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ROZPRAWA DOKTORSKA

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Wpływ antybiotyków na proces fermentacji metanowej osadów ściekowych, bioróżnorodność mikroorganizmów i zjawisko antybiotykooporności

The influence of antimicrobials on the anaerobic digestion of sewage sludge, microbial biodiversity, and the spread of antibiotic resistance

Praca wykonana w Katedrze Inżynierii Ochrony Wód i Mikrobiologii Środowiskowej, pod kierunkiem promotor prof. dr hab. inż. Moniki Harnisz oraz promotor pomocniczej dr Pauliny Rusanowskiej

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4. **Czatzkowska M**., Wolak I., Korzeniewska E., Harnisz M. 2022. Anaerobic Digestion in the Presence of Antimicrobials—Characteristics of Its Parameters and the Structure of Methanogens. Applied Sciences, 12, 17, 8422, https://doi.org/10.3390/app12178422
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Spis treści

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Załącznik 1

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Załącznik 2

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Załącznik 4

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Streszczenie

WSTĘP

Światowe zużycie i popyt na energię stale rosną, jednak zasoby takie jak węgiel, gaz ziemny i ropa naftowa nie stanowią zrównoważonego źródła energii. Z uwagi na rosnący poziom zaludnienia, koniecznym staje się zwiększenie nakładów energetycznych, co wpływa bezpośrednio na wzrost zainteresowania energią odnawialną. Biogaz, uzyskiwany na drodze fermentacji metanowej, stanowi jedną z najbardziej obiecujących alternatyw bioenergetycznych dla energii opartej na paliwach kopalnych (Holm-Nielsen et al., 2009; Scarlat et al., 2018). Co ważne, z przyjaznego środowisku paliwa, jakim jest biogaz, wytworzone może zostać najbardziej wydajne biopaliwo, czyli biometan (Bowe, 2013). Globalna liczba biogazowni na całym świecie wciąż rośnie, a potencjał rozwoju branży biogazowej jest ogromny i obejmuje każdy kraj. Aktualnie na całym świecie działa około 50 milionów mikro-bioreaktorów oraz łącznie 132,000 małych, średnich i dużych komór fermentacyjnych (World Biogas Association, 2019).

Fermentacja metanowa jest atrakcyjną praktyką przetwarzania odpadów, umożliwiającą ich zagospodarowanie przy jednoczesnym odzysku energii. Ten czteroetapowy, beztlenowy proces wymaga aktywności różnorodnych populacji mikroorganizmów, odpowiedzialnych za przebieg każdej z poszczególnych faz. Wśród nich wymienić można drobnoustroje hydrolizujące, acidogenne, acetogenne oraz metanogeny, bezpośrednio odpowiedzialne za produkcję metanu (Światczak et al. 2017). Niestety, znacznym utrudnieniem dla powszechnego stosowania tego procesu są problemy związane z jego optymalizacją i odpowiednio wysoką efektywnością. Główną przyczyną inhibicji fermentacji metanowej są różnorodne substancje, obecne w znacznych stężeniach w odpadach poddawanych stabilizacji beztlenowej. Wśród inhibitorów procesu można wymienić między innymi substancje przeciwdrobnoustrojowe (Rusanowska et al. 2019; Meegoda et al. 2018; Scarlat et al. 2018). Globalne spożycie antybiotyków w latach 2000-2015 wzrosło o 65%, z kolei na rok 2030 prognozowany jest 200% wzrost konsumpcji leków w porównaniu z rokiem 2015 (Klein et al., 2018). Intensywne spożywanie leków przez ludzi oraz ich nadmierne wykorzystanie w sektorze weterynaryjnym prowadzi do przedostawania się do środowiska antybiotyków w formie niezmienionej lub w postaci produktów ich transformacji. Z tego powodu leki przeciwdrobnoustrojowe mogą akumulować się w substratach poddawanych stabilizacji beztlenowej.

Fermentacja metanowa jest jedną z głównych strategii stabilizacji osadów ściekowych pochodzących z oczyszczalni ścieków (wastewater treatment plants – WWTPs) (Grobelak et al. 2019). Ilość osadów ściekowych wytwarzanych w WWTPs stale rośnie, a na całym świecie corocznie produkowane są one w setkach milionów ton. Zanieczyszczenia obecne w ściekach dopływających do WWTPs, w tym zanieczyszczenia mikrobiologiczne i pozostałości substancji przeciwdrobnoustrojowych, kumulują się w osadach ściekowych. Osady ściekowe mogą charakteryzować się występowaniem antybiotyków w koncentracjach wahających się od <1 do kilku tysięcy μg kg^{-1} (Czatzkowska et al., 2022). Co więcej, zarówno w ściekach, jak również w osadach ściekowych, stwierdza się występowanie ogromnej liczby drobnoustrojów, w tym mikroorganizmów chorobotwórczych. Szczególnie niebezpieczne wśród nich są bakterie posiadające mechanizmy oporności na substancje przeciwdrobnoustrojowe (Manaia et al. 2018). Jedną z przyczyn występowania bakterii posiadających jeden lub więcej genów oporności na antybiotyki (antibiotic resistance genes - ARGs), jest przede wszystkim niewłaściwe stosowanie substancji przeciwdrobnoustrojowych w medycynie ludzkiej i weterynaryjnej. Zjawisko wymiany struktur genetycznych między drobnoustrojami, w tym również ARGs, predysponuje do szerzenia w środowisku zjawiska antybiotykooporności (antimicrobial resistance - AR). Z uwagi na powszechne stosowanie antybiotyków w ostatnich dziesięcioleciach, ARGs definiuje się jako nowe zanieczyszczenia, zagrażające bezpieczeństwu i zdrowiu publicznemu (Becerra-Castro et al. 2015; Xu et al. 2017; Barancheshme and Munir 2019). Co ważne, z uwagi na obecność w ściekach i osadach ściekowych subinhibitorowych stężeń substancji przeciwdrobnoustrojowych, WWTPs sprzyjają zwiększonej selekcji bakterii posiadających specyficzne ARGs (Sun et al. 2019). Rodzaje i koncentracje antybiotyków oraz ARGs, a także liczebność i skład społeczności drobnoustrojów obecnych w osadach ściekowych może być zróżnicowana i zależy bezpośrednio od jakości ścieków przyjmowanych przez WWTPs, a także od rodzaju procesów wykorzystywanych do ich oczyszczania.

Jak napisano powyżej, fermentacja metanowa stanowi metodę zagospodarowania osadów ściekowych - produktu ubocznego procesu oczyszczania ścieków, generowanego na całym świecie w ogromnych ilościach – umożliwiającą jednoczesny odzysk energii z tegoż odpadu. Efektywność tego procesu może zostać obniżona wskutek obecnych w substracie substancji przeciwdrobnoustrojowych. Przyczynę zakłóceń fermentacji metanowej stanowić może między innymi obniżona aktywność różnych grup mikroorganizmów, biorących udział w stabilizacji beztlenowej (Chen et al., 2008; Scarlat et al. 2018). Ekspozycja osadów ściekowych na inhibitory fermentacji metanowej może skutkować niestabilnością całego procesu i obniżoną

efektywnością produkcji metanu. Co więcej, prawie połowa produkowanych w UE osadów ściekowych wykorzystywana jest w rolnictwie i trafia do gleb (Campo et al., 2021). Wykorzystywanie przefermentowanych osadów ściekowych jako nawozu organicznego może predysponować do przedostawania się ARGs na pola uprawne, skąd wraz ze spływami wód gruntowych i uprawami trafiać mogą one ostatecznie do organizmów ludzi i zwierząt (Xiao et al., 2021). W celu zapewnienia możliwie najwyższej wydajności procesu, niezwykle ważna jest kontrola zanieczyszczeń obecnych w substracie, jak również monitoring parametrów panujących w komorach fermentacyjnych; z kolei ograniczenie szerzenia AR w środowisku wymaga kontroli jakości pofermentu, uwzględniającej analizę występowania ARGs.

CEL PRACY

Celem badań podjętych w ramach niniejszej pracy doktorskiej było określenie wpływu obecności antybiotyków w osadzie ściekowym poddanym fermentacji metanowej, zarówno na (1) wydajność produkcji metanu, jak również (2) los ARGs oraz rozpowszechnianie zjawiska AR, (3) zmiany jakościowe i ilościowe w konsorcjach mikroorganizmów odpowiadających za właściwy przebieg procesu oraz (4) występowanie mikroorganizmów metanogennych.

HIPOTEZY BADAWCZE

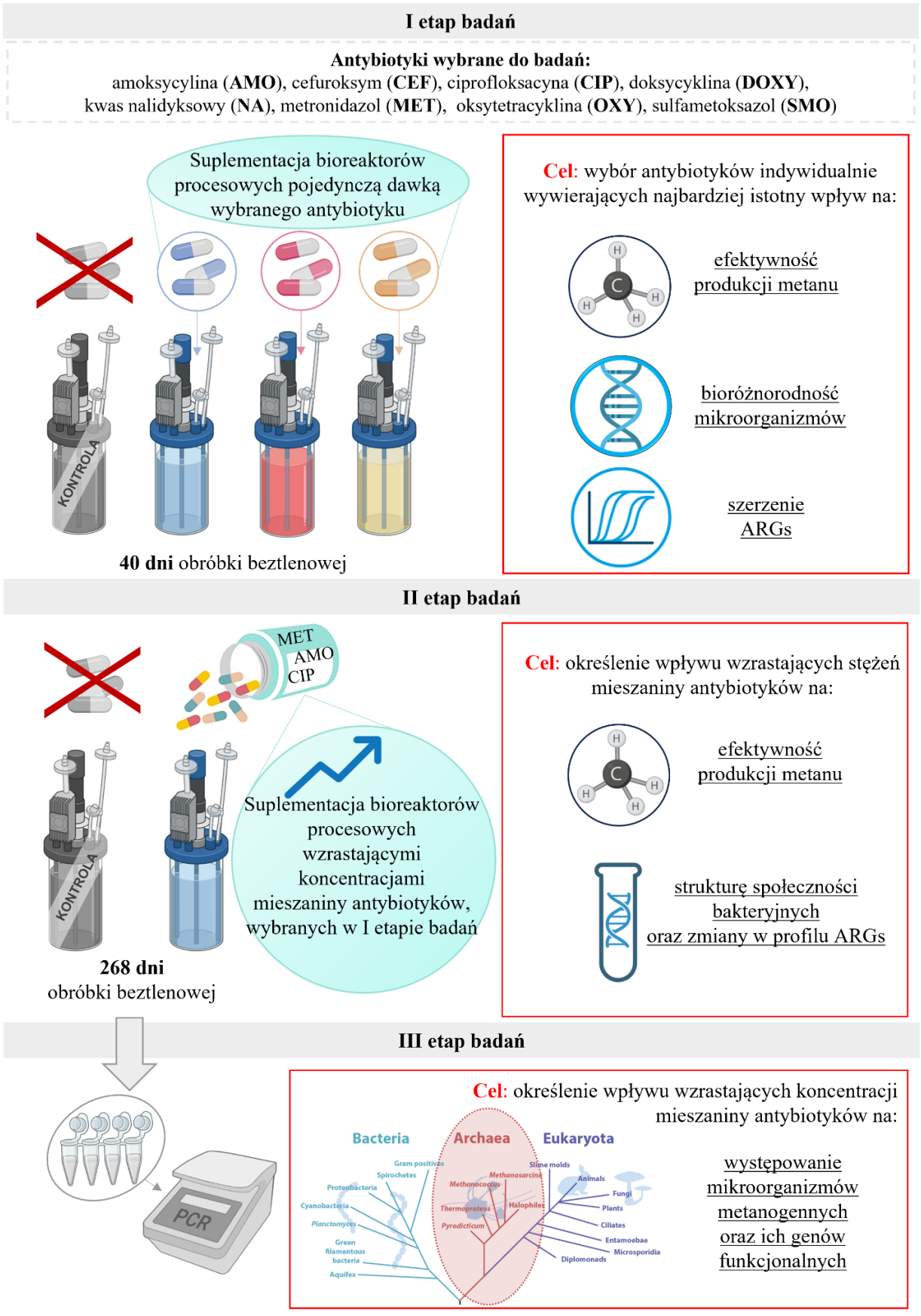
1. Ekspozycja osadów ściekowych na antybiotyki wpływa na efektywność produkcji metanu podczas ich stabilizacji beztlenowej.
2. Stabilizacja beztlenowa osadów ściekowych nie eliminuje ARGs.
3. Obecność antybiotyków w osadzie ściekowym poddanym fermentacji metanowej oddziałuje na zmiany w strukturze społeczności bakterii właściwych.
4. Substancje przeciwdrobnoustrojowe obecne w osadach ściekowych wywierają wpływ na aktywność i bioróżnorodność mikroorganizmów metanogennych zaangażowanych w proces stabilizacji beztlenowej.

ZAKRES BADAŃ, METODYKA I WYNIKI

Inhibitory fermentacji metanowej mogą wpływać zarówno na aktywność samych metanogenów, jak również innych grup mikroorganizmów zaangażowanych w stabilizację beztlenową. Antybiotyki, zaliczane do organicznych inhibitorów niespecyficznych, przedostają się do ścieków w tysiącach ton każdego roku na całym świecie. Ciągłe wzbogacanie ścieków w substancje przeciwdrobnoustrojowe powoduje ich wykrywanie również w osadach ściekowych. Inhibicja stabilizacji beztlenowej osadów ściekowych przez poszczególne

antybiotyki uzależniona jest od ich rodzaju i stężenia, a także współwystępowania w substracie innych substancji przeciwdrobnoustrojowych. Pogłębianie wiedzy na temat procesu fermentacji metanowej, a także jego inhibitorów - w tym antybiotyków - zaowocowało opracowaniem pracy przeglądowej, opartej na 168 artykułach naukowych (**Załącznik 1**). Dokonano w niej identyfikacji i charakterystyki zarówno grup mikroorganizmów zaangażowanych w poszczególne etapy biometanizacji, jak również inhibitorów procesu i mechanizmów ich działania. Opracowanie pracy przeglądowej miało na celu identyfikację inhibitorów fermentacji metanowej, na temat których literatura naukowa dysponuje nadal dość skąpymi informacjami. W artykule zwrócono szczególną uwagę na lukę w wiedzy dotyczącej hamowania aktywności mikrobiomu zaangażowanego w poszczególne etapy fermentacji metanowej. Praca ta stanowiła podwaliny do weryfikacji postawionych w rozprawie hipotez, a także dalszych badań, których etapy przedstawiono na Rycinie 1. W ramach prezentowanej rozprawy doktorskiej, zaplanowano eksperyment, który podzielono na trzy etapy:

I. Celem pierwszego etapu badań było wyznaczenie substancji przeciwdrobnoustrojowych, wywierających najbardziej istotny wpływ na fermentację metanową. W tym celu do wsadu bioreaktorów indywidualnie zadawano antybiotyki. W analizach uwzględniono zarówno efektywność produkcji metanu, jak również strukturę drobnoustrojów oraz losy ARGs podczas beztlenowej stabilizacji osadów ściekowych;
II. Trzy substancje przeciwdrobnoustrojowe, wyselekcjonowane na podstawie I etapu badań, dawkowano jednocześnie do substratu poddawanego fermentacji metanowej. Drugi etap badań podzielony został na serie eksperymentalne, z których każda kolejna charakteryzowała się zwiększeniem koncentracji każdego z antybiotyków, wchodzących w skład suplementowanej mieszaniny. Celem tego etapu badań było określenie długoterminowego wpływu wzrastających stężeń mieszaniny antybiotyków na efektywność produkcji metanu, strukturę populacji drobnoustrojów i profile ARGs;
III. W ostatnim etapie badań skupiono się na uzupełnieniu uprzednio uzyskanych wyników rezultatami analizy zmienności występowania metanogenów oraz charakterystycznego dla nich genu funkcjonalnego w trakcie długoterminowej ekspozycji wsadu bioreaktora na mieszaninę antybiotyków o wzrastającym stężeniu.



Ryc. 1. Schemat obrazujący etapy badań prezentowanych w ramach rozprawy doktorskiej.

I etap badań

Na podstawie raportu European Centre for Disease Prevention and Control (ECDC 2018) do badań wyselekcjonowano najczęściej stosowane w leczeniu ludzi substancje przeciwdrobnoustrojowe; metronidazol (MET), amoksycylinę (AMO), cefuroksym (CEF), oksytetracyklinę (OXY), doksycyklinę (DOXY), sulfametoksazol (SMO), ciprofloksacynę (CIP) i kwas nalidyksowy (NA). W celu określenia ich indywidualnego wpływu na fermentację metanową osadów ściekowych, przygotowano szereg bioreaktorów wypełnionych 25 g substratu i 175 g inokulum, które stanowiły odpowiednio osad ściekowy i osad beztlenowy z komory fermentacyjnej, pobrane z miejskiej oczyszczalni ścieków „Łyna" w Olsztynie. Do każdego z bioreaktorów wprowadzono wybraną substancję przeciwdrobnoustrojową, w dawce określonej eksperymentalnie na podstawie badań wstępnych. Eksperyment prowadzono w dwóch powtórzeniach, przy uwzględnieniu bioreaktora kontrolnego, w którym wsad bioreaktora nie został poddany ekspozycji na antybiotyk. Fermentację metanową prowadzono przez 40 dni w warunkach mezofilnych, w temperaturze 37°C. Produkcję metanu mierzono za pomocą urządzenia służącego do pomiaru potencjału wytwórczego metanu AMPTS II. Jakość biogazu analizowano z wykorzystaniem chromatografu gazowego, wyposażonego w detektor przewodności cieplnej. Po zakończeniu fermentacji metanowej z każdego bioreaktora pobrano reprezentatywne próbki pofermentu. Próbki te poddano analizie pod kątem zawartości lotnych kwasów tłuszczowych (LKT). Dodatkowo, z próbek pofermentu wyizolowano DNA, z wykorzystaniem FastDNA™ Spin Kit for Soil (MP Biomedicals). Materiał genetyczny poddano sekwencjonowaniu regionu hiperzmiennego V3-V4 genu *16S r*RNA, z wykorzystaniem Illumina MiSeq, w celu określenia różnorodności mikrobiomu. Łańcuchowa reakcja polimerazy w czasie rzeczywistym (quantitative polymerase chain reaction – qPCR) została wykorzystana do analizy występowania w próbkach wybranych genów antybiotykooporności i genów kodujących integrazy, a także genów charakterystycznych dla rodzin metanogenów dominujących w osadzie beztlenowym (*Methanosarcinaceae- MSC* i *Methanosaetaceae – MST*) oraz funkcjonalnego genu metanogenów, katalizującego ostatnią fazę metanogenezy (*mcr*A). Wyniki tego eksperymentu przedstawiono w artykule naukowym, stanowiącym **Załącznik 2** do rozprawy doktorskiej, poddającym weryfikacji wszystkie postawione hipotezy badawcze.

