure to both antimicrobial substances may increase the likelihood of selection and co-selection of AR [28]. The co-occurrence of HM resistance genes and ARGs on the same MGEs, such as integrons, is referred to as co-resistance. Co-resistance significantly facilitates the spread and proliferation of resistance in microbial communities. Cross-resistance is yet another mechanism responsible for the co-selection of bacterial resistance to antimicrobials and HMs. In cross-resistance, antibiotics and HMs initiate the same biochemical pathways, leading to the emergence of HM-driven AR co-regulation as the final mechanism in the co-selection of resistance to drugs and HMs. In this process, high concentrations of HMs in the environment increase bacteria's resistance to antibiotics by regulating the expression of specific genes, thus reducing microbial susceptibility to these compounds [16].

3.4. Statistical Analysis

Principal Component Analysis (PCA) of ARGs, Pharmaceuticals and HMs

Principal component analysis was used using all data from all samplings as variables to assess the overall patterns and variances of antibiotic concentrations, ARG, and HM

levels in the BP digestate samples. The PCA produced three main data clusters (Figure 3). On the PCA-presented mean loadings for ARGs, the content of antibiotics and HMs, and mean PC scores for biogas plants: A—biogas plant 1 (BP1), B—biogas plant 2 (BP2) and C—biogas plant 3 (BP3). The results for BP2 (B) revealed strong correlations between the values of DOX, bla_{OXA}, *sul*2, *intI*1, *intI*2, HMs, and TRI. The second cluster was formed by BP1, with strong correlations between the values of ENF, SFD, CLR, CLD, CIP, TC, *cfx*A, *tet*A, *tet*M, *sul*1, and bla_{TEM}. However, strong correlations with HM values were not observed. The values noted in each cluster suggest that resistance-inducing antibiotics, ARGs, and HMs had a common source. It can be concluded that the biogas plant processing both sewage sludge and liquid manure is a reservoir of drugs, ARGs, and HMs. The results of the PCA also indicate, with high probability, that HMs could contribute to an increase in ARG concentrations in the BP processing cattle manure (BP2) and promote the spread of AR. According to previous research [82], the presence of strong correlations between ARGs in digestate suggests that ARB could be present already in raw substrates. In the present study, the antibiotics detected in digestate samples could have exerted selection pressure during the AD process.

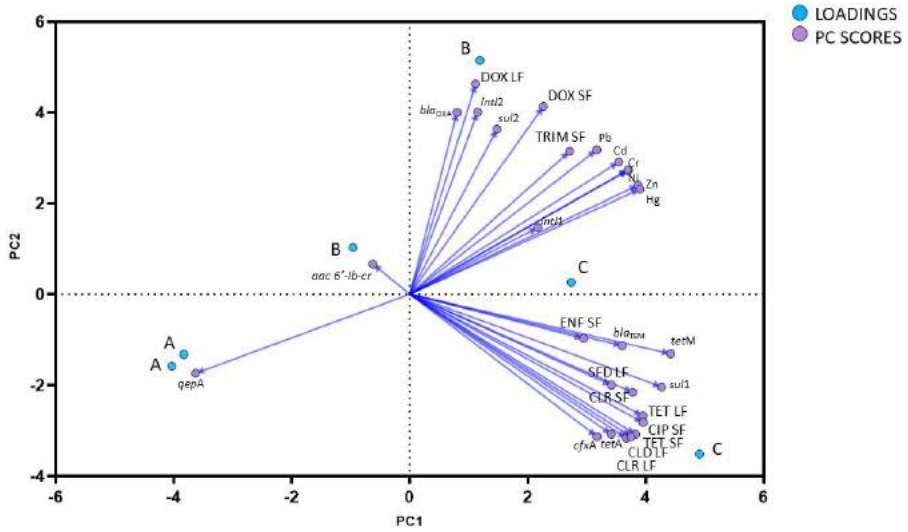


Figure 3. Principal Component Analysis (PCA) based on the distribution of ARGs and integrase genes, pharmaceuticals, and heavy metals (HMs) in all investigated samples. Mean loadings for ARGs and the content of antibiotics and HMs, and mean PC scores for biogas plants: A—biogas plant 1 (BP1), B—biogas plant 2 (BP2) and C—biogas plant 3 (BP3).

