The impact of selected antimicrobial drugs on biogas production, the prevalence of antibiotic resistance genes, and the microbiome involved in the anaerobic digestion of cattle slurry

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 determine 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.