W opisanych badaniach fermentacja metanowa została istotnie zahamowana w bioreaktorach, w których wsad suplementowany był MET, AMO, OXY, DOXY i CIP. MET, stosowany w leczeniu zakażeń drobnoustrojami beztlenowymi, był najsilniejszym inhibitorem,

a jego wprowadzenie do reaktora obniżyło zawartość metanu w biogazie do 12,8 ± 4,0% i zmniejszyło szybkość produkcji metanu do 21,85 L $kg^{-1}d^{-1}$. Zahamowanie metanogenezy doprowadziło do akumulacji LKT. Stężenia siedmiu z dziewięciu analizowanych LKT były istotnie wyższe w pofermencie z dodatkiem MET niż w próbce z bioreaktora kontrolnego. Poza MET, największe działanie inhibitujące stabilizację beztlenową osadów ściekowych wykazały AMO i CIP. Dodatek AMO do wsadu reaktora znacznie obniżył zawartość metanu w biogazie (o około 44%), natomiast wprowadzenie CIP zmniejszyło produkcję metanu (o około 40% w stosunku do bioreaktora kontrolnego).

Analiza sekwencji DNA w próbkach ze wszystkich bioreaktorów wykazała, że dominującymi typami mikroorganizmów w pofermencie byli przedstawiciele typów *Bacteroidetes*, *Firmicutes* i *Proteobacteria*. W porównaniu do kontroli, największy spadek liczby odczytów specyficznych dla *Bacteroidetes* odnotowano w próbkach pofermentu poddanego ekspozycji na MET. W tych samych próbkach zaobserwowano istotny wzrost udziału *Firmicutes* i *Proteobacteria* (odpowiednio o 6% i 6,8% w stosunku do kontroli). Ekspozycja wsadu bioreaktora na AMO i CIP istotnie zmniejszyła natomiast udział przedstawicieli rzędu *Syntrophobacteriales*, co może świadczyć o zahamowaniu acetogenezy.

Analizy oparte o metodę qPCR wykazały, że występowanie genu *mcr*A w pofermencie poddanym ekspozycji na MET, CEF i NA było niższe w porównaniu do kontroli. Jednakże analiza obfitości występowania tego funkcjonalnego dla metanogenów genu nie odzwierciedlała rzeczywistego stopnia efektywności produkcji metanu w obecności środków przeciwdrobnoustrojowych. W przefermentowanym osadzie ściekowym wystawionym na działanie MET, CEF i NA stwierdzono istotne względem kontroli zmniejszenie występowania genów charakterystycznych dla metanogennych rodzin *Methanosarcinaceae* i *Methanosaetaceae*. Powyższe obserwacje mogą świadczyć o pewnym stopniu wrażliwości metanogenów na wymienione związki przeciwdrobnoustrojowe.

Obecność antybiotyków wpłynęła na częstość występowania wytypowanych do analiz ARGs, powodując wzrost liczby kopii genów kodujących oporność wobec β-laktamów, tetracyklin i fluorochinolonów. Wykazano jednak, że presja selekcyjna wywierana przez substancje przeciwdrobnoustrojowe nie była specyficzna wobec genów kodujących oporność na ich poszczególne klasy. Wykorzystanie analizy korelacji pozwoliło wykazać, że geny kodujące integrazę odgrywają ważną rolę w przenoszeniu ARGs podczas fermentacji metanowej.

II etap badań

W ramach kontynuacji prezentowanych w I etapie badań przeprowadzono kolejny eksperyment, podjęty w celu oceny jednoczesnego, długoterminowego wpływu środków przeciwbakteryjnych na fermentację metanową osadów ściekowych. Badania objęły trzy substancje przeciwdrobnoustrojowe, które indywidualnie wywarły największy istotny wpływ na stabilizację beztlenową, z uwzględnieniem aspektów mikrobiologicznych procesu – MET, AMO i CIP. Przeprowadzony eksperyment przedstawiono w artykule naukowym, stanowiącym **Załącznik 3** do rozprawy doktorskiej, poddając weryfikacji pierwsze trzy hipotezy badawcze.

W niniejszej pracy, osad ściekowy, zaszczepiony osadem beztlenowym z komory fermentacyjnej, pobrany z tej samej WWTP, poddano stabilizacji beztlenowej w fermentatorach półciągłych przepływowych, utrzymywanych w warunkach mezofilnych (37°C). Komory zasilano substratem zawierającym mieszaninę MET, AMO i CIP, a także osadem ściekowym niepoddanym ekspozycji na antybiotyki (kontrola). Eksperymenty dla każdego z substratów przeprowadzono w dwóch powtórzeniach. Badanie składało się z sześciu serii eksperymentalnych, które różniły się stężeniami antybiotyków aplikowanymi do substratu. Początkowe stężenie każdego z trzech antybiotyków w mieszaninie dozowanej do osadów ściekowych podczas pierwszej serii doświadczalnej było zbliżone do stężeń tych leków w ściekach wpływających do oczyszczalni, z której pobrano osad ściekowy. Stężenia antybiotyków dodawanych do komory fermentacyjnej zwiększano po dwukrotnej wymianie objętości hydraulicznej komór fermentacyjnych. Każda z serii eksperymentalnych trwała średnio 45 dni, a cały eksperyment trwał 268 dni. W trakcie badań cotygodniowo dokonywano regularnego pomiaru ilości wytwarzanego metanu (AMPTS II), a jakość biogazu analizowano w chromatografie gazowym wyposażonym w detektor przewodności cieplnej. W tych samych odstępach czasu pobierano próbki w celu pomiaru LKT. Próbki przeznaczone do izolacji i sekwencjonowania DNA pobierano z wsadu bioreaktorów procesowych (zawierających substrat suplementowany antybiotykami) na początku, w środku i na końcu każdej serii doświadczalnej, natomiast z bioreaktora kontrolnego dokonano poboru jednej próbki podczas pierwszej, czwartej i ostatniej serii doświadczalnej. Do analizy bioróżnorodności mikroorganizmów oraz monitoringu występowania genów antybiotykooporności zastosowano czułą, molekularną metodę sekwencjonowania metagenomowego (Illumina NovaSeq).

Na podstawie analizy produkcji metanu zaobserwowano, że przedłużona ekspozycja na kombinację środków przeciwdrobnoustrojowych może wpływać na aklimatyzację mikroorganizmów wewnątrz bioreaktora, co sprzyja adaptacji drobnoustrojów. Tendencje

produkcji metanu w bioreaktorze kontrolnym i procesowym wykazywały podobny trend, jednak wydajność produkcji metanu w bioreaktorze, w którym wsad suplementowany był mieszaniną MET, AMO i CIP, była istotnie niższa, w porównaniu do kontroli. W prezentowanych badaniach nie stwierdzono istotnych różnic w stężeniach poszczególnych LKT pomiędzy próbkami wsadu pobranego z bioreaktorów procesowych i kontrolnych.

Analiza danych pozyskanych w wyniku sekwencjonowania wykazała istotne różnice w strukturze mikrobiomu pomiędzy próbkami wsadu kontrolnego i suplementowanego w trakcie eksperymentu. Ekspozycja na wzrastające dawki mieszaniny antybiotyków wywołała zmiany w strukturze populacji drobnoustrojów. Bakterie należące do typu *Acidobacteria* dominowały w próbkach osadu beztlenowego pobranych zarówno z bioreaktorów kontrolnych, jak i procesowych, lecz ich liczebność różniła się istotnie pomiędzy substratem poddanym działaniu antybiotyków, a kontrolą. Zaobserwowano, ze ekspozycja wsadu bioreaktora na AMO (36 μg/mL), CIP (16 μg/mL) i MET (16 μg/mL) znacząco hamowała wzrost przedstawicieli *Acidobacteria*. Podczas całego procesu częstość występowania *Candidatus Cloacimonetes* i *Proteobacteria* była podobna we wsadzie kontrolnym i suplementowanym antybiotykami, podczas gdy liczebność operacyjnych jednostek taksonomicznych (operational taxonomic unit – OTU) charakterystycznych dla *Bacteroidetes* różniła się istotnie między próbkami. Początkowo wysoka liczebność przedstawicieli *Firmicutes* w próbkach wsadu z bioreaktora procesowego zmniejszała się pod wpływem dalszej ekspozycji na mieszaninę substancji przeciwdrobnoustrojowych.

Ekspozycja na mieszaninę antybiotyków istotnie zwiększyła liczbę OTU charakterystycznych dla *Archaea* w analizowanych próbkach, jednak zmiany te nie wpłynęły na efektywność produkcji biogazu. Analiza liczebności OTU charakterystycznych dla metanogenów pozwoliła na identyfikację w próbkach wsadu czterech głównych rzędów metanogenów: *Methanosarcinales* i *Methanomicrobiales* z klasy *Methanomicrobia*, *Methanomassiliicoccales* z klasy *Thermoplasmata* i *Methanobacteriales* z klasy *Methanobacteria*. W czasie trwania całego eksperymentu obserwowano istotne różnice w obfitości przedstawicieli *Methanosarcinales* i *Methanomassiliicoccales*, zarówno w próbkach z bioreaktora procesowego, jak i kontrolnego. Występowanie OTUs charakterystycznych dla tych rodzajów metanogenów wykazywało silnie ujemną, wzajemną korelację. Wnioskowano, że dynamiczne zmiany w zbiorowiskach mikroorganizmów i ich adaptacja do zmieniających się warunków środowiskowych są niezbędne dla stabilnej pracy beztlenowych komór fermentacyjnych.

Próbki wsadu pobrane zarówno z bioreaktorów procesowych, jak i kontrolnych charakteryzowały się przewagą genów wielolekooporności oraz genów nadających oporność na antybiotyki MLS (>20 ppm). W próbkach licznie występowały również geny oporności na tetracyklinę i bacytracynę (5–15 ppm). Ponownie dowiedziono, że presja selekcyjna wywierana przez antybiotyki suplementowane do wsadu bioreaktorów procesowych nie była specyficznie ukierunkowana na geny kodujące oporność wobec konkretnych klas środków przeciwdrobnoustrojowych. Całkowita obfitość ARGs zmniejszyła się pod koniec stabilizacji beztlenowej, zarówno w próbkach wsadu z bioreaktorów procesowych, jak i kontrolnych. Co ważne, częstość występowania genów oporności wielolekowej wzrosła w próbkach wsadu bioreaktorów procesowych, podczas gdy w próbkach kontrolnych zaobserwowano tendencję odwrotną. Należy podkreślić, że przenoszenie genów oporności wielolekowej między mikroorganizmami stanowi poważne zagrożenie dla zdrowia publicznego na całym świecie.

III etap badań

Pomimo, iż sekwencjonowanie metagenomowe próbek z poprzedniego etapu badań umożliwiło identyfikację sekwencji charakterystycznych dla przedstawicieli *Archaea*, ich liczba była istotnie niższa w porównaniu do liczby odczytów charakterystycznych dla bakterii właściwych. W związku z tym, ostatni etap badań ukierunkowano na pogłębienie wiedzy dotyczącej wpływu, jaki długoterminowa suplementacja osadów ściekowych mieszaniną antybiotyków o wzrastającym stężeniu wywiera na mikroorganizmy metanogenne.

Materiał genetyczny wyizolowany w II etapie badań poddano ocenie występowania genów charakterystycznych dla metanogenów z rodzin *Methanosarcinaceae* (*MSC*) i *Methanosaetaceae* (*MST*), należących do rzędu *Methanosarcinales*. Do analiz wykorzystano technikę qPCR. Wyniki badań wstępnych pozwoliły na wykluczenie w osadzie beztlenowym istotnego udziału przedstawicieli metanogennych rodzin z innych rzędów, należących do domeny *Archaea*. Aktywność mikroorganizmów metanogennych oceniono poprzez określenie stężenia genu kodującego reduktazę metylokoenzymu M (*mcr*A), katalizującego ostatni etap fermentacji metanowej - metanogenezę. Wyniki tego eksperymentu przedstawiono w artykule naukowym, stanowiącym **Załącznik 4** do rozprawy doktorskiej. W tej pracy zweryfikowano ostatnią, czwartą hipotezę badawczą.

Na podstawie wyników molekularnej analizy efektywności stabilizacji beztlenowej, uwzględniającej koncentrację funkcjonalnego genu *mcr*A, zaobserwowano, że stężenia tego genu w próbkach wsadu z bioreaktorów procesowych i kontrolnych, różniły się w całej serii

eksperymentalnej. Wykazano, że produkcja metanu nie była skorelowana z występowaniem genu *mcr*A podczas długotrwałej stabilizacji beztlenowej, bez względu na ekspozycję na leki bądź jej brak. Po raz kolejny potwierdzono, że zmiany w wydajności uzysku metanu nie mogą być wiarygodnie mierzone na podstawie obfitości genu *mcr*A, pomimo faktu, że gen ten został zaproponowany przez innych naukowców jako marker do monitorowania aktywności metanogenów.

Zarówno w badanych, jak i kontrolnych próbkach wsadu, liczba kopii genów charakterystycznych dla *Methanosarcinaceae* pozostawała stabilna w czasie trwania eksperymentu, za wyjątkiem ostatniej, szóstej serii eksperymentalnej, w której liczebność genów *MSC* wzrosła o 2 rzędy wielkości w 1 gramie pofermentu. Świadczy to o stabilnym wzroście przedstawicieli *Methanosarcinaceae* podczas fermentacji osadów, niezależnie od suplementacji antybiotykami. Z kolei liczebność genów *MST*, charakterystycznych dla rodziny *Methanosaetaceae*, bardzo istotnie różniła się pomiędzy próbkami suplementowanymi, a kontrolnymi ($P \leq 0.001$). Co ważne, w ostatniej serii eksperymentalnej, liczebność genów *MST* w badanych i kontrolnych próbkach wsadu bioreaktora była zbliżona, co sugeruje, że konsorcja tych drobnoustrojów przystosowały się do wzrastających stężeń leków. Wnioskowano, że obie rodziny metanogenów dobrze zaadaptowały się do warunków panujących w bioreaktorze procesowym, jednak bez względu na ekspozycję na leki, to przedstawiciele *Methanosaetaceae* zdominowali poferment.

PODSUMOWANIE

Wyniki przeprowadzonych badań dostarczyły szerokiej gamy informacji dotyczących wpływu substancji przeciwdrobnoustrojowych na proces fermentacji metanowej, zarówno w ujęciu technologicznym, jak i mikrobiologicznym. Przedstawione badania pozwoliły na osiągnięcie zamierzonych celów oraz pozytywne zweryfikowanie wszystkich hipotez, postawionych w ramach rozprawy doktorskiej. Wykazano, że:

(1) niektóre leki przeciwdrobnoustrojowe, szeroko stosowane w medycynie, mogą obniżać efektywność fermentacji metanowej osadów ściekowych.

(2) obecność środków przeciwdrobnoustrojowych podczas fermentacji metanowej wywiera istotny wpływ na profil ARGs. Ekspozycja na MET wywołała najbardziej istotne zmiany w stężeniach ARGs, w szczególności poprzez zwiększenie koncentracji genów kodujących oporność wobec β-laktamów, tetracyklin i fluorochinolonów, a także obniżenie stężenia genów nadających oporność na leki z grupy MLS. Presja selekcyjna

wywierana przez antybiotyki nie była specyficzna wobec konkretnych typów ARGs. Co więcej, badania zwróciły uwagę na problem związany z nieskutecznością eliminacji ARGs w wyniku stabilizacji beztlenowej osadów ściekowych oraz zagrożenie związane z szerzeniem zjawiska AR.

(3) ekspozycja osadów ściekowych na środki przeciwdrobnoustrojowe podczas stabilizacji beztlenowej istotnie wpływa na strukturę zbiorowisk drobnoustrojów, w tym metanogenów. MET indukował największe zmiany w bioróżnorodności drobnoustrojów, znacznie zmniejszając udział *Bacteroidetes*, a zwiększając liczbę OTUs charakterystycznych dla *Firmicutes* i *Proteobacteria*. Przedstawiciele *Methanosaetaceae* dominowali wśród mikroorganizmów metanogennych podczas suplementacji indywidualnymi lekami, jak również w trakcie symultanicznej ekspozycji na MET, AMO i CIP .

(4) ilościowa analiza genów specyficznych dla mikroorganizmów metanogennych, takich jak funkcjonalny gen *mcr*A, nie odzwierciedla wartości parametrów fermentacji metanowej, a tym samym rzeczywistej wydajności procesu w obecności środków przeciwdrobnoustrojowych.

Przeprowadzone eksperymenty wykazały potrzebę prowadzenia dalszych badań w celu określenia wpływu obecności inhibitorów, takich jak antybiotyki, na aktywność drobnoustrojów fermentacji metanowej, co w przyszłości umożliwi zapewnienie optymalnych warunków wzrostu i rozwoju mikroorganizmów odpowiedzialnych za poszczególne etapy stabilizacji beztlenowej. Takie badania powinny opierać się na nowoczesnych metodach molekularnych, między innymi na sekwencjonowaniu metagenomowym.

Abstract

INTRODUCTION

The global consumption and demand for energy continue to increase, but fossil fuels such as coal, natural gas, and oil are not sustainable energy sources. The supply of energy carriers has to increase to cater to rapid population growth, which is directly responsible for the recent interest in renewable energy. Biogas produced during anaerobic digestion is one of the most promising alternatives to fossil fuels in energy generation (Holm-Nielsen et al., 2009; Scarlat et al., 2018). Biogas is environmentally friendly, and it can be converted to biomethane, which is the most efficient type of biofuel (Bowe, 2013). The number of biogas plants continues to increase around the world, and the biogas sector has a massive growth potential in all countries. At present, nearly 50 million micro-scale digesters and 132,000 small, medium, and large-scale digesters are operated around the world (World Biogas Association, 2019).

Anaerobic digestion is an attractive waste processing method that promotes effective waste management and energy recovery. This four-stage processes takes place in the presence of various microbial populations, including hydrolyzing, acidogenic, acetogenic, and methane-producing bacteria, which are responsible for different stages of fermentation (Świątczak et al. 2017). However, the efficiency of anaerobic digestion is difficult to optimize, which prevents the widespread implementation of this process. Anaerobic digestion is inhibited mainly by substances that are present in large quantities in stabilized waste, including antimicrobial compounds (Rusanowska et al. 2019; Meegoda et al. 2018; Scarlat et al. 2018). Global antibiotic consumption increased by 65% between 2000 and 2015, and a further increase of 200% is expected by 2030 (Klein et al., 2018). High antibiotic consumption in human medicine and the overuse of antimicrobials in the veterinary sector promote the spread of the parent compounds and their metabolites in the environment. As a result, antimicrobials can accumulate in substrates that are subjected to anaerobic digestion.

Anaerobic digestion is one of the key strategies for stabilizing sewage sludge from wastewater treatment plants (WWTPs) (Grobelak et al. 2019). Wastewater treatment plants produce increasing quantities of sewage sludge, and hundreds of millions tons of sewage sludge are being generated each year around the world. Incoming raw wastewater contains various pollutants, including microbial contaminants and drug residues, which are accumulated in sewage sludge. Antibiotic concentrations in sewage sludge can range from <1 to several thousand μg kg^{-1} (Czatzkowska et al., 2022). In addition, both wastewater and sewage sludge

are also massively colonized by microorganisms, including pathogens. Bacteria harboring mechanisms of antimicrobial resistance (AR) pose a particular threat (Manaia et al. 2018). The misuse of antibiotics in human and veterinary medicine leads to the emergence of bacteria with one or more antibiotic resistance genes (ARGs). The exchange of genetic structures, including ARGs, between microorganisms contributes to the spread of AR in the environment. Due to the widespread use of antibiotics in recent decades, ARGs have been classified as a new source of pollution that poses a threat to public health and safety (Becerra-Castro et al. 2015; Xu et al. 2017; Barancheshme and Munir 2019). In addition, wastewater and sewage sludge contain subinhibitory concentrations of antimicrobials, and WWTPs promote increased selection pressure on bacteria harboring specific ARGs (Sun et al. 2019). The types and concentrations of antibiotics and ARGs, and the abundance and composition of microbial communities in sewage sludge can vary, depending on the quality of incoming raw wastewater and the applied wastewater treatment technology.