The results of the PCA suggest that soil fertilization with digestate from agricultural BPs can increase the abundance of various ARGs, metal resistance genes (MRGs), drugs, drug residues and the associated microorganisms in the soil environment, and that digestate can significantly contribute to the spread of ARGs in the environment.

3.5. Implications for the Environment

Digestate is a rich source of organic matter and nutrients, and it is often used as organic fertilizer. However, digestate is also a vector of veterinary antibiotics, which are transmitted to the soil ecosystem and plants [17] because the spread of antibiotics and ARGs in the environment is not limited to a single ecosystem. Antibiotics are transported between all ecological niches that form a specific network [83] which also carries ARGs, leading to artificial selection of microorganisms that initially are not resistant to antibiotics. Selection pressure from human activities and antibiotics influences evolutionary processes in the environment. A strong phylogenetic relationship was observed between human pathogens and pathogens colonizing livestock [84]. The spread of ARGs in the food chain can undermine the efficacy of antibiotics for treating infectious diseases in both livestock

and clinic and hospital patients. The wide spread of ARGs in the environment gives serious cause for concern. Another study demonstrated that manure storage in the proximity of animal pens increased resistance to chlortetracycline and tetracycline in soil bacteria [85]. Manure and digestate storage sites are permanent reservoirs of ARB and ARGs.

Abiotic factors can also contribute to the spread of ARGs. Physical factors such as wind, surface runoffs, flowing river water, and human activity can also increase AR in pathogenic bacteria in the environment [16,86–88]. Animals are raised on farms in close proximity to humans, and antibiotics are widely used as growth promoters to increase productivity and cater to the nutritional demands of the world's rapidly growing population, which increases the risk of ARGs transferring to the animals' gut microbiota. The substrates processed in agricultural BPs, including manure, green waste, and sewage sludge, are reservoirs of antibiotics and ARGs. The present study demonstrated that antimicrobials present in digestate samples in microgram quantities can be transferred to the environment and promote the evolution of bacteria able to grow at drug concentrations as high as 1000 mg/L. These results are comparable with the PNEC values estimated by some authors [89,90]. Drugs can significantly influence bacterial growth, the structure and composition of soil microbial communities, soil quality, and the quality of crops contaminated with high concentrations of antibiotics and ARGs [91].

Despite the fact that digestate is the end product of the AD process, the analyzed digestate samples were characterized by high concentrations of ARGs and antibiotics. These observations suggest that digestate-based fertilizers should be applied at intervals longer than two months to restore background levels of ARGs in digestate-modified soils. This strategy would improve agricultural practices, inhibit the spread of ARGs, and decrease their concentrations in the environment [92–95].

Effective strategies for controlling the use of antibiotics in agriculture are the simplest solution to reducing the concentrations of drugs and ARGs in cattle slurry, AD substrates, digestates, and farmland. Such strategies should be urgently implemented to minimize the health risks and adverse economic impacts of agricultural products containing drugs, ARB, and ARGs. Bioremediation and microbial electrolysis techniques are promoted in the agricultural sector to remove antibiotic residues and reduce AR. Heavy metals can be extracted from soil or wastewater with kaolin and sulfate-reducing bacteria. An integrated global strategy is needed to control the use of antibiotics in hospitals and the livestock sector, and novel methods should be introduced to optimize wastewater treatment, composting, and bioremediation of soil pollutants.