As previously mentioned, anaerobic digestion is an effective method of managing sewage sludge, a by-product of the wastewater treatment process which is generated in massive quantities around the world. The energy accumulated in sewage sludge is recovered during anaerobic digestion. The efficiency of this process can be compromised by the presence of antimicrobial substances in the substrate. Decreased activity of different microbial groups that participate in anaerobic digestion can also undermine digestion efficiency (Chen et al., 2008; Scarlat et al. 2018). The presence of inhibitors in sewage sludge can destabilize anaerobic digestion and decrease methane output. Nearly half of the sewage sludge produced in the EU is used in agriculture as organic fertilizer and is introduced to the soil environment (Campo et al., 2021). Digestate-based fertilizers can promote the spread of ARGs in cultivated fields; ARGs are then transferred from the soil to groundwater and crops, and they ultimately reach humans and animals (Xiao et al., 2021). The pollutants present in the substrate should be controlled, and processing parameters in reactors should be closely monitored to maximize the efficiency of anaerobic digestion. In turn, the quality of the resulting digestate, including the presence of ARGs, should be analyzed to reduce the spread of AR in the environment.

AIM OF THE STUDY

The aim of the study, whose results constituted the basis for this doctoral dissertation, was to determine the effect of antibiotics present in sewage sludge subjected to anaerobic digestion on (1) efficiency of methane production, (2) fate of ARGs and the spread of AR, (3)

qualitative and quantitative changes in the consortia of microorganisms that participate in anaerobic digestion, and (4) prevalence of methanogens.

RESEARCH HYPOTHESES

5. Exposure of sewage sludge to antibiotics affects the efficiency of methane production during anaerobic digestion.
6. Anaerobic digestion of sewage sludge does not eliminate ARGs.
7. The presence of antibiotics in sewage sludge subjected to anaerobic digestion induces changes in the structure of Eubacteria communities.
8. Antimicrobials present in sewage sludge affect the activity and biodiversity of methanogens that participate in anaerobic digestion.

SCOPE OF THE STUDY, RESEARCH METHODS AND RESULTS

Inhibitors of anaerobic digestion may affect the activity of both methanogens and other groups of microorganisms that participate in anaerobic digestion. Each year, thousands of millions tons of antibiotics, classified as non-specific organic inhibitors, are detected in wastewater around the world. Due to the continuous supplementation of wastewater with antimicrobials, these substances are also identified in sewage sludge. The inhibition of anaerobic digestion of sewage sludge by antibiotics is affected by their type and concentration, and the co-occurrence of other antimicrobial agents in the substrate. Recent research findings regarding anaerobic digestion and its inhibitors, including antibiotics, were summarized in a review based on 168 articles (**Appendix 1**). Microbial groups involved in each stage of biomethanation, inhibitors of the process and mechanisms of their action were identified and characterized. The aim of the review was to identify the inhibitors of anaerobic digestion, which remain insufficiently investigated in the scientific literature. Particular emphasis was placed on a knowledge gap regarding the inhibition of the activity of the microbiome involved in each stage of anaerobic digestion. The review provided a basis for verifying the research hypotheses formulated in the dissertation, and paved the way for future research whose stages are presented in Figure 1. An experiment conducted as part of doctoral research was divided into three stages:

IV. The aim of the first stage of research was to identify antimicrobials that exert the most significant effect on anaerobic digestion. For this purpose, individual antibiotics were added to the feedstock in bioreactors. The efficiency of methane production, the structure of microbial communities and the fate of ARGs during the anaerobic digestion of sewage sludge were determined;

V. Three antimicrobials, selected in the first stage of the study, were simultaneously applied to the substrate subjected to anaerobic digestion. The second stage of research was divided into experimental series, and the concentrations of all antibiotics in the mixture were increased in each series. The aim of this stage was to determine the long-term impact of increasing concentrations of the tested antibiotic mixture on the efficiency of methane production, structure of microbial populations and ARG profiles;

VI. The last stage of research focused on complementing previous findings with the results of an analysis of variation in the prevalence of methanogens and their characteristic functional genes during long-term exposure of the substrate in a bioreactor to increasing concentrations of the tested antibiotic mixture.

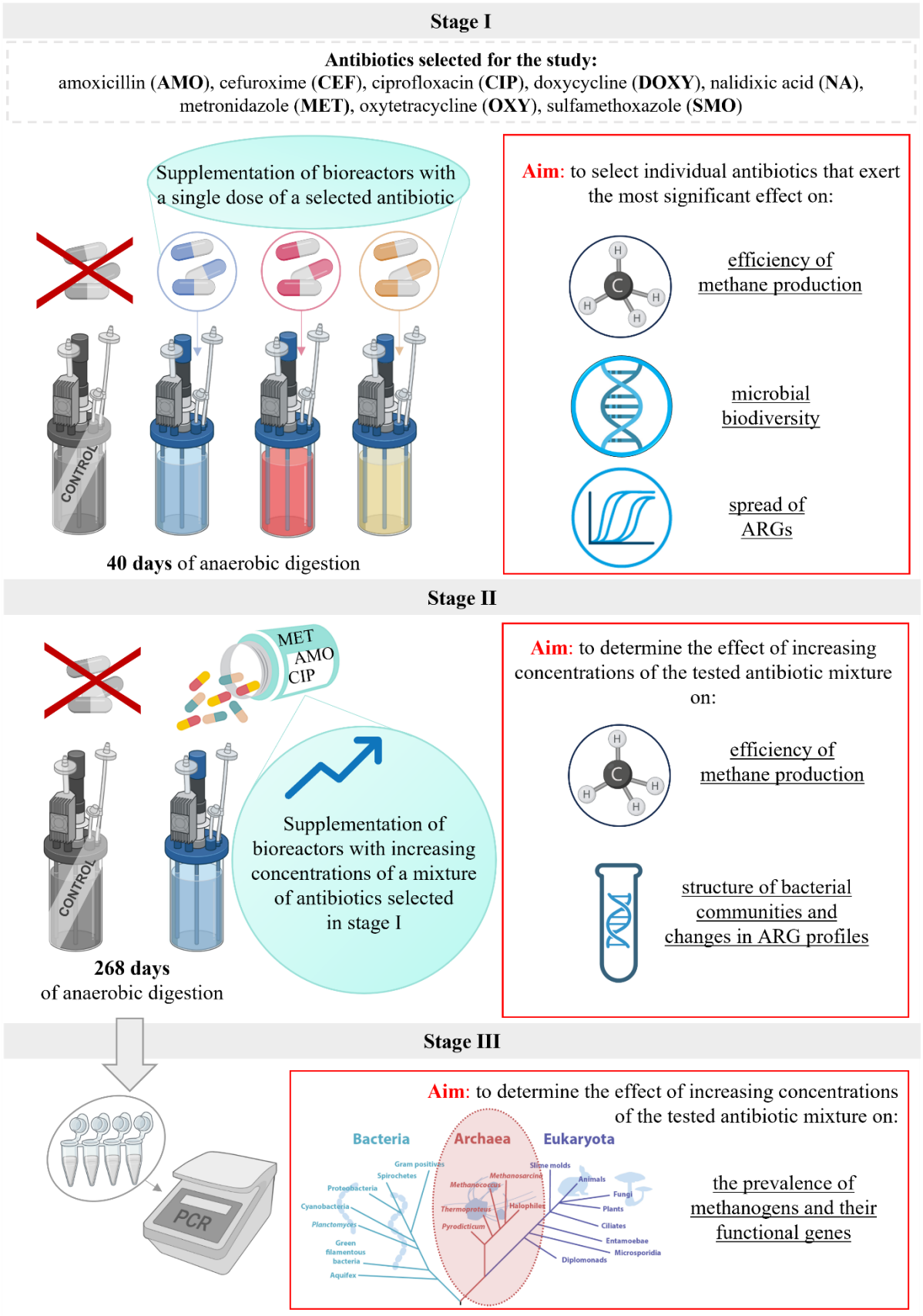


Figure 1. Stages of research described in the doctoral dissertation.

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Stage I

Based on a report of the European Center for Disease Prevention and Control (ECDC 2018), antimicrobial drugs that are most widely used in human medicine were selected for the study: metronidazole (MET), amoxicillin (AMO), cefuroxime (CEF), oxytetracycline (OXY), doxycycline (DOXY), sulfamethoxazole (SMO), ciprofloxacin (CIP) and nalidixic acid (NA). In order to determine their individual effects on the anaerobic digestion of sewage sludge, bioreactors were filled with 25 g of the substrate and 175 g of the inoculum, i.e. sewage sludge and anaerobically digested sludge from a fermentation tank, respectively, collected in the "Łyna" Municipal WWTP in Olsztyn. Selected antimicrobials at doses determined experimentally during a preliminary study were added to substrates in bioreactors. The experiment was performed in duplicate. In the control bioreactor, the substrate was not exposed to antibiotics. Anaerobic digestion was conducted for 40 days under mesophilic conditions at a temperature of 37°C. Methane production was measured with the AMPTS II. Biogas quality was analyzed in a gas chromatograph equipped with a thermal conductivity detector. After anaerobic digestion, representative digestate samples were collected from each bioreactor. The samples were analyzed to determine the concentrations of volatile fatty acids (VFAs). In addition, DNA was isolated from digestate samples with the use of the FastDNA™ Spin Kit for Soil (MP Biomedicals). The genetic material was subjected to sequencing of the hypervariable region V3-V4 of the *16S r*RNA gene with the use of the Illumina MiSeq instrument, to determine microbial diversity. Real-time quantitative polymerase chain reaction (qPCR) was applied to analyze the concentrations of selected ARGs, genes encoding class 1 and 2 integrases, genes specific to the families of methanogens that predominate in anaerobically digested sludge (*Methanosarcinaceae* - *MSC* and *Methanosaetaceae* - *MST*), and the functional methyl-coenzyme M reductase gene (*mcr*A), which catalyzes the final step of anaerobic digestion - methanogenesis. The results of this experiment were presented in a research article (**Appendix 2** to this dissertation), and all research hypotheses were verified.

In the described study, anaerobic digestion was significantly inhibited in the bioreactors where the substrate was supplemented with MET, AMO, OXY, DOXY and CIP. Metronidazole, which is used to treat infections caused by anaerobic bacteria, was the strongest inhibitor, and its introduction to the bioreactor decreased the methane content of biogas to 12.8±4.0% and reduced the methane production rate to 21.85 L kg^{-1} d^{-1}. The inhibition of methanogenesis led to the accumulation of VFAs. The concentrations of seven out of the nine analyzed VFAs were significantly higher in the digestate supplemented with MET than in the sample from the control

bioreactor. Amoxicillin and CIP were also potent inhibitors of anaerobic digestion of sewage sludge. The addition of AMO to the bioreactor significantly reduced the methane content of biogas (by approx. 44%), whereas the introduction of CIP decreased methane production (by approx. 40%, relative to the control bioreactor).

An analysis of DNA sequences in the samples from all bioreactors revealed that *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were the dominant bacterial phyla in the digestate. In comparison with the control treatment, the greatest decrease in the number of *Bacteroidetes*-specific reads was noted in digestate samples exposed to MET. A significant increase in the proportions of *Firmicutes* and *Proteobacteria* (by 6% and 6.8% relative to the control treatment, respectively) was also observed in these samples. The exposure of the substrate to AMO and CIP significantly decreased the proportion of Syntrophobacteriales, which may point to the inhibition of acetogenesis.

Analyses involving qPCR demonstrated that the concentration of the *mcr*A gene in digestate samples exposed to MET, CEF and NA was lower than in the control treatment. However, it was found that the abundance of this functional gene does not fully reflect the actual efficiency of methane production in the presence of antimicrobials. The total concentration of genes specific to the families *Methanosarcinaceae* and *Methanosetaceae* decreased significantly (relative to the control treatment) in digestate samples exposed to MET, CEF and NA, which could suggest that methanogens are somewhat sensitive to these antimicrobials.

The presence of antibiotics affected the prevalence of the analyzed ARGs, leading to an increase in the copy numbers of genes encoding resistance to β-lactams, tetracyclines and fluoroquinolones. It was found, however, that the selective pressure exerted by antimicrobials was not specific to the corresponding ARGs. The correlation analysis revealed that genes encoding class 1 and 2 integrases play an important role in the transfer of ARGs during anaerobic digestion.

Stage II

The research described in stage I was continued, and another experiment was conducted to determine a long-term, simultaneous impact of antimicrobials on the efficiency of anaerobic digestion of sewage sludge. Three antibiotics, MET, AMO and CIP, which individually exerted the most significant effect on anaerobic digestion, including the microbiological aspects of the process, were analyzed. The results of this experiment were presented in a research article (**Appendix 3** to this dissertation), and the first three research hypotheses were verified.

In the second stage of the study, sewage sludge was inoculated with anaerobically digested sludge from a fermentation tank, collected in the same WWTP, and it was subjected to anaerobic digestion in semi-continuous flow digesters, under mesophilic conditions (37°C). The digesters were fed substrates containing a mixture of MET, AMO and CIP or sewage sludge not exposed to antibiotics (control). The experiments were performed in duplicate for each substrate. The study consisted of six experimental series that differed in the concentrations of antibiotics applied to the substrate. The initial concentration of each of the three antibiotics in the mixture dosed to sewage sludge during the first experimental series was similar to the concentrations of the respective drugs in the wastewater flowing into the WWTP from which the sludge was obtained. The concentrations of the antibiotics added to the digester were increased after doubling the hydraulic volume of each digester. Each of the experimental series lasted 45 days on average, and the entire study lasted 268 days. Throughout the study, methane production was measured (AMPTS II) every week, and biogas quality was analyzed in a gas chromatograph equipped with a thermal conductivity detector. The concentrations of VFAs in the collected samples were also determined at weekly intervals. Samples for DNA isolation and sequencing were collected from process bioreactors (containing substrate supplemented with antibiotics) at the beginning, in the middle and at the end of each experimental series, whereas one sample was collected from the control bioreactor during the first, fourth and last experimental series. Microbial biodiversity and prevalence of ARGs were determined using a sensitive molecular method of metagenomic sequencing (Illumina NovaSeq).

An analysis of methane production revealed that prolonged exposure to a combination of antimicrobials could influence microbial acclimation inside the bioreactor, thus contributing to the adaptation of microorganisms. Methane production in process and control bioreactors followed a similar trend, but its efficiency was significantly lower in the process bioreactor where the substrate was supplemented with a mixture of MET, AMO and CIP than in the control bioreactor. No significant differences in the concentrations of individual VFAs were found between sewage sludge samples collected from process and control bioreactors.

The sequencing analysis revealed significant differences in the structure of microbiota between samples of control and supplemented sewage sludge during the experiment. Exposure to increasing doses of the tested antibiotics induced changes in the structure of microbial populations. Bacteria of the phylum *Acidobacteria* were predominant in samples of anaerobically digested sewage sludge collected from both control and process bioreactors, but their abundance differed significantly between substrates exposed to antibiotics and the control

substrate. It was found that substrate exposure to AMO (36 µg/mL), CIP (16 µg/mL) and MET (16 µg/mL) significantly inhibited the growth of *Acidobacteria*. During the entire process, the prevalence of *Candidatus Cloacimonetes* and *Proteobacteria* was similar in control and antibiotic-supplemented substrates, whereas the abundance of operational taxonomic units (OTUs) characteristic of *Bacteroidetes* differed significantly between samples collected from process and control bioreactors. The high initial abundance of *Firmicutes* in the process bioreactor decreased under exposure to a mixture of the tested antibiotics.

Exposure to a mixture of the tested antibiotics significantly increased the abundance of OTUs characteristic of *Archaea* in the analyzed samples, but these changes did not affect the efficiency of biogas production. An analysis of the abundance of OTUs characteristic of methanogens supported the identification of four main orders of methanogens in substrate samples: *Methanosarcinales* and *Methanomicrobiales* of the class *Methanomicrobia*, *Methanomassiliicoccales* of the class *Thermoplasmata*, and *Methanobacteriales* of the class *Methanobacteria*. Throughout the experiment, significant differences were observed in the abundance of *Methanosarcinales* and *Methanomassiliicoccales* in samples collected from both process and control bioreactors. The abundance of OTUs characteristic of these methanogens was bound by a significant negative correlation. It was concluded that dynamic changes in microbial communities and their adaptation to changing environmental conditions are essential for the stable operation of anaerobic digesters.

Substrate samples collected from both process and control bioreactors were characterized by a predominance of genes conferring resistance to multiple drugs and MLS antibiotics (>20 ppm). Tetracycline and bacitracin resistance genes were also abundant (5-15 ppm) in the analyzed samples. It was confirmed again that selective pressure exerted by antibiotics added to the substrate in process bioreactors did not specifically target genes encoding resistance to various classes of antimicrobials. Total ARG abundance decreased in both process and control digestates at the end of anaerobic digestion. However, it should be noted that the prevalence of multidrug resistance genes increased in samples of antibiotic-supplemented samples, whereas a reverse trend was observed in control samples. It should also be stressed that the transfer of multidrug resistance genes between bacteria poses a serious public health threat around the world.

Stage III

Although the metagenomic sequencing of samples collected in the previous stage of the study supported the identification of sequences characteristic of *Archaea*, their number was significantly lower than the number of Eubacteria-specific reads. Therefore, the last stage of research focused on expanding the existing knowledge on the effect exerted by long-term supplementation of sewage sludge with a mixture of antibiotics at increasing concentrations on methanogenic microorganisms.

The genetic material isolated in the second stage of the study was evaluated to determine the prevalence of genes characteristic of methanogens of the families *Methanosarcinaceae* (*MSC*) and *Methanosaetaceae* (*MST*), belonging to the order *Methanosarcinales*. The analyses were performed using the qPCR technique. The results of a preliminary study enabled to exclude a significant proportion of methanogenic families of other orders in the domain *Archaea* in anaerobically digested sludge. The activity of methanogenic microorganisms was assessed based on the concentration of the methyl-coenzyme M reductase gene (*mcr*A), which catalyzes the final step of anaerobic digestion - methanogenesis. The results of this experiment were presented in a research article (**Appendix 4** to this dissertation), and the fourth research hypothesis was verified.

A molecular analysis of the efficiency of anaerobic digestion, including the concentration of the functional *mcr*A gene, demonstrated that the concentration of this gene in substrate samples collected from process and control bioreactors varied throughout the entire experimental series. It was found that methane production was not correlated with the prevalence of the *mcr*A gene during long-term anaerobic digestion, regardless of exposure to antibiotics. It was confirmed again that changes in methane yields cannot be reliably measured based on the abundance of the *mcr*A gene despite the fact that this gene has been proposed by other researchers as a bioindicator for monitoring methanogen activity.

In both experimental and control substrate samples, the copy numbers of genes characteristic of *Methanosarcinaceae* remained stable during the experiment. The only exception was the last, sixth experimental series, where the abundance of *MSC* genes increased by two orders of magnitude in 1 gram of digestate. This points to a stable increase in *Methanosarcinaceae* counts during the anaerobic digestion of sewage sludge, regardless of antibiotic supplementation. In turn, highly significant ($P \leq 0.001$) differences were noted in the abundance of *MST* genes, characteristic of the family *Methanosaetaceae*, between antibiotic-

supplemented and control samples. It should be stressed that in the last experimental series, the abundance of *MST* genes was similar in experimental and control substrate samples, which suggests that microbial consortia adapted to the increasing concentrations of antibiotics. It was concluded that both methanogen families well adapted to the conditions in the process bioreactor, but irrespective of antibiotic exposure, *Methanosaetaceae* predominated in the digestate.

SUMMARY AND CONCLUSIONS

The results of the study provided new information on the impact of antimicrobials on anaerobic digestion, in both technological and microbiological contexts. The research objectives were achieved, and all hypotheses formulated in the doctoral dissertation were positively verified. It was found that:

(1) some antimicrobials, widely used in human medicine, may decrease the efficiency of anaerobic digestion of sewage sludge.

(2) the presence of antimicrobials during anaerobic digestion exerts a significant effect on ARG profiles. Exposure to MET induced the most significant changes in ARG concentrations, in particular through increasing the concentrations of genes encoding resistance to β-lactams, tetracyclines and fluoroquinolones, and decreasing the concentrations of genes encoding resistance to MLS antibiotics. The selective pressure exerted by antimicrobials was not specific to the corresponding ARGs. Moreover, the study highlighted the problem associated with ineffective elimination of ARGs during the anaerobic digestion of sewage sludge and threats posed by the spread of AR, which are very important considerations.

(3) the exposure of sewage sludge to antimicrobials during anaerobic digestion significantly affects the structure of microbial communities, including methanogens. The greatest changes in microbial diversity were induced by MET, which decreased the proportion of *Bacteroidetes*, and increased the abundance of OTUs characteristic of *Firmicutes* and *Proteobacteria*. *Methanosaetaceae* dominated among methanogenic microorganisms during supplementation with individual antibiotics and during simultaneous exposure to MET, AMO and CIP.

(4) the results of a quantitative analysis of genes specific to methanogenic microorganisms, such as the functional *mcr*A gene, do not fully reflect the parameters of anaerobic digestion, i.e. the actual efficiency of methane production in the presence of antimicrobials.

The conducted experiments revealed the need for further research to determine the effect of inhibitors, such as antibiotics, on the activity of microorganisms responsible for each stage of anaerobic digestion, which in the future will make it possible to ensure optimal conditions for their growth and development. Such research should involve the use of modern molecular techniques, including metagenomic sequencing.

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Załącznik nr 1

Appendix 1

Czatzkowska M., Harnisz M., Korzeniewska E., Koniuszewska I.

Inhibitors of the methane fermentation process with particular emphasis on the microbiological aspect: A review.

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REVIEW

Inhibitors of the methane fermentation process with particular emphasis on the microbiological aspect: A review

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Abstract

Methane fermentation is an attractive practice in waste processing, which enables one to both control pollution and recover energy. This kind of anaerobic digestion is exposed to inhibitors, which can retard the process and cause failure. The mechanism causing toxicity of these substances and their impact on the efficiency of the process are already known, but there is still not much information about their influence on methane fermentation microorganisms' activity and the composition of microbiota. In this review, based on 168 articles, we present a summary of the up-to-date research on the inhibition of anaerobic processes by some specific toxicants: ammonia, sulfides, ions of light metals, heavy metals, antibiotics, ethylene and acetylene, chlorophenols, halogen aliphatic hydrocarbons, aliphatic nitro compounds, and long-chain fatty acids. This review principally focuses on the impact of these inhibitors on the microorganisms involved in the process. More accurate recognition of methane fermentation inhibition mechanisms, with particular emphasis on the microbiological aspect, can help to improve the efficiency of the process.

KEYWORDS

anaerobic digestion, inhibitors, methane production efficiency, methanogens

1 | INTRODUCTION

Biomethanation is defined as a process of converting complex organic matter under anaerobic conditions mostly to methane and carbon dioxide, with possible emission of trace amounts of hydrogen sulfide, hydrogen, and carbon monoxide. Methane fermentation requires the activity of various populations of microorganisms, responsible for a proper course of consecutive process phases.[1-3] The following phases of biodegradation are distinguished according to the subsequent organic substance conversions (Figure 1): (a) hydrolysis, where complex organic compounds, such as carbohydrates, proteins, and lipids, undergo hydrolytic transformations with the catalytic participation of enzymes. These processes lead to the production of mostly simple sugars, higher fatty acids, glycerol, and amino acids. Two phyla dominate among hydrolyzing bacteria; Bacteroidetes and Firmicutes, and they include most of the known species.[4-6] (b) Acidic fermentation, where acidogenic bacteria convert products of the hydrolysis to VFAs, which include acetate, propionate, butyrate and isobutyrate, and valerate and isovalerate. Besides VFAs, alcohols, lactate, formate, CO_2, and H_2 are produced. These two stages are carried out by bacteria of the genera *Bacillus* sp., *Pseudomonas* sp., *Clostridium* sp., *Bifidobacterium* sp., and, to a lesser

extent, by *Streptococcus* sp. and *Enterobacterium* sp. and others.[7,8] (c) Acetogenesis, where acetogenic bacteria (including *Syntrophomonas* sp., *Syntrophobacter* sp.) produce acetic acid. Methanogens can use acetate, formate, H_2, CO_2, and methyl compounds directly, but other intermediates formed by acidogenesis have to be additionally biodegraded by other microorganisms, which enables methanogens to use them in order to produce methane. Syntrophic acetogenesis is the process in which these intermediates are further biotransformed to form acetate, H_2, and CO_2. With regard to thermodynamics, this is one of the most difficult stages. What is needed here is the syntrophy of acetogenic and methanogenic bacteria, where one group of microorganisms produces and the other consumes hydrogen. Hydrogenotrophic methanogens live in syntrophy with acetogens and consume H_2 provided from the latter.[7,9,10] A recent study has shown direct interspecies electron transfer performed by some microorganisms using electrically conductive pili. Electrons can be transferred in this way from *Geobacter* to *Methanosaeta*, for example.[4,10-12] (d) Methanogenesis, where methanogenic microorganisms under anaerobic conditions convert products of the preceding phases, releasing methane, carbon dioxide, and water. Hydrogenotrophic methanogens are critical for anaerobic digestion because of their ability to scavenge H_2 and keep the partial pressure low. The most frequently observed hydrogenotrophic methanogens in anaerobic digesters belong to the genera *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Methanospirillum*, and *Methanothermobacter*. Acetoclastic methanogens belong to the genera *Methanosaeta* and *Methanosarcina*.[9,10,13-15]

Methane fermentation is an attractive practice in waste processing, which enables us to both control pollution and recover energy. As reported by Scarlat et al,[17] in 2015, in Europe alone, there were about 17 000 biogas plants of different sizes and types, and the total biogas production reached more than 650 PJ of primary energy. Biogas production has achieved an important growth recently. However, the same researchers state that there are frequent problems due to the low efficiency of methane production (eg, caused by a decrease in the activity of various groups of microorganisms involved in anaerobic digestion, including methanogenes) and instability of the entire process, which prevents the widespread implementation of this technology. The major reason why methane fermentation process is inhibited is the diversity of substances present in various concentrations in different types of waste.[2,17,18] This review article is dedicated to the identification of factors and mechanisms causing the inhibition of methane fermentation, with particular emphasis on the microbiological aspect. First, however, the characteristics of the process of biogas production and methanogenic microorganisms are discussed.

1.1 | General introduction to the anaerobic digestion process

The global energy consumption and demand for power are constantly growing. Meanwhile, most of the resources, such as coal, natural gas, or crude oil, are not sustainable energy sources. The contemporary critical phase in the human population growth requires increasingly larger energy inputs. These circumstances substantiate the growing interest in renewable energy.[19]

Except for solar and wind energy, biogas is among the most promising bioenergy alternatives to the energy based on fossil fuels. Many types of biodegradable waste can be used as feedstocks for biogas production, thus relieving the pressure on the natural environment and limiting the total area of landfills.[20] Biodegradable waste most often consists of by-products from agricultural production (including

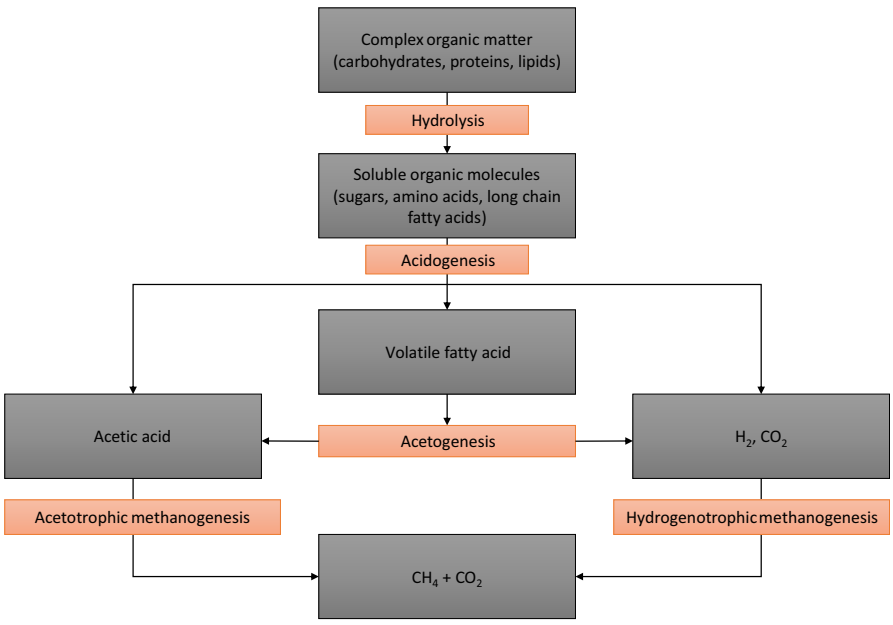

FIGURE 1 Process of methane fermentation according to Chen et al[16]

postharvest residues, excess biomass, roots, and leaves), waste from the agricultural and food processing industries (pressed fruit pomace, extracts, pulp, sediments, filtration, and extraction leftovers), and from abattoirs and meat processing plants.[21] Other substrates used for methane fermentation are sewage sludge and municipal waste as well as dedicated energy crops (maize, amaranthus, sorghum, oilseed rape, sugar beet, fodder beet, and others), algae and seaweeds (water base) and by-products from the production of ethanol and biodiesel.[22,23]

Animal farms generate waste and by-products, which have various impacts on the natural environment. They can also serve as feedstocks for methane fermentation.[19] Slurry is certainly the type of raw material considered for methane production that is available in large quantities in many parts of the world.[24] Data on methane productivity from different feedstocks submitted to fermentation are given in Table 1.

Methane can be an alternative to fossil fuels in thermal and electric power generation, but it can also serve as a fuel for vehicles.[25] By replacing a natural fossil fuel with a renewable one, we can produce a beneficial influence on the environment and achieve greater diversification of sources of energy.[20] Sustainable development of the human population requires both restraining our addiction to fossil fuels and limiting environmental pollution, and methane fermentation is one of several technologies able to achieve both aims.[25]

The site and method of biogas production have a significant influence on its quality and quantity. Biogas from different sources can have different methane content and therefore different values of energy parameters as well as the content of pollutants. It has been demonstrated, for example, that biogas from landfills is characterized by a highly variable methane content, and biogas from fermentation tanks at wastewater treatment plants as well as on farms is more stable. Typical landfill biogas contains between 25% and 67.9% of methane, and its calorific value ranges between 16.0 and 23.5 MJ/m^3. The content of methane in biogas from WTPs is between 57% and 67%, and its calorific value varies from 20.5 to 23.4 MJ/m^3.[26-28] Biogas with the highest calorific value, from 18.7 to 30.6 MJ/m^3, can be obtained from agricultural biogas plants,[26] where the methane content varies within 56%-70%.[27,28] The methane content in biogas obtained during cow dung slurry fermentation ranges

TABLE 1 Biogas and methane yield from different types of substrates

Type of organic waste	Organic content (%)	Biogas production (mL/g)	Methane yield	Unit of methane yield measurement	References
Cellulose	—	—	73.4	%	Barlaz et al[141]
Hemicellulose	—	—	17.1		
Fruit and vegetable waste	—	—	326	mL/g	Li et al[142]
Grass silage	—	—	238		
Wheat straw	—	—	305		
Cotton stock	—	—	192.4		
Chicken manure	—	80	272		Li et al[142]
Pig/cow manure	—	25-30	138		Cassie et al[143]
Food scarps	—	265	—		Cassie et al[143]
Corn silage	—	190	—		
Brewery waste	—	120	—		
Bakery waste	—	714	—		
Stomach and intestine content	15-20	—	40-60		Angelidaki et al[144]
Fish-oil sludge	80-85	—	450-600		
Source sorter organic household waste	20-30	—	150-240		
Whey	7-10	—	40-55		
Soya oil/Margarine	90	—	800-1000		
Sewage sludge	3-4	—	17-22		
Concentrated sewage sludge	15-20	—	85-110		
Forage mix	86-91	—	297-370		Balat and Balat[30]
Maize	96-97	—	247-375		
Barley	90-93	—	382-506		
Rye	91-93	—	403-404		
Sugar beet	90	—	504		

from 53% to 59%.[29] The biogas yields received from animal manure and animal slurry vary from 370 m^3 per ton dry matter cattle manure to 450 m^3 per ton dry matter pig manure.[30]

Unlike natural gas, biogas can contain various types of pollutants. These can be chemical (sulfides, ammonia, chlorine, and fluorine compounds, silanes), mechanical (eg, sillicon, dust), and biological (bacteria, fungi) pollutants.[26,27] The contaminants found in biogas may have an adverse impact on its quality and combustion.[26] Recognizing and understanding the aspects connected with this problem could certainly support attempts to create strategies for developing the technology and reinforce its credibility as an alternative energy source.[31] The continued improvement of existing biomethanation technologies and the development of new technologies can enhance the effectiveness and stability of these processes.[25]

Methane fermentation is a process in which technical solutions must respond to the following considerations: (a) only the organic fraction undergoes degradation, (b) the nature of the biological process imposes certain restrictions, such as the process's temperature, pH, composition of feedstocks, presence of toxic substances, (c) anaerobic digestion requires a sealed container (reactor), and (d) the product (biogas) contains other components apart from methane and carbon dioxide.[32]

1.2 | Microorganisms involved in methane production

Archaeal methane metabolism has a significant role in the global carbon cycle, with methane produced by archaea corresponding to over a half of all methane produced in the world per year.[33] Methane is produced by methanogenic archaea in the last step of organic matter fermentation under anaerobic conditions.[9,10,13,14,34] All methane-synthesizing microorganisms have a specific functional gene, *mcr*A, which encodes the α-subunit of methyl-coenzyme M reductase and is a better tool for analysing their biodiversity changes than *16S* rRNA. The analysis of methanogens and their analysis based on *16S* rRNA as a marker gene is limited because methanogenic Archaea are not monophyletic.[33,35,36] Several orders of methanogens have been recognized: *Methanosarcinales*, *Methanococcales*, *Methanomicrobiales*, *Methanobacterales*, *Methanopyrales,* and *Methanocellales*.[33,37,38]

The overall cell structure of the Archaea representatives resembles the structure of a bacterial cell. The cytoplasm lacks mitochondria, lysosomes, endoplasmatic reticulum, or the Golgi apparatus. The cell is typically enveloped by a cell wall and membrane. The cell wall in archaea does not contain peptidoglycan (murein), and its stability and stiffness depend on the presence of other polymers.[39] A paracrystalline protein cover layer, commonly referred to as the S-layer, is present in almost all described archaea. S-layers are formed of only one or two proteins and create various lattice structures.[40] This is a superficial, 5- to 25-nm-thick layer that envelopes the cell, thus helping it to maintain the proper shape and protecting it from unfavorable changes in the environment. It is fairly smooth on its outer surface, with a more corrugated internal surface. In some archaea, S-layer proteins are the sole cell cover component, while in others the cell cover consists of various polymers, including the polysaccharides pseudomurein and methanochondroitin, and can also include additional S-layer proteins.[39,41] Same as in certain bacteria, the S-layer of archaea is composed of proteins and/or glycoproteins, distinguished by a large content of acidic and hydrophobic amino acids.[39] However, the structure of archaea is clearly different from that of bacteria. Representatives of the Archaea also have some specific surface structures, including archaella, pili, hami, and cannullae.[42] Many microorganisms from the domain Archaea have intercellular organelles of motion, which used to be called flagella, like organs of locomotion in bacteria. Nowadays, it is known that these organelles have a structure different from that of bacterial flagella or of cillia, characteristic for many cells of eukaryotic organisms. These organelles are now referred to as archaella. Unlike bacterial flagella, archaella do not have rings that would enable them to anchor in the cell wall and membrane.[43] Archaea are characterized by a considerable natural resistance to antibiotics, the presence of nucleotides in tRNA molecules, absent in cells of other microorganisms, and by an atypical structure of RNA polymerase, dependent on the DNA. Genes linked to cellular divisions and metabolism in archaea resemble the ones occurring in the genome of bacteria, whereas genes participating in the processes of replication, transcription and translation are more similar to their counterparts in eukaryotic cells.[39,44] Archaea reproduce asexually, by cell division or budding, and they exchange genetic material in a way similar to generalized transduction in bacteria, but also through the processes of conjugation and transformation. This is possible because archaea, like bacteria, possess additional genetic material in the form of plasmids. Most of the Archaea recognized until now multiply by cell division.[39] Although the last two decades have witnessed an enormous progress in our knowledge and understanding of cellular structures, including the complete structure of archaeal cells, some of the functions and mechanisms responsible for stability in extreme ambient conditions still await clarification.[45]

Microorganisms which belong to the domain Archaea are isolated from various environments, often from particularly extreme habitats. Archaea are typical microbiota of oceans, seas, lakes, soils, the rumen, and also biogas reactors. This domain is characterized by a large share of thermophilic and hyperthermophilic organisms, isolated from hot springs and from hydrothermal chimneys situated on the bottom of the

oceans.[46,47] Other archaea are representatives of typical psychrophilic and psychrotrophic organisms, isolated from waters and soils at temperatures close to 0°C.[48,49] Another group of archaea is composed of halophilic organisms, growing in habitats with extremely high salinity, there are also ones dwelling in habitats with extremely high or low pH (alkaliphiles and acidophiles).[46,50,51] A particularly interesting group of archaea consists of methane synthetizing anaerobic organisms (methanogenic archaea), isolated from benthic sediments in water bodies, peats, and mines.[46] Methanogenic archaea are also a constituent part of the microbiome of many animals and humans. Methanogenic organisms colonize mostly the digestive tract, including the large bowel.[52]

Archaea can produce methane in three ways (Figure 2), different in the carbon substrates and sources of reduction potential.[14,53,54] The most common methanogenesis among methanogenic archaea is the hydrogenotropic pathway, where carbon dioxide is reduced with the partcipation of hydrogen as an electron donor. It consists of seven stages, leading to the production of methane. Another substrate used on this pathway is formate, which is the source of both carbon and electrons. The two other types of methanogenesis are the acetic pathway and methylotrophic pathway, which occur among representatives of the order *Methanosarcinales*.[37,55] In the former pathway, acetic acid is decomposed to carbon dioxide and a methyl group. CO is gradually oxidized, which coincides with the release of electrons, necessary to reduce the methyl group to methane. The methyltropic pathway has been also observed among representatives of the *Methanobacteriales*. In its thus far best explored variant, one-carbon compounds (ie, methylamines or methanol) are used simultaneosuly as a donor and acceptor of electrons. One C-1 molecule of the compound is oxidized to obtain electrons, which serve to reduce three consecutive molecules until the final product, that is, methane, is obtained.[55,56] The process of methane synthesis is participated by many unique co-enzymes (tetrahydromethanopterine, methanofurane, co-enzyme F420, HS-coenzyme B, co-enzyme M) and electron carriers (ie, methanophenazine).[57,58] Additionally, over 200 genes are responsible for encoding the synthesis of co-enzymes, enzymes, and prosthetic groups partcipating in the process of reducing carbon dioxide to methane and its coupling with ADP phosphorylation.[59] It is maintained that the primordial group in the evolution consisted of hydrogenotrophic methanogens. This is confirmed by the presence of genes responsible for production of methane in all species, in an almost unchanged form.[55]

It is worth underlining that methanogenesis can occur in a wide range of temperatures, and its efficiency depends primarily on the conditions in which particular representatives of Archaea dwell. As Mikucki et al[60] report, methane is synthesized at different temperatures. Mesophilic as well as thermophilic species are responsible for its production. Methane can be synthesized by hyperthermophilic species, for example, *Methanococcus jannaschii* and *Methanopyrus kandleri*. For instance, the mesophilic species *Methanoculleus submarinus* synthesizes methane as hydrates at a temperature as low as 15-16°C.[60] Some psychrophilic methanogens have also been reported, such as *Methanogenium frigidum* and *Methanosarcina lacustris*.[37,61,62] It is therefore evident that methanogens play a significant role in the carbon cycle in nature, by synthesizing methane from various simple inorganic and organic compounds.[39]

Table 2 presents microorganisms responsible for conducting consecutive stages of biogas production.