4. Conclusions

Studies evaluating the presence and concentrations of various antibiotic classes and HMs in complex matrices, such as digested cattle manure, maize silage, sewage sludge, slaughterhouse waste, green waste, and confectionary press cake, pose an analytical challenge because different protocols have to be applied depending on digestate fraction and composition. Moreover, the composition of digestate samples can vary considerably because many agricultural BPs process mixed substrates with different proportions of plant biomass and manure. Industrial residues such as slaughterhouse waste also pose a considerable challenge for researchers. Despite these problems, this study demonstrated that most of the analyzed antibiotics and HMs were not degraded during the AD process. Digestate samples were highly contaminated with various drugs and HMs whose concentrations were influenced by the composition of the substrates processed by the examined BPs. Attention should also be paid to factors that contribute to the spread of AR in plant substrates. Unlike sewage sludge and cattle manure digestates, plant-based digestates did not contain drugs, but were characterized by high concentrations of many ARGs and selected HMs. Digestate can be regarded as a reservoir of ARGs due to the high abundance of various ARGs. Digestate samples were free of beta-lactams, but contained numerous genes encoding resistance to this class of antibiotics, which could point to high levels of AR already in the substrates. High concentrations of integrase genes also suggest that MGEs

participate in the transmission of AR in digestate. The results of this study indicate that the use of digestate as organic fertilizer increases the risk of soil contamination with antibiotics, HMs, and ARGs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph20032672/s1, Table S1: Procedures used in the determination of antimicrobials in digestate samples; Table S2: Parameters of the qPCR reaction; Table S3: Antibiotic concentrations in digestate samples; Table S4: The presence and absolute abundance of antibiotic resistance genes (ARGs) in digestate samples (abbreviations are explained in Table S3 (number of copies/g of digestate); Figure S1: Spearman's non-parametric correlation analysis between antibiotic concentrations and concentrations of ARGs, integrase genes, physicochemical parameters (TN–total nitrogen; TP–total phosphorus), and concentration of HMs (Cd-cadmium, Pb–lead, Ni–nickel, Cr–chromium, Zn–zinc, and Hg–mercury) (other abbreviations are explained in Table S1 and Table S3). Sig-nificant statistical results are marked with an asterisk: * for $p < 0.05$; ** for $p < 0.01$; *** $p < 0.001$).

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Supplementary Materials

Digestate from agricultural biogas plants as a reservoir of antimicrobials and antibiotic resistance genes – implications for the environment

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Table S1. Procedures used in the determination of antimicrobials in digestate samples.

Class of antimicrobials	Compound	Fraction	References
betalactams	AMO	S	[1]
		L	[2]
	AMP	S	[1]
		L	[2]
tetracyclines	TC	S	[1]
		L	[2]
	OXY	S	[1]
		L	[2]
	DOX	S	[1]
		L	[2]
	CHLOR	S	[1]
		L	[2]
sulphonamides	SMX	S	[1]
		L	[2]
	SFD	S	[3]
		L	[2]
fluoroquinolones	ENF	S	[1]
		L	[2]
	CIP	S	[1]
		L	[2]
macrolides	CLR	S	[3]
		L	[2]
lincosamides	CLD	S	[3]
		L	[2]
dihydrofolic acid reductase inhibitor	TRI	S	[3]
		L	[2]

AMO – amoxicillin ; AMP – ampicillin; TC – tetracycline; OXY – oxytetracycline; DOX – doxycycline; CHLOR – chlortetracycline; SMX – sulfamethoxazole; SFD – sulphonamide; ENF – enrofloxacin; CIP – ciprofloxacin; CLR – clarithromycin ; CLD – clindamycin; TRI – trimethoprim; S – solid fraction; L – liquid fraction

Table S2. Parameters of the qPCR reaction.

Gene	Primer (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)	Reference

*cfx*A	CGTAGTTTTGAGTATAGCTTT GATGTTGCCTATATATGTC	802	56	[1]
*bla*TEM	AGTGCTGCCATAACCATGAGTG CTGACTCCCCGTCGTGTAGATA	431	61	[2]
*bla*OXA	ATTATCTACAGCAGCGCCAGTG TGCATCCACGTCTTTGGTG	296	61	
*tet*A	GCTACATCCTGCTTGCCTTC GCATAGATCGCCGTGAAGAG	211	54	[3]
*tet*M	ACAGAAAGCTTATTATATAAC TGGCGTGTCTATGATGTTCAC	171	55	
aac 6′-Ib-cr	TTGCAGTGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	57	[4]
*qep*A	GCCGGTGATGCTGCTGA CAGTAACAGCGCACCAA	204	63	[5]
*sul*1	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	163	55.9	[4]
*sul*2	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	191	57.5	
*int*I1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	54	[5]
*int*I2	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	233	50	
*erm*F	TAGATATTGGGGCAGGCAAG GGAAATTGCGGAACTGCAAA	126	58	[6]

Table S3. Antibiotic concentrations in digestate samples.