2 | INHIBITORS OF METHANE FERMENTATION

Inhibitors of methane fermentation can be divided into specific and nonspecific ones. Specific inhibitors cause the process to stop by affecting only the group of methanogenic microorganisms, active in the last stage of fermentation, whereas nonspecific inhibitors influence the activity of both methanogens and other groups of microorganisms. There are numerous studies reporting on various chemical substances which inhibit methane production by archaea, at different densities of microbial populations and concentrations of inhibitors.[16,63,64]

2.1 | Ammonia

Although ammonia is an essential nutrient for the growth of bacteria, if present in very high concentrations it can inhibit methanogenesis during anaerobic digestion. According to Yenigun and Demirel,[65] ammonia is considered to be a potential inhibitor during biogas production, especially in composite substrates, such as manure or the organic fraction of municipal waste. Ammonia is generated during the biological degradation of nitrogenous matter, mostly proteins, and urea. Ammonia ions (NH_4^+) and free ammonia (NH_3) are the two main forms of inorganic ammonia nitrogen in aqueous solution.[65] It is suggested that free ammonia is the main cause of the inhibition of methanogensis because it can freely permeate through cell membranes.[64] The relative concentration of molecular ammonia (NH_3) and ammonia in the form of the ammonium ion (NH_4^+) depends on the pH and temperature. An increase in pH and temperature values favors the formation of toxic molecular ammonia.[66] Several mechanisms of free ammonia inhibition after diffusion into a cell have been described: change of the intracellular pH, proton imbalance, rise in maintenance energy demand, and inhibition of specific enzymatic reactions.[67,68]

It is commonly maintained that ammonia concentrations of approximately 200 mg/L are beneficial for anaerobic processes because nitrogen is an essential nutrient for anaerobic microorganisms. However, large concentrations of

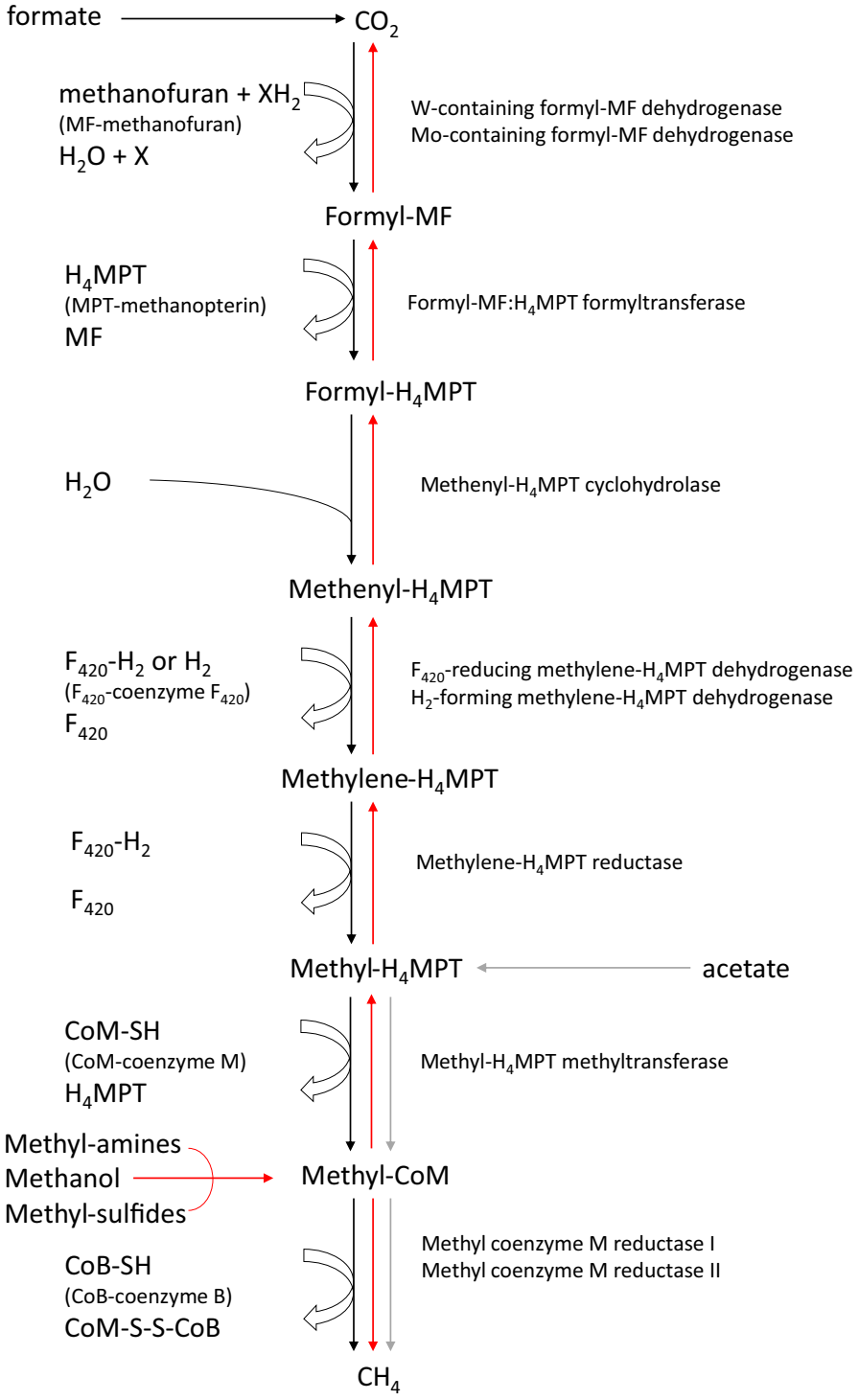

FIGURE 2 Pathways of methanogenesis according to Bapteste et al.[55] Hydrogenotrophic (gray arrows), aceticlastic (black arrows) and methylotrophic (red arrows)

total ammonia nitrogen can limit microbial activities.[69] The literature also provides information about the sensitivity of methanogens to ammonia nitrogen. As reported by Yenigun and Demirel,[65] the influence of ammonia on the maximum rise in the growth of hydrogen-consuming methanogenic microorganisms was investigated at different pH levels and temperatures. The maximum noninhibited rate of the growth of methanogens in sewage sludge was 0.126 hour^{-1} at pH equal 7.0 and temperature of 37°C. The maximum growth rate under these conditions was depressed to nearly a half of this value at 350 mmol/L ammonia. Besides, it has been shown that an increase in pH from 7.0 to 7.8 at 37°C seemed to have reinforced the inhibitory action of ammonia. During anaerobic digestion of liquid manure, the activity of methanogens was inhibited at high concentrations of total nitrogen, which was confirmed by changes in the parameters of acetate consumption. In a study on the fermentation of poultry litter, the maximum rate of growth of acetogenic bacteria was

TABLE 2 Microorganisms responsible for conducting individual stages of the methane fermentation process

Stages of methane fermentation	Bacteria	References
Hydrolysis	*Baceriodes* sp. *Bacillus* sp. *Bifidobacterium* sp. *Cellulomonas* sp. *Clostridium* sp. *Enterobacterium* sp. *Erwinia* sp. *Micrococcus* sp. *Peptococcus* sp. *Pseudomonas* sp. *Ruminococcus* sp. *Streptococcus* sp. *Thermomonospora* sp.	Li et al[145] Lo et al[146] Venkata et al[147] Wiącek and Tys[148] Yu et al[25] Ziemiński and Frąc[8]
Acidogenesis	*Aerobacter* sp. *Alcaligenes* sp. *Bacillus* sp. *Bacteroides* sp. *Butyribacterium* sp. *Clostridium* sp. *Escherichia* sp. *Flavobacterium* sp. *Micrococcus* sp. *Propionibacterium* sp. *Pseudomonas* sp. *Ruminococcus* sp.	
Acetogenesis	*Acetobacterium* sp. *Methanobacterium propionicum* *Methanobacterium suboxydans* *Pelobacter* sp. *Pelotomaculum* sp. *Smithllela* sp. *Sporomusa* sp. *Syntrophobacter* sp. *Syntrophomonas* sp. *Syntrophus* sp.	Ariesyady et al[149] Bertsch et al[150] De Bok et al[151] de Bok et al[152] Detman et al[153] Imachi et al[154] Li et al[145] Schmidt et al[155] Sousa et al[156] Wiącek and Tys[148] Ziemiński & Frąc[8]
Methanogenesis	*Methanobacterium thermoautotrophicum* *Methanococcoides burtonii* *Methanococcus jannaschii* *Methanococcus voltae* *Methanocorpusculum sinense* *Methanogenium cariaci* *Methanolacina paynteri* *Methanopyrus candleri* *Methanosaeta concilii* *Methanosarcina barkeri* *Methanosarcina mazei* *Methanosarcina thermophila* *Methanospirillum hungatei* *Methanothermobacter thermautotrophicus* *Methanothermobacter thermoflexus* *Methanothermobacter wolfei* *Methanosarcina flavescens* *Methanobacterium formicicum*	Albers and Meyer[41] de Bok et al[152] Jarrell et al[157] Korzeniewska et al[158] Kouzuma et al[159] Lira-Silva et al[160] Liu et al[63] Mikucki et al[60] Yenigun and Demirel[65] Zhang et al[161] Ziemiński and Frąc[8]

(Continues)

observed at a concentration of total nitrogen between 7700 and 10 400 mg/L and pH between 7.8 and 7.93.[65,70-72] It has been reported that pH and total nitrogen concentration are the factors that inhibit acetogenic bacteria.[65] In a study by Hendriksen and Ahring[73] dealing with the impact of ammonia on methanogenic microorganisms consuming hydrogen, including *M thermoautotrophicum*, *Methanobacterium thermoformicicum*, and *Methanogenium* sp., initial inhibition was detected at a total nitrogen concentration in a range of 3000-4000 mg/L, and when it rose to 6000 mg/L, the growth of microorganisms declined by 50%. Moreover, slow growth and formation of aggregates of *M thermoformicicum* were noticed by the same authors at a total nitrogen concentration equal 9000 mg/L. Based on the research results, it was concluded that thermophilic methanogens are less sensitive to ammonia than their mesophilic forms.[65,73]

The literature describes a wide range of inhibitory concentrations of ammonia, with inhibitory concentrations of ammonia nitrogen in a range of 1700 do 14 000 mg/L causing a 50% reduction in the production of methane.[74] Sung and Liu informed that methanogenic activity in soluble nonfat dry milk digestion was heightened at TAN concentrations lower than 1500 mg/L, whereas methane fermentation was obviously inhibited at TAN concentrations higher than 4000 mg/L.[74,75]

The differentiating role in the inhibition due to ammonia concentrations can be attributed to the type of substrate submitted to fermentation, environmental conditions (temperature, pH), and acclimation periods. When waste with high concentrations of ammonia nitrogen is being processed, pH affects the growth of microorganisms and the form of nitrogen that appears under such conditions.[76] The accumulation of volatile fatty acids (VFA) causes a decrease in pH, and therefore, it reduces concentrations of ammonia but raises the content of ammonia ions. According to Chen et al,[75] ionized ammonium nitrogen is an important inhibitor during food waste methane fermentation with uncontrolled pH. The inhibition effect occurred with the ammonium concentration of 2000 mg/L. The inhibition effects of high ammonium concentrations on anaerobic digestion led to VFA increase and pH decrease. These factors repressed the acetoclastic pathway and activity of *Methanosaeta* sp. The same authors reported that the ammonium concentration of 6000 mg/L inhibited the metabolism of the hydrogenotrophic methanogens, such as *Methanobacterium* sp. and *Methanospirillum* sp.[75] Interactions between the form of ammonia nitrogen, VFA, and pH can lead to "the inhibition of the established state," where the fermentation process runs in a stable manner but generates less methane.[77] As for temperature, in general, a higher temperature of the process has a beneficial effect on the rate of metabolic changes achieved by microorganisms, but it also causes an increase in the concentration of toxic free ammonia. The study of Hansen et al[78] has demonstrated that fermentation of waste with a high content of ammonia is less stable and more strongly inhibited at thermophilic rather than at mesophilic temperatures. However, the acclimation of microorganisms as a factor can influence the rate of inhibition of methane fermentation by ammonia. Adaptation can result from internal changes among dominant methanogenic species and consequently within the whole population of methanogens.[64] Conclusions derived from the research into ammonia effect on anaerobic digestion are summarized in Table 3.

Two physicochemical methods can be applied to remove ammonia from a feedstock: ammonia stripping with air, and chemical titration. Both methods have proven to be feasible at high ammonia concentrations and complex compositions of substrates.[79] A popular approach to limiting the inhibition of methane fermentation due to ammonia consists in the dilution of a feedstock (mostly slurry) up to the final ammonia concentration of 0.5% to 3.0%. However, the resultant increase in the volume of waste to be processed makes this method economically unattractive.[80] Another approach is to increase the retention time of a substrate in a reactor. It has been found that the methane productivity in a continuous stirred-tank reactor (CSTR) could be improved when a stirrer is switched on half an hour before and after feeding the substrate. This solution is thought to be promising because it is easy and economically viable.[78]

2.2 | Sulfides

Sulfate is a common component of many types of industrial wastewater. In anaerobic reactors, sulfate is reduced to sulfides by sulfate-reducing bacteria (SRB). Sulfate is reduced by two major groups of SRB, that is, the ones which reduce such compounds as lactate to acetate and CO_2, and the ones which completely decompose acetate to CO_2 and HCO_3^-. Sulfate-reducing bacteria are highly varied in terms of metabolic pathways. The compounds which can be partly or completely degraded by SRBs comprise branched and long-chain fatty acids, ethanol and other alcohols, organic acids, and aromatic compounds. Because of the various ways in which substrate can be used, SRBs compete for organic substrates or hydrogen with other fermentation microorganisms, that is, methanogens and acetogens, acidogens, and hydrolytic bacteria.[81-83] The outcome of such competition between SBRs and other anaerobic microbes determines the concentration of sulfides in the reactor. Sulfides in different concentrations can be toxic not only to methanogens but also to sulfate-reducing bacteria themselves.[64] Thus, degradation of sulfates in sewage sludge is a highly undesirable process because of both the depressed methane productivity and the unpleasant odor due to H_2S release.[84,85]

As reported by Chen et al,[64] H_2S is the most toxic form of sulfide because it is capable of diffusing through cell

TABLE 3 The impact of ammonia on biogas production during anaerobic digestion, based on selected publications

Substrate	Temperature (°C)	pH	Critical or specified TAN concentration (mg/L)	Critical or specified FAN concentration (mg/L)	Organisms affected/ present	References
Chicken manure	35-73	—	—	>250 (100% inhibition)	—	Bujoczek et al[162]
Soluble nonfat dry milk	55	—	4000 10 000 (100% inhibition)	—	—	Sung and Liu[74]
Livestock waste	55	7.2-7.3	3000-4000	—	*Methanosarcina* sp.	Angelidaki and Ahring[163]
Sewage sludge	35	—	6000	—	*Methanobacterium* sp. *Methanobacterium* sp. *Methanosarcina* sp.	Sawayama et al[164]
Food waste	37	—	2000 6000	—	*Methanosaeta* *Methanobacterium* sp. *Methanospirillum* sp.	Chen et al[75]
Swine waste	25	—	≥3500	—	*Methanosarcina* sp.	Angenent et al[165]
Synthetic wastewater	35	8.0	6000 (100% inhibition)	>700 (100% inhibition)	*Methanosarcina* sp.	Calli et al[166]
Sodium acetate	—	—	7000 (acclimated)	—	*Methanosarcinaceae* spp.	Fotidis et al[167]
		—	5000 (nonacclimated)	—	*Methanococcales* spp.	
Cattle excreta + olive mill waste	37/55	—	1300	—	*Methanosarcina* sp.	Goberna et al[168]

Note: Critical concentration—the concentration at which inhibition begins.