			Samples of digestate from biogas plants					
Antibiotic	Fraction	Parameters	BP1W	BP1S	BP2W	BP2S	BP3W	BP3S
SFD	LF	Av.	136.5	98.2	106.8	n.d	n.d	n.d
		SD.	21.6	14.3	8.8	-	-	-
	SF	Av.	n.d	n.d	n.d	n.d	n.d	n.d
		SD	-	-	-	-	-	-
TC	LF	Av.	368.7	275.6	n.d	n.d	n.d	n.d
		SD	27.2	32.3	-	-	-	-
	SF	Av.	1164.4	464.8	n.d	n.d	n.d	n.d
		SD	216.3	104.8	-	-	-	-
DOX	LF	Av.	<LOQ	620.4	854.6	1555.9	n.d	n.d
		SD	-	34.2	142.7	305.9	-	-
	SF	Av.	218.1	800.2	396.7	1282.5	n.d	n.d
		SD	26.4	131.1	69.5	193.8	-	-
CIP	LF	Av.	n.d	n.d	n.d	n.d	n.d	n.d
		SD	-	-	-	-	-	-
	SF	Av.	145.3	92.4	n.d	n.d	n.d	n.d
		SD	21.1	13.4	-	-	-	-
ENF	LF	Av.	n.d	n.d	n.d	n.d	n.d	n.d
		SD	-	-	-	-	-	-
	SF	Av.	147.1	387.9	n.d	n.d	n.d	n.d
		SD	21.8	56.2	-	-	-	-
CLR	LF	Av.	208.9	56.97	n.d	n.d	n.d	n.d
		SD	27.2	8.3	-	-	-	-

CLD	SF	Av.	62.8	70.7	n.d	n.d	n.d	n.d
		SD	9.8	9.4	-	-	-	-
	LF	Av.	268.4	88.6	n.d	n.d	n.d	n.d
		SD	29.1	8.7	-	-	-	-
TRI	SF	Av.	n.d	n.d	n.d	n.d	n.d	n.d
		SD	-	-	-	-	-	-
	LF	Av.	n.d	n.d	n.d	n.d	n.d	n.d
		SD	-	-	-	-	-	-
	SF	Av.	66.8	226.1	<LOQ	261.9	n.d	n.d
		SD	7.6	42.5	-	53.2	-	-

BP – biogas plant; 1,2,3 – Number of BP; W – winter; S – spring; SFD - sulfadiazine; TC - tetracycline; DOX - doxycycline; CIP - ciprofloxacin; ENF - enrofloxacin; CLR - clarithromycin; CLD - clindamycin; TRI - trimethoprim; Av. – Average (ng/g in solid fraction or ng/L In liquid fraction); SD – standard deviation; n.d – not detected;

Table S4. The presence and absolute abundance of antibiotic resistance genes (ARGs) in digestate samples (abbreviations are explained in Table S3) (number of copies/g of digestate).

	Targeted genes and class of antibiotics											
	betalactams			tetracyclines		fluoroquinolones		sulfonamides		MLS group	Integrase genes	
Sample	*cfx*A	*bla*TEM	*bla*OXA	*tet*A	*tet*M	*aac* 6′-Ib-cr	*qep*A	*sul*1	*sul*2	*erm*F	*int*I1	*int*I2
BP1S	4.98E3	1.07E4	2.60E3	2.47E5	1.26E6	9.50E3	2.34E5	5.19E10	1.63E5	4.13E7	4.37E5	4.37E5
BP1W	3.33E5	7.78E4	3.05E3	2.22E6	2.25E6	5.02E3	5.10E4	8.89E10	7.2E5	7.08E7	7.24E6	8.14E6
BP2S	4.98E3	3.11E4	4.25E4	7.87E4	9.65E5	2.60E3	1.60E4	2.11E10	3.14E6	1.00E8	6.94E6	5.84E7
BP2W	2.79E3	3.21E4	1.16E3	1.28E4	2.89E5	4.25E4	1.60E4	2.9E9	1.42E5	1.06E7	8.76E6	9.39E6
BP3S	1.50E3	3.46E3	3.70E3	1.10E4	2.29E5	3.70E3	6.15E4	2.98E9	1.19E5	2.30E7	5.19E5	1.39E5
BP3W	5.73E2	2.83E3	1.67E3	1.21E4	1.64E5	3.05E3	3.31E5	2.45E9	6.25E4	2.47E7	2.78E5	1.11E5

S – spring; W – winter

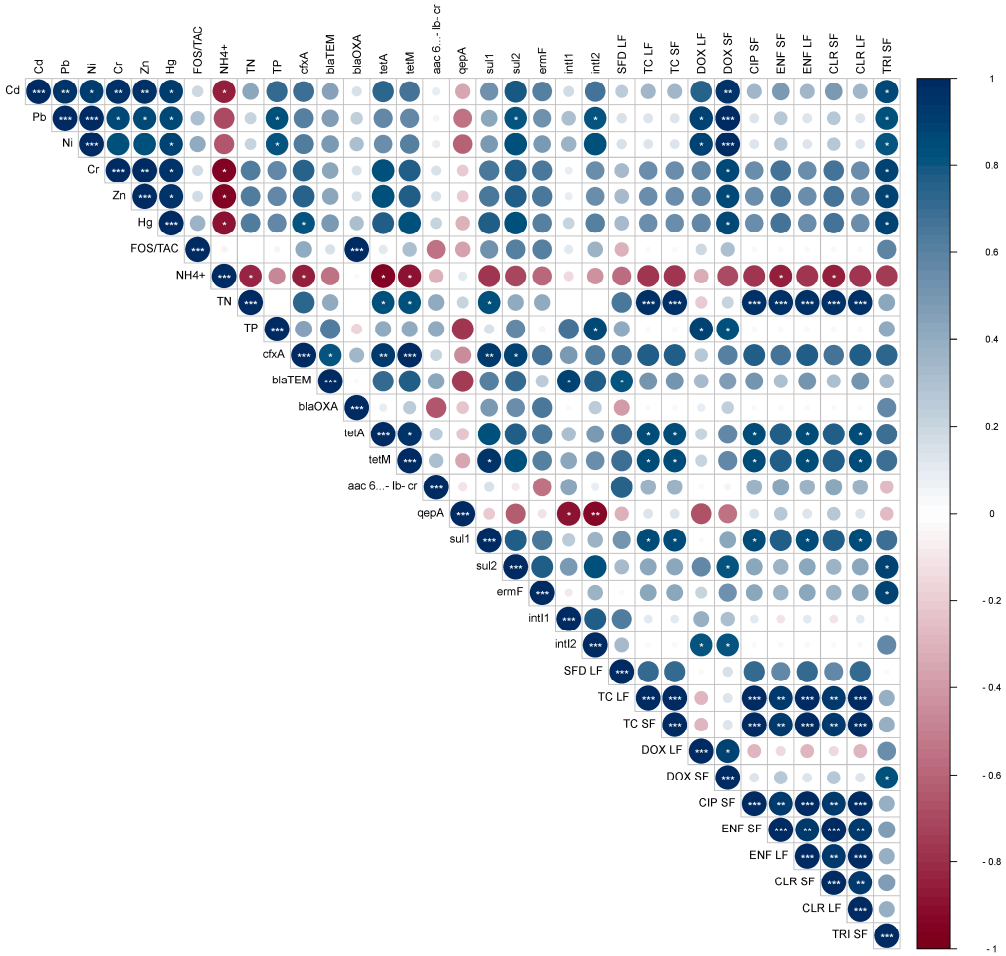


Figure S1. Spearman's non-parametric correlation analysis between antibiotic concentrations and concentrations of ARGs, integrase genes, physicochemical parameters (TN – total nitrogen; TP – total phosphorus), and concentration of HMs (Cd - cadmium, Pb – lead, Ni – nickel, Cr – chromium, Zn – zinc, and Hg – mercury) (other abbreviations are explained in Table S1 and Table S3). Significant statistical results are marked with an asterisk: * for p<0,05; ** for p<0.01; *** p<0.001).