Abbreviations: FAN, free ammonia nitrogen; TAN, total ammonia nitrogen.

membranes and, as a result, it causes denaturation of proteins by forming disulfide bridges between polypeptide chains, thus disturbing metabolism. Moreover, the presence of H_2S in biogas significantly decreases the potential use of biogas and its economic value because the H_2S is an acidic and toxic gas, which causes powerful corrosion on pipes, combustors and instruments. Therefore, the H_2S in biogas must be removed before its use, which minimizes the corrosion.[86-88]

In order to control the methanogenesis inhibitory effect of sulfides, certain processes are implemented that remove these compounds from the substrate. A possible measure to prevent the toxicity of sulfides is by diluting a stream of wastewater, although an unwanted consequence is the enlarged total volume of the wastewater that undergoes processing. An alternative solution is to remove sulfides during the entire wastewater processing. Technologies include physicochemical solutions (stripping), chemical reactions (coagulation, oxidation, titration), and biological conversions (partial oxidation of sulfur to its elemental form).[89] A commonly used procedure to remove sulfides is to add iron salt solutions to the wastewater, which results in the precipitation of sulfide from the solution. As reported Ahmad et al,[90] the maximum sulfide elimination efficiency of the Fe^{+2}/Fe^{+3} treatment was around 70%. These researchers found the sulfide precipitation method promising for effective sulfidic wastewater treatment in various industries.[90] According to Krayzelova et al,[91] various processes are available to remove high sulfide content from biogas, too. There are physicochemical (which involve high costs and energy) and biological methods. The latter one, more economically advantageous, rely on the oxidation of sulfides to sulfates, thiosulfates, and elemental sulfur. The same authors state that microaeration is one of the available biological methods that has recently gained much attention owing to its simplicity and high efficiency. Microaeration takes place in the anaerobic digester and involves the dosing of little amounts of air into it. In effect, sulfides oxidize to elemental sulfur as a result of the activity of sulfide oxidizing bacteria (SOB), which includes, for example, *Thiobacillus* sp.[91,92]

2.3 | Ions of light metals

Toxicity of salts towards microorganisms has been investigated in microbiology for decades. High concentrations of

salts lead to the dehydration of bacterial cells due to a change in osmotic pressure. Although salt cations in a solution must be always bound to anions, it has been found that the toxicity of salts is largely determined by ions with a positive charge.[64,93,94] Ions of light metals, including sodium, potassium, calcium, and magnesium, are present in fermentation tanks. As Chen et al[64] reported, they can be released as a result of the decomposition of organic substances in the substrate or added with pH-regulating substances. While moderate concentrations stimulate the growth of microorganisms, excessive amounts of light metal ions will decelerate the multiplication of microbes, cause inhibition of their activity and have a toxic influence, as a result of which they can eventually destabilize cell membranes, disrupt functions of buffers and inhibit the production of biogas.[64] At relatively low salinities (about 100-150 g/L), processes like the transformation of acetate and higher fatty acids, reduction of sulfate, acetotrophic, and hydrogenotrophic methanogenesis are difficult.[51]

Data from the literature concerning the effect of aluminum on methane fermentation are very scarce. It has been implicated, however, that the inhibitory effect of aluminum may arise from its competition with iron and magnesium, or from the adhesion to the membranes/walls of bacterial cells, which may have an impact on the growth of bacteria. Cabirol et al[95] showed that the activity of acetogenic and methanogenic microorganisms becomes inhibited when $Al(OH)_3$ has been added to a fermentation mixture. After the exposure to $Al(OH)_3$ in a concentration of 1000 mg/L for 59 days, the specific activity of methanogenic and acetic microorganisms decreased by 50% and 72%, respectively.

As Chen et al[64] reviewed, very little is known about the toxicity of calcium ions in an anaerobic system. It has been demonstrated that the optimal concentration of Ca^{2+} needed for methanation of acetic acid is 200 mg/L. Calcium ions produced a moderate inhibitory effect when present in concentrations of 2500-4000 mg/L, although strong inhibition was demonstrated at a concentration of 8000 mg/L. High levels of potassium ions in fermentation tanks are also undesirable. The passive influx of K^+ ions neutralizes the membrane potential. It has been shown that low potassium concentrations (below 400 mg/L) cause an increase in the methane fermentation productivity, in both thermophilic and mesophilic processes. At higher concentrations of this ion, there is an inhibitory effect, clearly seen in thermophilic processes.[64]

Wastewater with a high sodium concentration is generated in the food-processing industry.[96] At low concentrations, sodium is essential for methanogens, probably due to its role in the generation of adenosine triphosphate or in NADH oxidation. The optimal conditions for the growth of mesophilic methanogens include a concentration of sodium ions up to 350 mg/L. Higher concentrations of sodium are likely to affect the activity of microorganisms and disturb their metabolism,[97] except for halophilic archaea (haloarchaea) which belong to the order Halobacteriales and thrive in environments with salt concentrations nearing saturation.[98] A comparison of the sensitivity of bacteria able to decompose volatile fatty acids showed that sodium was more toxic to acidogenic than to acetogenic microorganisms.[96] Gradual adaptation of methanogens to high sodium concentrations can improve their tolerance and shorten the lag phase before the onset of methane production.[99]

2.4 | Heavy metals

Heavy metals are an important class of compounds with an inhibitory effect towards methanogens. The impact of heavy metals on the activity of cultures of methanogens is well described in literature.[100,101] The development of several industries, like manufacture of glass and ceramics, metal plating, mining, as well as production of paper, pesticides, and storage batteries, has raised the heavy metals concentration in wastewater.[101,102] The presence of heavy metals in larger concentrations is detectable in industrial and municipal wastewater as well as in sewage sludge. The most common heavy metals are zinc (Zn), lead (Pb), copper (Cu), mercury (Hg), cadmium (Cd), chromium (Cr), iron (Fe), nickel (Ni), cobalt (Co), and molybdenum (Mo).[100,101,103] The main characteristic of heavy metals is that—unlike many other toxic substances—they are not biodegradable and can accumulate in cells. The toxicity of heavy metals is one of the main causes of disruptions and low productivity during methane fermentation processes. An important effect of a disturbance during anaerobic digestion induced by the presence of heavy metals is the reduction in biogas production and the accumulation of intermediate organic compounds.[101,104] The toxic effect of heavy metals arises from the way they interfere with the functions and structures of bacterial enzymes by binding with thiol and other groups of protein molecules or by replacing the naturally occurring metals in enzymatic prosthetic groups.[105] It is known that heavy metals inhibit the activity of anaerobic microorganisms, including acidogenic,[106-108] acetogenic,[109] and methanogenic ones[102,103,110] as well as sulfate reducing bacteria.[111] The heavy metal concentrations that cause a 50% decrease (IC_{50}) values in the hydrogen production by acidogenic bacteria were as follows: 3300 mg/L for Cd, 3000 mg/L for Cr, 30-350 mg/L for Cu, 1300 mg/L for Ni, >500-1500 mg/L for Zn, and > 5000 mg/L for Pb.[106,108] The activity of methanogens was inhibited in 50% by concentrations of: 36 mg/L, 27 mg/L, 8,9-20,7 mg/L, 35 mg/L, and 7,7 mg/L for Cd, Cr, Cu, Ni, and Zn, respectively.[103,110] An inhibitory effect of heavy metals on methanogenic microorganisms was also confirmed by the study of Sarioglu et al,[102] who evaluated the effect of Cu, Ni, Zn, and Pb

during biomethanization of wastewaters from a yeast factory. The decline in methane production for heavy metal concentrations over 0.16 mmol/L of Cu, 0.17 mmol/L of Ni, 0.15 mmol/L of Zn, and 0.05 mmol/L of Pb was observed.

Many heavy metals are contained in the structure of essential enzymes, which drive numerous anaerobic reactions. Whether heavy metals stimulate or inhibit anaerobic microorganisms depends on their total concentrations in substrates or on their chemical forms.[107] Toxicity of heavy metals largely depends on ambient parameters, too, eg pH, redox potential, and others.[112]

2.5 | Organic compounds

As reported by Chen et al,[64] a wide range of organic compounds can inhibit anaerobic processes. Organic substances which are weakly dissolved in water or adsorbed on the surface of sediments can accumulate in large concentrations within fermentation tanks. The accumulation of non-polar organic compounds in bacterial membranes makes the membranes demonstrate a disrupted gradient of ions, which may eventually lead to the cell's lysis. The same authors informed that factors which influence the toxicity of organic compounds include the concentration of a toxic substance, the concentration of biomass, exposure duration, age of a cell, acclimation, and temperature. Same as with other inhibitory substances, the adaptation of microorganisms to the presence of organic substances is an important factor to consider in an evaluation of their inhibitory effect. Mutually related mechanisms have been proposed through which such adaptation can be achieved. These are (a) enrichment of reactors with microorganisms which can degrade toxic compounds, (b) induction of specific degradation enzymes, and (c) genetic engineering. Acclimation of microorganisms participating in methane fermentation enhances their tolerance to the presence of toxic organic substances and biodegradability of these substances.[64]

There is still little knowledge about the exact mechanism of action of most of these organic inhibitors, and the literature on this issue is scarce and requires more credibility, especially in the microbial aspect.

2.5.1 | Antibiotics

Every year, thousands of tonnes of antibiotics and products of their metabolism enter wastewater treatment plants, having been excreted by humans and animals, or disposed of if unused.[113,114] Antibiotics present in waste can induce the inhibition of waste treatment processes, including methane fermentation.[115,116] Antibiotics can affect microorganisms in different ways. The action of these compounds can rely on the inhibition of DNA replication, RNA transcription, SOS response, or ATP generation. Antibiotics can also impair cell division, protein translation (by inhibition of aminoacyl tRNA binding to ribosome or the setback of elongation and translocation steps), and cell wall synthesis or nucleotide biosynthesis.[117-119] Ionophore antibiotics accumulate in the bacteria's cell membranes and interfere with the ion gradients required to generate a protonmotive force and transport nutrients.[120] A study by Sanz et al[116] revealed how different methanogen populations are inhibited by different antibiotics. The researchers chose several antimicrobial agents: ampicillin, chloramphenicol, erythromycin, hygromycin B, kanamycin, novobiocin, rifampicin, chlortetracycline, gentamicin, neomycin, penicillin G, spectinomycin, streptomycin, tylosin, and doxycycline. The study showed some regularity: (a) some antibiotics, such as the macrolide erythromycin, are characterized by any inhibitory effect on the process of biogas production,(b) some antimicrobial agents, with different specificities (especially the aminoglycosides), have partial inhibitory effects on biomethanization and decrease methane production by suppressing the activity of bacteria which degrade propionic acid and butyric acid; and (c) the protein synthesis inhibitors, like chlortetracycline and chloramphenicol, strongly inhibit methane fermentation. The majority of the chosen antibiotics inhibited the activity of acetogenic bacteria. Chloramphenicol and chlortetracycline are able to cause complete inhibition of the acetoclastic methanogenic archaea. Rusanowska et al[115] conducted a study to determine to what extent methane fermentation of sewage sludge could be inhibited due to β-lactams, tetracyclines, fluoroquinolones, sulfonamides, and metronidazole contained in this feedstock. According to amounts of generated biogas, no significant differences were determined between the control and the analyzed samples. In another study, Aydin et al[121] analyzed a long-term effect of mixtures of antibiotics: (a) erythromycin, tetracycline and sulfamethoxazole (ETS), and (b) sulfamethoxazole and tetracycline (ST) on communities of anaerobic microorganisms, and the influence of these antibiotics on processes in bioreactors. It was demonstrated that the activity of acetogens in the presence of either of the antibiotic combinations was higher than that of methanogens. The biogas productivity and the stability of a bioreactor were higher in a bioreactor fed a feedstock with the ETS rather than with the ST set of antibiotics. Mutual interactions and activities of acetogens and methanogens were of key importance to the processes occurring in both bioreactors. Coban et al,[122] too, showed mutual relationships between structures of microbial assemblages and the presence of an antibiotic (oxytetracycline) in fermentation tanks, which had a direct impact on the production of biogas.

Mitchell et al[123] found no effect of sulfamethazine or ampicillin on the total yield of biogas once the concentration of these antibiotics in the substrate reached 280 and 350 mg/L, respectively. However, an inhibitory effect of ampicillin on biogas production was observed at its earlier stages. On

the other hand, tylosin at concentrations between 130 and 913 mg/L decreased the biogas yield by 10%-38%, whereas the presence of florfenicol in a bioreactor at a concentration of 6.4, 36, and 210 mg/L lowered the output of biogas by 5%, 40%, and 75%, respectively. Reyes-Contreras and Vidas[124] analyzed the effect of the methanogenic toxicity of chlortetracycline in different concentrations and demonstrated that this antibiotic at a concentration of 10 mg/L inhibited the activity of methanogenic bacteria by 50%. Moreover, values of volatile fatty acids (VFA) achieved at the termination of the experiment showed that the presence of chlortetracycline in the bioreactor also affected the efficiency of methanogenesis.

2.5.2 | Ethylene and acetylene

It has been demonstrated that ethylene, the simplest unsaturated hydrocarbon from the homologous series of alkenes, at its concentration of 0.07% in the gaseous form inhibits by 50% the production of methane by pure cultures of *Metanospirillum hungatei*, *Methanothrix soehngenii* and *Methanosarcina barkeri*. This inhibition is reversible, and the activity of methanogens is completely recuperated after ethylene has been removed from the bioreactor. Acetylene, which is the simplest unsaturated hydrocarbon among alkines, also shows an inhibitory influence on methanogenesis. Acetylene inhibited methane production even more efficiently: 50% inhibition was noted with 0.015%[125,126] (Schink, 1985).

2.5.3 | Chlorophenols

Chlorophenols comprise monochlorophenols (CPs), dichlorophenols (DCP), trichlorophenols (TCP), tetrachlorophenols (TeCPs), and pentachlorophenol (PCP). Chlorophenols are popular as pesticides, herbicides, antiseptics, and fungicides. They are also used as wood preservatives, or added to glues, paints, plant fabrics, and leather goods. These compounds are toxic to anaerobic microorganisms. Their high hydrophobicity promotes the adhesion of these compounds onto the bacterial membranes, which produces an effect by interfering with the gradient of protons of the cell membranes and the transduction of energy in cells.[16,127]

Based on the research, it can be stated that PCP is the most toxic chlorophenol, and there is evidence indicating that the toxicity of chlorophenols is associated with hydrophobicity through a linear dependence between the logarithm of the partition coefficient *n*-octanol/water (log *P*) and the EC_{50} values.[127] There are many reports indicating various degrees of inhibition caused by organic compounds which belong to the above group. A concentration of PCP within 0.5-10 mg/L inhibited the activity of acidogenic and methanogenic populations.[128] It was demonstrated in an experiment conducted by Jin and Bhattacharya[129] that TCP were more toxic than DCP and CP. The toxicity induced by DCP and TCP is associated with the degradation of both propionate and acetate and depended on where in the benzene ring chlorine atoms were substituted. The inhibitory activity of chlorophenols seems to be directly connected with the preservation of the division into lipophilic groups. Disturbances of the membrane gradient of protons caused by this group of compounds, as well as transduction of cellular energy, result in certain irregularities in cellular catabolic and anabolic reactions.[64]

2.5.4 | Halogen aliphatic hydrocarbons

Most halogen aliphatic hydrocarbons, which are products of halogen reactions with chain hydrocarbons, are potent inhibitors of methanogenesis. Bromine compounds are stronger inhibitors towards methanogens than their chlorinated analogues. It has also been shown that tri- and tetrachloride forms of these compounds are more toxic than dichloride forms. Compared to their saturated counterparts, unsaturated chlorinated aliphatic hydrocarbons are less toxic.[64]

2.5.5 | Aliphatic nitro compounds

Aliphatic nitro compounds are reactive toxic substances, which include nitrobenzene, nitrophenol, aminophenol, and aromatic amines. Their reactive toxicity is due to specific chemical interactions with enzymes and disturbances they cause in metabolic pathways.[130]

A greater number of nitro groups do not have any substantial influence resulting in an elevated toxicity of nitrobenzens. On the other hand, the presence of more than one amino group in aminophenoles adds to the inhibitory effect on methane fermentation induced by these compounds. At the same time, an additional amino group in aniline led to a lesser inhibition of the said process.[131]

Anderson et al[132] noted that methane production was markedly reduced by additions of aliphatic nitro compounds during ruminal fermentation, and maximal inhibition was reached at concentrations of 12 mmol/L of nitroethane.

2.5.6 | Long chain fatty acids

Methane fermentation of substrates with a high content of the fatty fraction is often inhibited by long-chain fatty acids. These compounds are highly toxic to methane fermentation microorganisms, retarding their growth and making the cell membranes rupture due to absorption.[125]

Inhibition of a methane fermentation process by long chain fatty acids (LCFA) depends on the type of LCFA, population

of microorganisms, and temperature.[133] It has been revealed that thermophilic microorganisms involved in methane fermentation are more sensitive to long chain fatty acids than mesophilic microorganisms, most probably because of having a different composition of cell membranes.[134] Oleic, palmitic, and stearic acids have been described as LCFAs with the most potent inhibitory effect on thermophilic microorganisms.[135] If the microbial population's activity is disturbed by LCFAs, inhibition of anaerobic digestion occurs, which induces accumulation of volatile fatty acids (VFA) and disturbs methane production.[136,137]

The mechanism of inhibiting methanogenesis by long-chain fatty acids mainly consists in the adsorption of LCFAs to the cell membrane or wall, and affecting the metabolic transport.[138,139] This decelerates the production of methane. The mechanism can be prevented by providing a competitive, synthetic adsorbent (eg, bentonite).[140] Due to detergent properties, LCFAs can solubilize the lipid bilayer or membrane proteins, leading to enzyme activity inhibition, electron transport chain disruption or even cell lysis. The LCFAs structure influences its inhibitory effect. LCFAs with longer carbon chains affect microbial activity more than LCFAs with shorter carbon chains. The inhibition of LCFAs is positively correlated with the number of double bonds in the LCFAs.[136]

Among the factors that can counteract the inhibitory influence of the presence of organic compounds on the process of methane fermentation is the adaptation of the microorganisms engaged in this process. Studies based on the degradation of oleic acid in bioreactors with immobilized substrate showed that acclimation of microorganisms had a positive influence on their resistance to oleate and improved the ability to degrade the substrate. It was also demonstrated that addition of calcium diminishes the inhibitory effect of long chain fatty acids by forming insoluble salts.[138]

3 | SUMMARY

Methane fermentation is an efficient method of processing waste, as it enables us to reduce the volume of waste and to generate renewable energy such as biogas. Depending on the origin of the waste, its composition can include inhibitory and toxic substances. All the factors described in this paper that inhibit the course of methane fermentation are often mutually connected. Thus, it is extremely important to establish proper parameters in a bioreactor's fermentation tank so as to ensure the highest possible efficiency of this process. This review paper is based on 168 articles, of which 15.5% had been published prior to the year 2000 (Figure S1). Many subsequent publications on the inhibition of the methane fermentation process are still based on outmoded data. Furthermore, nearly all cited papers deal with the effect of inhibitors on parameters of biogas generation; meanwhile, our knowledge about inhibition of the microbiota engaged at particular steps of methane fermentation is still rather scanty. Ammonia is the only type of a methane fermentation inhibitor for which the literature provides information on the impact on the activity of microorganisms involved in the process, as well as changes in the structure of their population. In the case of the other inhibitory compounds mentioned in this review, these data are very scarce and require verification (ions of light metals, heavy metals, antibiotics, ethylene and acetylene, chlorophenols), or the literature does not provide any information about them (sulfides, halogen aliphatic hydrocarbons, aliphatic nitro compounds, long-chain fatty acids). Therefore, more research is required in order to identify the influence of inhibitory and toxic substances present in waste on the activity of methane fermentation microbiota, which will allow us to ensure the optimal conditions for the growth and development of these microorganisms. Such research should rely on some modern research tools, for example, NGS sequencing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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OŚWIADCZENIE

Oświadczam, że w pracy:

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mój wkład polegał na analizie oraz interpretacji danych literaturowych dotyczących podejmowanego zagadnienia. Samodzielnie wykonałam przegląd literatury oraz przygotowałam pierwszą wersję manuskryptu. Graficznie opracowałam figury i tabele oraz uczestniczyłam w korekcie pracy przed złożeniem do druku.

Małgorzata Czatzkowska

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mój wkład polegał na opracowaniu koncepcji artykułu, jego tematyki i kształtu. Dodatkowo, nadzorowałam tworzenie manuskryptu, proces recenzji oraz korektę pracy przed złożeniem do druku.

Monika Harnisz

Olsztyn, 12.07.2023

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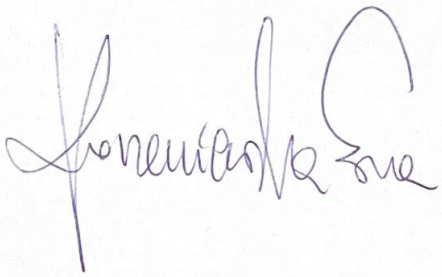
mój wkład polegał na udzieleniu konsultacji i opiece nad tworzeniem pierwszej wersji manuskryptu.



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OŚWIADCZENIE

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mój wkład polegał na udziale w dyskusji dotyczącej tematyki i kształtu artykułu.

Załącznik nr 2

Appendix 2

Czatzkowska M., Harnisz M., Korzeniewska E., Rusanowska P., Bajkacz S., Felis E., Jastrzębski J. P., Paukszto Ł., Koniuszewska I.

The impact of antimicrobials on the efficiency of methane fermentation of sewage sludge, changes in microbial biodiversity and the spread of antibiotic resistance

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Research Paper

The impact of antimicrobials on the efficiency of methane fermentation of sewage sludge, changes in microbial biodiversity and the spread of antibiotic resistance

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ABSTRACT

The study was designed to simultaneously evaluate the influence of high doses (512–1024 μg/g) the most commonly prescribed antimicrobials on the efficiency of anaerobic digestion of sewage sludge, qualitative and quantitative changes in microbial consortia responsible for the fermentation process, the presence of methanogenic microorganisms, and the fate of antibiotic resistance genes (ARGs). The efficiency of antibiotic degradation during anaerobic treatment was also determined. Metronidazole, amoxicillin and ciprofloxacin exerted the greatest effect on methane fermentation by decreasing its efficiency. Metronidazole, amoxicillin, cefuroxime and sulfamethoxazole were degraded in 100%, whereas ciprofloxacin and nalidixic acid were least susceptible to degradation. The most extensive changes in the structure of digestate microbiota were observed in sewage sludge exposed to metronidazole, where a decrease in the percentage of bacteria of the phylum *Bacteroidetes* led to an increase in the proportions of bacteria of the phyla *Firmicutes* and *Proteobacteria*. The results of the analysis examining changes in the concentration of the functional methanogen gene (*mcr*A) did not reflect the actual efficiency of methane fermentation. In sewage sludge exposed to antimicrobials, a significant increase was noted in the concentrations of β-lactam, tetracycline and fluoroquinolone ARGs and integrase genes, but selective pressure was not specific to the corresponding ARGs.

1. Introduction

The activated sludge technology is a popular biological method of wastewater treatment, and the produced sewage sludge, including surplus activated sludge, has to be appropriately managed. Sewage sludge is stabilized mainly through anaerobic treatment (Grobelak et al., 2019). During methane fermentation, complex organic matter is decomposed under anaerobic conditions, which leads to the release of methane and carbon dioxide. Methane fermentation is an alternative method of energy generation which consists of (1) hydrolysis, (2) acidogenesis, (3) acetogenesis and (4) methanogenesis. Each of its four interdependent stages require specific microorganisms that are responsible for biogas production (Swiatczak et al., 2017; Czatzkowska et al., 2020).

Methane fermentation of sewage sludge can be affected by various factors leading to low methane production efficiency and destabilization of the entire process. These disruptions can be caused by the low activity of selected microbial groups that participate in anaerobic digestion. The efficiency of methane fermentation is compromised mainly by the presence of inhibitory substances in sewage sludge (Meegoda et al., 2018; Scarlat et al., 2018). Various chemical substances that decrease the efficiency of methane fermentation have been researched. Antimicrobials are most widely used as organic inhibitors of methane fermentation (Chen et al., 2008, 2014; Czatzkowska et al., 2020). Each year, thousands of tons of antimicrobial substances and their

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metabolites are excreted by humans and animals, and they are carried with sewage to wastewater treatment plants (Boxall, 2004; Bound and Voulvoulis, 2005). Wastewater-borne antimicrobials are also detected in sewage sludge. Sulfonamides, quinolones and MLS drugs (macrolides-lincosamides-streptogramins) are most frequently identified in sewage sludge (Kumar et al., 2005). Antimicrobials can affect microorganisms in various ways, including through the inhibition of RNA transcription, DNA replication or ATP generation. This group of inhibitors can also retard protein translation, cell division, and the synthesis of nucleotides and the cell wall (Kohanski et al., 2010; Schmidt et al., 2018). The prevalence of antimicrobial substances in bioreactors can directly influence methane production (Rusanowska et al., 2018).

Despite considerable progress in bioreactor engineering and design, the microbiological aspects of fermentation continue to pose a challenge. The efficiency of anaerobic treatment is most often compromised by the accumulation of volatile fatty acids (VFAs), which are one of the products of methane fermentation and disrupt the equilibrium between microbial groups that participate in the process (Chen et al., 2008). A knowledge of microbial communities that participate in methane fermentation can be useful in predicting and preventing system failures (Amani et al., 2010; Alvarado et al., 2014). The *mcr*A gene has been proposed to monitor the activity of methanogenic microorganisms (Alvarado et al., 2014). This gene encodes the methyl-coenzyme M reductase, and it is characteristic of methanogenic metabolism. The *mcr*A gene was considered to be a molecular marker for this group of microorganisms (Aydin et al., 2015). Only one or two copies of the *mcr*A gene have been found in sequenced methanogen genomes, as opposed to an average of four copies of *16S r*RNA. The above suggests that the *mcrA* gene is a more accurate tool for evaluating the number of methanogens (Lee et al., 2009; Koniuszewska et al., 2021).

Methane fermentation is one of the main strategies for stabilizing sewage sludge from municipal wastewater treatment plants (WWTPs). Antibiotic misuse in human and veterinary medicine contributes to the emergence of bacteria that possess one or more antibiotic resistance genes (ARGs). Antibiotics have been widely used in recent decades, and ARGs constitute a new category of pollutants that endanger public safety and health (Becerra-Castro et al., 2015; Xu et al., 2017; Barancheshme and Munir, 2019). Microorganisms are capable of exchanging genetic structures, including ARGs, in a process known as horizontal gene transfer (HGT). Wastewater contain subinhibitory concentrations of antimicrobial substances, and WWTPs also create a supportive environment for HGT and the selection of bacteria with specific ARGs (Sun et al., 2019). Numerous pharmaceuticals have also been detected in wastewater sewage sludge (Bisognin et al., 2019; Yang et al., 2020). The fate of antimicrobials during anaerobic digestion was analyzed in several studies (Spielmeyer et al., 2015; Oberoi et al., 2019). Research has demonstrated that biodegradation and biosorption are the dominant antimicrobial removal pathways during anaerobic digestion (Oberoi et al., 2019; Zhou et al., 2020). However, changes in the concentrations of various classes of antimicrobials during methane fermentation of sewage sludge have not been sufficiently investigated under identical experimental conditions. The present study aims to fill in this knowledge gap.

The efficiency of methane fermentation in removing ARGs from sewage sludge has been extensively explored in the literature (Wang et al., 2019; Redhead et al., 2020). However, the results of research studies are often contradictory. In general, ARGs concentration are affected by numerous factors, including the thermal conditions of the anaerobic process. Some authors have concluded that anaerobic treatment under thermophilic conditions is more effective in eliminating ARGs and integrase-encoding genes than fermentation under mesophilic conditions (Ghosh et al., 2015; Diehl and LaPara, 2010). In turn, other researchers have argued that individual ARGs are equally or even more effectively removed during mesophilic anaerobic digestion (Ma et al., 2011; Huang et al., 2019). Research has also shown that individual trends are not specific to particular ARGs. In a study by Zhang et al. (2015), sewage sludge was subjected to anaerobic digestion under various conditions. The concentrations of *aad*A, *mac*B and *sul*1 genes increased under thermophilic conditions, whereas the frequency of *sul*1 and *tet*M genes increased under mesophilic conditions. Several authors have also investigated the impact of antimicrobials on ARGs in sewage sludge processed in bioreactors. Xu et al. (2019) and Ni et al. (2020) reported that the presence of tetracyclines, sulfonamides and MLS antimicrobials in sewage sludge increased the diversity and abundance of ARGs during anaerobic digestion. However, the effects of all antimicrobial groups on ARGs have never been studied comprehensively under identical experimental conditions.

Therefore, this study was designed to evaluate the influence of the most widely used antimicrobial drugs (ECDC 2018) on the efficiency of anaerobic digestion of sewage sludge. The antimicrobial classes selected for the experiment (nitroimidazoles, β-lactams, tetracyclines, sulfonamides and fluoroquinolones) could affect various groups of microorganisms that participate in all four stages of anaerobic treatment. To the best of our knowledge, this is the first study to analyze the impact of metronidazole on methane fermentation. Metronidazole is used in the treatment of infections caused by anaerobic bacteria, including species of the phylum *Bacteroidetes*, which is one of the largest bacterial phyla involved in methane fermentation. Moreover, we believe that the impact of the all most common classes of antimicrobial drugs on methane fermentation has never been investigated concurrently to date.

Considering the above, the aim of the present study was to determine the impact of selected antimicrobials on: (1) the efficiency of methane fermentation, (2) qualitative and quantitative changes in microbial consortia responsible for methane fermentation, (3) the presence of methanogenic microorganisms, and (4) the fate of ARGs and the spread of antibiotic resistance (AR). The efficiency of antimicrobials degradation during anaerobic digestion was also evaluated. The results of the study provide new insights into the role of antimicrobials on methane fermentation, both from the technological and the microbiological point of view. The present findings also highlight the risks associated with the spread of AR.

2. Materials and methods

2.1. Substrate and inoculum

Sewage sludge from a municipal WWTP in Olsztyn, Poland was used as the substrate in this study. The analyzed sewage sludge had the following characteristics: 47.3 ± 1.6 mg total solids (TS)/g, 34.7 ± 0.5 mg volatile solids (VS)/g, pH of 6.66 ± 0.2, 1.6 ± 0.4 mg total phosphorus (TP)/g TS, and 3.3 ± 0.8 mg total nitrogen (TN)/g TS. Anaerobically digested sludge from a fermentation tank in the analyzed WWTP was used as the inoculum. The inoculum had the following characteristics: 55.7 ± 1.5 mg TS/g, 42.8 ± 2.3 mg VS/g, pH of 8.01 ± 0.4, 0.6 ± 0.2 mg TP/g TS, and 2.1 ± 0.4 mg TN/g TS.

2.2. Methane fermentation

The effect of metronidazole (MET), amoxicillin (AMO), cefuroxime (CEF), oxytetracycline (OXY), doxycycline (DOXY), sulfamethoxazole (SMO), ciprofloxacin (CIP) and nalidixic acid (NA) was determined with the Automatic Methane Potential Test System II (Bioprocess Control) in batch experiments. Antimicrobial doses were determined experimentally. Low doses were tested initially in our previous study, but they did not influence biogas production (Rusanowska et al., 2018). For this reason, high doses of antibiotics were used to obtain a pronounced response of the microorganisms involved in the process. The antimicrobial doses applied in bioreactors are presented in Table 1. Antimicrobial drugs that are most widely used in human medicine (ECDC 2018) were selected for the study. In the control bioreactor (CONT), the substrate was subjected to methane fermentation without the addition of antibiotics. Fermentation was carried out in 250 mL reactors filled with

Table 1
Type and concentration of antimicrobial substances [μg/g].

Type of antimicrobial substance	Group of antimicrobial substances	Concentration of antimicrobial substances [μg/g]
Metronidazole (MET)	Nitroimidazoles	512
Amoxicillin (AMO)	β-lactams	1024
Cefuroxime (CEF)		512
Oxytetracycline (OXY)	Tetracyclines	1024
Doxycycline (DOXY)		1024
Sulfamethoxazole (SMO)	Sulfonamides	512
Ciprofloxacin (CIP)	Fluoroquinolones	512
Nalidixic acid (NA)		512
CONTROL		–

25 g of the substrate and 175 g of the inoculum. The initial organic loading rate was 5 g VS/L. Methane fermentation was conducted for 40 days under mesophilic conditions at 37 °C. The contents were stirred every 10 min for 30 s at a speed of 100 rpm. Anaerobic conditions were achieved by continuous flushing of pure nitrogen through the sludge. The experiments were performed in duplicate. Methane production was measured with the AMPTS II kit. Biogas quality was analyzed in a gas chromatograph equipped with a thermal conductivity detector (GC-TCD) (Agilent 7890 A). Before and after fermentation, the samples were analyzed to determine the content of VFAs, pH, FOS/TAC ratio (the TAC value denotes the estimated buffer capacity of the sample, and the FOS value denotes the VFA content), and the content of TS, VS, TN and TP.

2.3. Analytical procedures

The content of VFAs was determined with the use of a previously described method (Kisielewska et al., 2015). The FOS/TAC ratio was determined with TitraLab AT1000 Series Titrator (Hatch). The content of TS and VS in biomass samples was determined in a gravimetric analysis. The content of TN and TP in mineralized samples was determined in Hach Lange cuvette tests. The measurements were performed in duplicate.

2.4. Determination of antimicrobial concentrations in bioreactors

At the completion of methane fermentation (after 40 days), representative digestate samples were collected from each bioreactor and analyzed to determine the concentrations of each antimicrobial substance.

2.4.1. Standards and chemicals

The analytical standards for MET, AMO, CEF, OXY, DOXY, SMO, CIP and NA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hypergrade water, acetonitrile and formic acid for LC–MS analyses were supplied by Merck (Darmstadt, Germany).

Analytical-grade acetonitrile, methanol, ethyl acetate and 25% ammonium hydroxide solution were supplied by CHEMPUR (Piekary Śląskie, Poland). Analytical-grade acetic acid was purchased from POCH S.A. (Gliwice, Poland), and citrate buffer (pH = 4.0) was supplied by Merck (Darmstadt, Germany).

2.4.2. Sample preparation

The samples were centrifuged (15 min, 4000 rpm) to separate liquid and solid fractions. Liquid fractions were filtered (0.22 μm) before LC-MS/MS, and some samples were diluted (when quantification results exceeded the limits on the calibration curves). Solid fractions were frozen at a temperature of − 22 °C and freeze-dried to completely remove the remaining liquid fraction. Freeze-dried solid fractions were homogenized. Approximately 0.2 g of the homogenized sample was weighed, and the analytes were isolated by solid-liquid extraction (SLE) with different solvents (for each of the analyzed antimicrobials). The samples containing MET, SMO and NA were extracted in three steps. The extraction solvents were 2% ammonium hydroxide in methanol, 2% acetic acid in methanol, and methanol. Samples containing AMO and CEF were extracted in two steps. The extraction solvents were 2% acetic acid in ethyl acetate and 2% acetic acid in methanol. Samples containing DOXY, OXY and CIP were extracted with a mixture of citrate buffer (pH = 4.0) and acetonitrile (1:1, *v/v*).

The analytes were isolated with 10 mL of each extraction solvent by shaking for 1 h at 750 rpm (Vibramax 100, Heidolph Instruments GmbH & Co.). The extracted samples were filtered, the supernatants from each extraction step were combined (from procedures involving two and three steps) and evaporated to dryness. The residues were dissolved in 15 mL of 5% methanol in water and purified by solid-phase extraction (SPE).

The extract was purified and concentrated by SPE (CHROMABOND® SPE-system, Macherey-Nagel, Germany) with the Oasis HLB cartridge (500 mg/6 mL, Waters). The cartridge was preconditioned with 6 mL of methanol, followed by 6 mL of water, and the extract was passed through the cartridge at 1 mL/min. The cartridge was dried for 15 min, and the analytes were eluted with 6 mL of 0.1% acetic acid in methanol. The eluate was evaporated to dryness, dissolved in 1 mL of 0.1% acetic acid in methanol, and filtered (0.22 μm) before LC-MS/MS analysis. All extractions were performed in triplicate.

2.4.3. Liquid chromatography-mass spectrometry

Liquid chromatography was performed in a Dionex UHPLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a degasser, a binary pump, an autosampler and a column oven. Antimicrobials were analyzed on a ZORBAX SB-C3 column (150 mm × 3.0 mm, 5 μm, Agilent Technologies, USA). The mobile phases were: (A) 0.1% formic acid in water, and (B) acetonitrile with the following gradients (for positively ionized compounds): 10–45% (B) for 3 min (flow rate of 0.8 mL/min); 45–90% (B) for 0.1 min; 90–100% (B) for 2 min, and 5.1–8 min. Mobile phase B was maintained at 10% (flow rate of 1.0 mL/min). The chromatographic separation of negatively ionized compounds involved the following gradients: 20–90% (B) for 2 min, and 2.1–4 min. Mobile phase B was maintained at 20% (flow rate of 1.0 mL/min). Column temperature was 30 °C and injection volume was 2 μL in both separation processes.

The LC system was coupled to a triple-stage quadrupole mass spectrometer (4500 QTrap, ABSciex) with an electrospray ion (ESI) source (TurboV, ABSciex). Nitrogen was used as the nebulizer gas. The MS/MS settings and the parameters of the ESI source were optimized by manual infusion with a syringe pump and by the flow injection of standards. Electrospray ionization voltage was set at + 4.0 kV or − 4.0 kV. Curtain and collision gas pressure were set at 20 and 4 psi, respectively, and the source heater was set at 500 °C. Nebulizer gas (ion source gas 1) pressure was 60 psi, and heater gas (ion source gas 2) pressure was 50 psi. The analytes were identified based on their chromatographic characteristics as well as the fragmentation products of multiple reaction monitoring (MRM). The analytes' specific retention times, two characteristic ion transitions, and the specific intensity ratios of the produced ions (< 20% deviation relative to analytical standard ratios) were compared with the corresponding standard parameters. Data were processed in Analyst software (version 1.5.1).

2.4.4. Calculation of the solid-liquid distribution coefficient and the removal efficiency of the selected antimicrobials

The solid-liquid distribution coefficient (K_d) can be used to describe the sorptive properties of a compound. It is assumed that under equilibrium conditions in two-component systems (i.e. liquid-suspended solid), the concentration of a given compound adsorbed onto suspended solids (X_p) is proportional to its concentration in the liquid phase (S). The above is expressed by Eq. (1) (Ternes and Joss, 2006):

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

where:

K_d solid-liquid distribution coefficient, L/gSS.

X_p concentration of the compound adsorbed onto suspended solids, per unit of dry weight of suspended solids, μg/gSS.

S concentration of the compound dissolved in the liquid phase, μg/L.

X concentration of the compound adsorbed onto suspended solids, per unit of reactor volume, μg/ L.

X_{SS} concentration of suspended solids in the reactor per L, gSS/L.

Under equilibrium conditions, the total concentration of the compound in the reactor (C) can be calculated with the use of Eq. (2) (Ternes and Joss, 2006):

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

where:

C total concentration of the compound in the entire reactor, μg/ L.

The parameters determined in Eq. (2) were used to calculate the total removal efficiency (R) of a given compound during digestion with the use of Eq. (3):

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

where:

R total removal efficiency, %.

C_0 total initial concentration of the compound in the reactor, μg/ L.

C_t total concentration of the compound in the reactor after digestion, μg/L.

2.5. Isolation of genomic DNA

Representative digestate samples were collected from each bioreactor at the completion of methane fermentation. Samples of 1 mL each were transferred to 2 mL Eppendorf tubes, centrifuged (9000 rpm, 4 °C/ 15 min), and the supernatant was discarded. The process was repeated. DNA was isolated from the pellet with the FastDNA™ Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. The quality and quantity of the acquired genetic material was verified with a spectrophotometer (Multiskan Sky, Thermo Scientific). DNA was isolated in two replicates in a total amount of 150 μL and was stored at − 20 °C until further analysis.