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Olsztyn, 15.06.2023

OŚWIADCZENIE

Oświadczam, że w pracy:

Wolak I., Bajkacz S., Harnisz M., Stando K., Męcik M., Korzeniewska E. Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment. *International Journal of Environmental Research and Public Health.* 2023; 20(3):2672. https://doi.org/10.3390/ijerph20032672

mój wkład polegał na współudziale w opracowaniu metodyki, przygotowaniu pierwszej wersji manuskryptu oraz wykonaniu analiz laboratoryjnych.

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mój wkład polegał na współudziale w opracowaniu metodyki i eksperymentu oraz zaplanowaniu analiz laboratoryjnych. Byłam również odpowiedzialna za pozyskanie środków finansowych i pomoc edytorską przygotowywanego manuskryptu.

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Wydział Chemiczny

Politechnika Śląska

Gliwice, 15.05.2023

OŚWIADCZENIE

Oświadczam, że w pracy:

Wolak I., Bajkacz S., Harnisz M., **Stando K.,** Męcik M., Korzeniewska E. Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment. *International Journal of Environmental Research and Public Health.* 2023; 20(3):2672. https://doi.org/10.3390/ijerph20032672

mój wkład polegał na współudziale w opracowaniu metodyki i eksperymentu oraz zaplanowaniu i wykonaniu analiz laboratoryjnych.

Stando

.....

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Olsztyn, 15.04.2023

OŚWIADCZENIE

Oświadczam, że w pracy:

Wolak I., Bajkacz S., Harnisz M., Stando K., **Męcik M.,** Korzeniewska E. Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment. *International Journal of Environmental Research and Public Health.* 2023; 20(3):2672. https://doi.org/10.3390/ijerph20032672

mój wkład polegał na współudziale w opracowaniu metodyki. Byłam również odpowiedzialna za wykonanie analiz laboratoryjnych, statystycznych oraz przygotowanie pierwszej wersji manuskryptu.

Magdalena Męcik

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Wydział Geoinżynierii

Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 15.06.2023

OŚWIADCZENIE

Oświadczam, że w pracy:

Wolak I., Bajkacz S., Harnisz M., Stando K., Męcik M., **Korzeniewska E.** Digestate from Agricultural Biogas Plants as a Reservoir of Antimicrobials and Antibiotic Resistance Genes—Implications for the Environment. *International Journal of Environmental Research and Public Health.* 2023; 20(3):2672. https://doi.org/10.3390/ijerph20032672

mój wkład polegał na współudziale w opracowaniu metodyki. Byłam również odpowiedzialna za pomoc edytorską przygotowywanego manuskryptu.

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Olsztyn, 08.07.2023

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Dorobek naukowy

Artykuły znajdujące się w bazie Journal Citation Reports (JCR):

	Impact Factor/ Liczba punktów MEN	Suma IF/ Suma punktów MEN
[1] Koniuszewska I., Korzeniewska E., Harnisz M., and Czatzkowska M., "Intensification of biogas production using various technologies: A review", *International Journal of Energy Research,* 2020:2:1–19. https://doi.org/10.1002/er.5338	4.672/100	46.099/ 1300
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Czasopisma naukowe nie posiadające współczynnika Impact Factor (IF):

Publikacje w recenzowanych materiałach z konferencji międzynarodowej, uwzględnionej w Web of Science:

Rozdziały w monografiach w języku polskim i angielskim:

W języku angielskim:

1. Potorski J, **Koniuszewska I**, Czatzkowska M, Harnisz M. Drug resistance in airborne bacteria isolated from waste management and wastewater treatment plants in Olsztyn. Publikacja indeksowana przez bazy literatury recenzowanej Scopus oraz Web of Science E3S Web Conf. 11th Conference on Interdisciplinary Problems in Environmental Protection and Engineering EKO-DOK 2019;100. DOI: https://doi.org/10.1051/e3sconf/201910000066.

W języku polskim:

1. **Koniuszewska I.** Wpływ mikroorganizmów na jakość piwa – ocena mikrobiologiczna surowców, produktu i procesu technologicznego. Publikacja w recenzowanej monografii naukowej pod redakcją dr W. Truszkowskiego. ISBN 978-83-943385-5-8, rozdział V:2019: 251-258 s.

Doniesienia na konferencjach:

1. Potorski J, **Koniuszewska I**, Czatzkowska M, Harnisz M. Drug resistance in airborne bacteria isolated from waste management and wastewater treatment plants in Olsztyn. 11th Conference on Interdisciplinary Problems in Environmental Protection and Engineering EKO-DOK 2019. DOI: https://doi.org/10.1051/e3sconf/201910000066
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4. **Koniuszewska I.** Międzynarodowe Seminarium Kół Naukowych 2019, 11-12.04.2019 r., Olsztyn. Prezentacja posteru pt. „Wpływ mikroorganizmów na jakość piwa – ocena mikrobiologiczna surowców, produktu i procesu technologicznego".
5. **Koniuszewska I**, Czatzkowska M, Amenda E. Prezentacja posteru pt. „ Występowanie bakterii opornych na ampicylinę w Zakładzie Gospodarki Odpadami Komunalnymi w Olsztynie". 5th Conference Environmental Protection and Energy in Gliwice, 2018.
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10. Korzeniewska Ewa, Monika Harnisz, **Izabela Wolak**, Małgorzata Czatzkowska, 2022. Long-term impact of antimicrobials on the efficiency and microbiome of anaerobic digestion. ACB-ISBE 2022 (The Asian Congress on Biotechnology (ACB) 2022 in conjunction with The 7th International Symposium on Biomedical Engineering (ISBE), organized by the Asian Federation of Biotechnology (AFOB) and the Research Center for Biomedical Engineering Universitas Indonesia (RCBE UI)), Bali, Indonesia, 2-6 October, 2022.
11. Korzeniewska E., Harnisz M., **Koniuszewska I.**, Czatzkowska M., Kiedrzyńska E., 2019. The dynamics of the environmental resistome in flowing water. 5th International Symposium on the Environmental Dimension of Antibiotic Resistance, EDAR 2019, 9–14 czerwiec 2019, Hong Kong, Chiny, P64.

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13. E. Korzeniewska, **I. Wolak**, M. Harnisz, Magdalena Męcik, K. Stando and S. Bajkacz. Digestate from agriculture biogas plants as a potential source of tetracycline antimicrobials and antibiotic resistance genes. 2nd International Conference on Sustainable Chemical and Environmental Engineering 14th – 18th June 2023, Limassol, Cyprus.

Projekty badawcze:

1. „Wykorzystanie bioaugmentacji do intensyfikacji produkcji biogazu z substratów organicznych o wysokiej koncentracji antybiotyków", finansowany przez Narodowe Centrum Nauki w Krakowie (umowa Nr 3/18.650.035-300/2017) Konkurs OPUS, realizacja od listopada 2017 – listopada 2019 r., stypendystka projektu.
2. Studencki Grant Rektora dla Studenckiego Naukowego Koła Mikrobiologów Molekularnych „Coccus", 2018 – 2020 r., członek zespołu badawczego.
3. „Poferment z biogazowni rolniczych jako rezerwuar lekooporności - realne zagrożenie dla środowiska?", finansowany przez Narodowe Centrum Nauki w Krakowie (umowa Nr 2020/37/N/NZ9/00431) Konkurs PRELUDIUM – 19, luty 2021 – lipiec 2023 r., kierownik projektu.

Staże zagraniczne:

1. Udział w międzynarodowej wymianie akademickiej PROM 11.06 – 8.07.2021, Leibniz Unversity of Hannover, Niemcy.

Szkolenia:

1. Praktyka i analiza reakcji qPCR (Roche Diagnostics Polska Sp. z o.o.), **2019**.
2. Podstawy analiz bioinformatycznych (ideas4biology Sp. z o.o.), **2020.**
3. Szkolenie operatorskie LightCycler96 (Roche Diagnostics Polska Sp. z o.o.), **2021.**