2.6. Genomic DNA sequencing

The diversity of the microbiome was analyzed by high-throughput sequencing of the hypervariable region V3-V4 of 16 S rRNA gene with the Illumina MiSeq instrument (Illumina Inc. USA) and the MiSeq Reagent v3 Kit (Macrogen, Korea) with 2 × 250 bp paired-end reads and primers 341F (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG) and 785R (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) designed by Klindworth et al. (2013). DNA quality was controlled in FastQC version 0.11.8 (Andrews, 2010) and Qualitative Insights Into Microbial Ecology (QIIME2) version 2019.4 (https://qiime2.org/). Adapter trimming and quality filtration were performed in both Trimmomatic version 0.38 (Bolger et al., 2014) and QIIME2. Noise was eliminated, reads were merged, and chimeras were removed with the DADA2 tool (Callahan et al., 2016) in QIIME2 environment. Sampling depth was set to 17,200. The 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/).

2.7. Quantification of methanogen-specific genes and ARGs

Various primer sets targeting different hypervariable regions of the 16S rRNA gene have been developed in an attempt to improve the effectiveness of sequencing the genetic material. Specific primers increase the specificity of sequences characteristic of the analyzed microbial groups (Ju et al., 2016). *Archaea*-specific primers are recommended to improve the quantification and identification of microorganisms belonging to this domain (Yu et al., 2005). In the present study, efforts were made to identify sequences specific to bacteria. For this reason, methanogenic microorganisms were analyzed by Real-Time PCR (qPCR). The concentrations of genes specific to the families *Methanosarcinaceae* (*MSC*) and *Methanosaetaceae* (*MST*) of the genus *Methanosarcinales* were determined in the qPCR assay. Based on the results of preliminary analyses, the presence of other genera of the domain *Archaea* (*Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*) was ruled out in the evaluated samples.

Before gene quantification, standard curves were plotted based on serial dilutions of samples with known copy numbers of the analyzed genes. Amplicons were cloned from positive controls in vector pCR2.1-TOPO (Invitrogen, Massachusetts, USA). The presence of genes characteristic of the families *MSC* and *MST* of the domain *Archaea*, the methyl-coenzyme M reductase (*mcr*A) gene that catalyzes the last phase of methanogenesis, and 16S rRNA was determined by qPCR. The same assay was conducted to determine the concentration of genes encoding resistance to β-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA}, *cfx*A), macrolide-lincosamide-streptogramin (MLS) antibiotics (*erm*F, *lin*A, *mef*A), quinolones (*qep*A, *aac(6')-Ib-cr*), tetracyclines (*tet*A, *tet*M, *tet*Q), sulfonamides (*sul*1), and the gene encoding the multidrug resistance efflux pump (*bex*A). The concentrations of genes encoding class 1 and 2 integrases (*int*l1, *int*l2) were also determined in the qPCR assay. Reaction conditions and the applied primers are described in detail in Table S1 (Supplementary materials). Digestate samples collected from each bioreactor were analyzed to determine the presence of the tested ARGs and to describe the effect of each antimicrobial substance on the spread of AR.

All qPCR assays were conducted in triplicate in the Roche Light Cycler 480 (Roche Applied Science, Denver, CO, USA) to ensure repeatability. Each reaction involved 15 μL of the reaction mix composed of 0.8 μL of gDNA, 0.75 μL of forward and reverse oligonucleotide primers each (with a concentration of 10 μM), 7.5 μL of SYBR Green I Master Mix (Roche Diagnostics) and sterile water. Both negative (without the DNA matrix) and positive controls (with known copy numbers of the analyzed genes) were used.

2.8. Statistical analysis

Methane fermentation parameters were analyzed for homogeneity of variance in Levene's test, and the significance of differences was determined in Tukey's HSD test. Differences were considered significant at $p < 0.05$. The rate constants of methane production (k) were determined by non-linear regression by plotting the volume of methane produced per the amount of the added VS versus time. The value of *k* determines the shape of the reaction curve. The methane production rate was calculated as the product of *k* and the maximum volume of methane produced per the amount of the added of VS. The model's fit to the experimental data was assessed by calculating the value of R^2 (Statistica 13.1, Statsoft).

The results of sequencing data analysis were processed in Microsoft Excel 2013. The differences in the size of bacterial populations between bioreactors were determined by hierarchical cluster analysis with the use of Ward's method. Cluster analyses were performed in the R environment (R 3.5.2), and the results were presented in a heatmap.

The relative concentrations of the evaluated ARGs, integrase genes,

and selected methanogen genes in digestate samples from each bioreactor were compared in Spearman's rank-order correlation analysis and the non-parametric Kruskal-Wallis test for several independent samples (Statistica 13.1, Statsoft). The analyses involved the results of three qPCR replicates. Diagrams and the remaining calculations were performed in Microsoft Excel 2013 and RStudio (v. 3.5.2).

The relationships between microbial biodiversity in bioreactors, the concentrations of ARGs and the remaining genes, the accumulation of VFAs, and biogas production were visualized in a principal component analysis (PCA) (Statistica 13.1, Statsoft).

3. Results and discussion

3.1. The impact of antimicrobials on the efficiency of methane fermentation

Anaerobic digestion can be described by the sequential steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis. The products of each step constitute the substrates for successive steps, and the concentration of VFAs, acetic acid and methane after fermentation indicate which step was less efficient or was inhibited. In the current study, methane fermentation was significantly inhibited in sewage sludge containing MET, AMO, OXY, DOXY, and CIP. Metronidazole was the most potent inhibitor, and its effects were statistically significant (Fig. S1). Less methane was produced in the reactor where MET was added to the substrate than in the reactor containing only anaerobic sludge (Table 2). This observation could be explained by the fact that MET is one of the mainstay drugs for the treatment of anaerobic infections (Rusanowska et al., 2018). Ansorg et al. (2003) reported that MET exerted an inhibitory effect on the fecal methanogenic community characteristic of the human digestive system. However, MET's effects on biogas production in bioreactors have not been studied to date. In this study, the introduction of MET to the reactor lowered the methane content of biogas to 12.8 ± 4.0% and decreased the methane production rate to 21.85 L kg^{-1} d^{-1} (Fig. S1). The inhibition of methanogenesis led to the accumulation of VFAs. The concentrations of seven out of the nine analyzed VFAs were higher in sewage sludge supplemented with MET than in the sample from the control bioreactor. This observation was confirmed by the statistical analysis. Acetic acid was present in the highest concentration. Volatile fatty acids are the main products of protein and carbohydrate hydrolysis during methane fermentation (Lu et al., 2014). The presence of VFAs also points to the inhibition of acetogenesis because propionic acid, isobutyric acid, isovaleric acid and isocaproic acid (5 g L^{-1}) were not used to produce acetic acid (Table 2). The rise in VFA content also significantly increased the FOS/TAC ratio ($p < 0.05$) (Table S2). Stable biogas production usually takes place at a pH between 7 and 8 and a FOS/TAC ratio below 0.4 (Drosg, 2013). Sludge samples from the reactor containing MET and the control reactor did not differ significantly in the content of nitrogen, phosphorus, TS and VS, or pH.

Amoxicillin and CIP were less potent inhibitors of methane fermentation than MET. The addition of AMO to the reactor significantly reduced the methane content of biogas, whereas the introduction of CIP decreased methane production (Table 2). Amoxicillin reduced the methane content of biogas to around 44%, and CIP decreased methane production by around 40% relative to the control reactor. Zhang et al. (2019) analyzed tylosin, CIP and sulfadimidine, and found that CIP induced the greatest decrease (approx. 22%) in methane production. In the present study, both AMO and CIP increased VFA levels (Table 2). Interestingly, AMO significantly increased the concentration of butyric acid to 253.00 ± 15.89 g L^{-1}. Acids such as butyric acid are produced during acidogenesis and are used in acetogenesis for the production of acetic acid. Acetic acid was also present in the digestate supplemented with AMO (0.83 ± 0.17 g L^{-1}), which indicates that it was not used for methane production. These results suggest that AMO inhibits acetogenesis and methanogenesis. Similar observations were made by Sun et al. (2009). However, CIP contributed to a significant increase in the concentrations of acetic acid (7.58 ± 0.82 g L^{-1}) and isovaleric acid (2.01 ± 0.41 g L^{-1}) whose presence is also indicative of the inhibition of acetogenesis and methanogenesis. Ciprofloxacin and AMO did not influence methane fermentation parameters, excluding the FOS/TAC ratio which was somewhat elevated in the presence of CIP (Table S2). However, the observed difference was not statistically significant.

Oxytetracycline and DOXY significantly influenced methane fermentation of sewage sludge. Methane production in the bioreactor containing OXY was 33% lower than in the control bioreactor, and the methane production rate was 62.07 L kg^{-1} d^{-1} (Fig. S1). Tian et al. (2018) observed that OXY inhibited acidogenesis and methanogenesis during anaerobic digestion at a concentration of approximately 18, 000 mg kg^{-1} (OTC dose, 1000 mg L^{-1}). In the present study, acetogenesis and methanogenesis were probably inhibited because methane generation efficiency was lower in reactors containing these antimicrobials than in the control reactor, and acetic acid was present in the digestate after anaerobic digestion (Table 2). Doxycycline suppressed methane production by approximately 38% compared to the control sludge, and the methane production rate was lowest in the bioreactor containing DOXY at 53.02 L kg^{-1} d^{-1} (Fig. S1) (except for the reactor with MET). The concentrations of VFAs did not increase significantly in

Table 2
The results of methane fermentation in bioreactors containing antimicrobials, the control reactor and sludge samples.

Antibiotic	Methane production (L/kg VS)	Methane content in biogas (%)	VFA concentration								
			Acetic acid (g/L)	Propionic acid (g/L)	Isobutyric acid (g/L)	Butyric acid (g/L)	Isovaleric acid (g/L)	Valeric acid (g/L)	Isocaproic acid (g/L)	Caproic acid (g/L)	Heptanoic acid (g/L)
MET	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
AMO	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
CEF	209.0±11.3	69.8±0.6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
OXY	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
DOXY	169.1 *±3.6	69.8±0.6	0.33±0.13	0.08±0.02	0.06±0.01	0.30±0.10	0.34±0.11	0.27±0.09	0.08±0.02	0.01±0.01	0.00
SMO	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
CIP	164.4 *±3.6	67.3±0.9	7.58 *±0.82	0.30±0.10	0.25±0.13	0.98±0.21	2.01 * ±0.41	0.12±0.06	0.06±0.05	0.05±0.02	0.01±0.01
NA	210.6 ±7.5	68.7±0.5	0.60±0.11	0.23±0.1	0.27±0.14	0.56±0.21	0.59±0.18	0.26±0.09	0.46±0.19	0.01±0.01	0.03±0.02
CONTROL	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
SLUDGE	69.1 *±11.4	65.6±1.3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* statistically significant changes relative to control.

the presence of DOXY. Doxycycline exerted a similar effect on anaerobic digestion to OXY. In a study by Sanz et al. (1996), DOXY reduced methane production by 25–45%, and the observed decrease was directly proportional to an increase in DOXY concentration in a range of 10 mg L^{-1} and 100 mg L^{-1}. Doxycycline inhibited mainly butyric-acid-consuming bacteria. In the present study, the effect of DOXY on VFAs was difficult to observe because all VFAs were present in low concentrations in the digestate. However, DOXY probably decreased the consumption of VFAs and, consequently, lowered the rate of methane production. Nalidixic acid exerts a similar effect, but methane generation was not significantly inhibited and was around 22% lower than in the control reactor. Cefuroxime and SMO did not significantly influence methane fermentation of sewage sludge.

A detailed interpretation of the influence of antimicrobials on changes in the consortia of microorganisms has been described in subchapters 3.3. and 3.4.

3.2. Removal of antimicrobials during anaerobic digestion

In an analysis of the efficiency of removal of eight selected antimicrobial substances during anaerobic digestion, AMO was the only substance that was not detected in the solid or liquid fraction of digested samples. Two other antimicrobials, MET and SMO, were not detected in the liquid fraction, and their concentrations in the solid fraction were determined at 0.26 μg/g and 2.91 μg/g, respectively. The remaining compounds were identified after anaerobic digestion in both the solid fraction and the liquid fraction. The results were used to calculate parameter K_d (with the use of Eq. (1)) and determine the sorption affinity of the tested antimicrobials for solid phase particles (Table 3).

It is generally assumed that sorption affinity for suspended solids is high in substances where log $K_d \geq 2.5$ (Ternes and Joss, 2006; Carballa et al., 2008). In the tested system, NA, DOXY and SMO displayed such strong affinity for the solid phase. According to Carballa et al. (2008), the values of K_d in digested sludge are not always similar to those noted in primary and secondary sludge because digested sludge differs in composition, structure and morphology. Sorption affinity is influenced by the properties of the tested substance, the mechanism of sorption, and the applied sludge pre-treatment method. For example, the log value of K_d for SMO in the experiment was similar to that calculated for secondary and digested sludge (Ternes and Joss, 2006; Carballa et al., 2008; Fernandez-Fontaina et al., 2013). In contrast, other researchers reported that CIP has very strong affinity for secondary and primary sludge (log K_d is equal to 4.4 and 3.4, respectively) (Ternes and Joss, 2006). CIP also shows affinity for the solid phase in digested sludge (log $K_d = 2.4$), but it is weaker than in secondary and primary sludge. The above can be explained by the sorption mechanism: at neutral pH, the CIP molecule is positively charged, and it is adsorbed due to electrostatic interactions between negatively charged surfaces of activated sludge microorganisms (Ternes and Joss, 2006). During anaerobic digestion, microbial cells are disintegrated and thermodynamic conditions change dynamically, which also induces changes in the interactions between CIP and the solid phase. However, Gonzalez-Gil et al. (2018a) demonstrated that a compound's affinity for the solid phase during anaerobic digestion does not significantly affect the efficiency with which selected micropollutants are removed because their biotransformation occurs in both liquid and solid phases. This observation was confirmed in the present study where the value of K_d was not correlated with removal efficiency. The total concentrations of the studied compounds in the experimental bioreactors and the removal efficiency of selected antibiotics (calculated with the use of Eqs. (2) and (3), respectively) are presented in Table 4. Only the antibiotic dose introduced to the reactor was considered in the calculations of the total initial concentrations of the tested compounds. Some of the drugs were present in the control reactor (and in the raw substrate), but their concentrations were relatively low, and they were disregarded in further analyses. Antimicrobial concentrations in raw substrate and the control reactor are presented in Supplementary materials (Table S3).

Metronidazole and CIP were least effectively degraded during anaerobic digestion, and their total removal efficiency was determined at 10.6% and 19.0%, respectively. Metronidazole, AMO, CEF and SMO were completely degraded. Sulfamethoxazole was also highly biotransformed in other studies (Gonzalez-Gil et al., 2018a; Gonzalez-Gil et al., 2018b). However, the results of this study suggest that such a high degree of SMO degradation could result from its biotransformation into a by-product with a very similar structure and the same molar mass. When transformed by the same fragment ions, the by-product of SMO differed only in retention time Fig. S2), and its analytical signal was very high. The structure of this by-product could not be clearly confirmed because the relevant analytical standard is not commercially available. However, the structure of the SMO by-product (TP1-SMO) was modeled based on the fragment ions obtained during LC-MS/MS Fig. 1). It could not be ascertained whether this transformation is reversible under given thermodynamic conditions.

According to some authors, biotransformation during anaerobic digestion is more likely to be limited by thermodynamic than kinetic constraints (Gonzalez-Gil et al., 2018b). However, the main transformation by-products of selected micropollutants should be identified, and the overall mass balance of individual antibiotics should be determined during and after the process to fully understand their impact. Such an approach would expand our understanding of the mechanism by which selected antibiotics influence biogas production in methanogenic conditions.

3.3. Diversity of methane fermentation microorganisms

High-throughput sequencing of the V3-V4 hypervariable region of the *16S rRNA* gene produced 863506 reads. Sequence reads were mapped to the reference sequences at the level of microbial phyla. The reads associated with OTUs that accounted for less than 1% of total reads

Table 3
K_d values calculated for selected antimicrobials in digested sewage sludge.

Compound	X_p, μg/gSS	S, μg/L	Moisture content, %	log K_d
MET	0.26	<LOD	97.9	2.3*
AMO	<LOD	<LOD	97.9	n.a.
CEF	0.30	53	97.8	0.7
OXY	1250.00	345000	97.8	0.6
DOXY	4550.00	13400	98.0	2.5
SMO	2.91	<LOD	98.0	2.5*
CIP	17400.00	72400	98.1	2.4
NA	20700.00	21200	97.9	3.0

X_p - concentration of compound adsorbed onto suspended solids; S - concentration of compound dissolved in the liquid phase; LOD – limit of detection; log K_d - log of the calculated value of K_d (expressed in L/kg);
n.a. – not available.
* - estimate based on LOD;

Table 4
Total concentration of antimicrobial substances in the experimental bioreactors and total removal efficiency of selected antimicrobials.

Compound	C_0, μg/L	C_t, μg/L	R, %
MET	512000	6.6	≈ 100.0
AMO	1024000	0.0	100.0
CEF	512000	59.5	≈ 100.0
OXY	1024000	372125.0	63.7
DOXY	1024000	103490,0	89.9
SMO	512000	68.0	≈ 100.0
CIP	512000	409960.0	19.9
NA	512000	457970.0	10.6

C_0 – total initial concentration of antimicrobial substance in the bioreactor; C_t – total concentration of antimicrobial substance in the bioreactor after digestion; R – total removal efficiency.

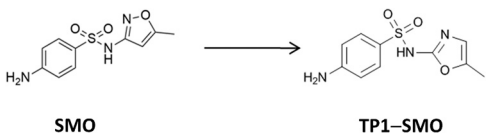

Fig. 1. Structure of the SMO by-product (TP1-SMO) synthesized during anaerobic digestion.

in a given sample were classified as "other" and combined with non-classified reads. The sequencing of the region non-specific for *Archaea* produced a small group of *Archaea*-specific reads. These reads were taken into account in the analysis because *Archaea* microorganisms play a key role in methane fermentation.

The 11 dominant bacterial phyla were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, *Tenericutes* and *Thermotogae,* which accounted for 84–91% of all sequences in the examined samples (Figs. 2, S3). The dominant phyla were represented mainly by bacteria characteristic of soil ecosystems (Janssen, 2006). These microorganisms rely on various sources of carbon, ranging from simple compounds to complex substrates (Chaves et al., 2019). An analysis of the sequences in the samples from all bioreactors revealed that *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were the dominant bacterial phyla. Similar observations were made in other studies analyzing microbial communities in sewage sludge from municipal WWTPs (Wan et al., 2011; Wang et al., 2012) and methane fermentation of sewage sludge (Walter et al., 2018). The antibiotic-induced changes in the structure of the above phyla were also presented at the level of bacterial orders (Fig. 3).

Bacteroidetes was the dominant phylum in sewage sludge samples collected from the control bioreactor (52.4%) as well as experimental bioreactors (39.9–59.6%). According to Hu et al. (2012), anaerobic conditions inside bioreactors contribute to an increase in *Bacteroidetes* species in sewage sludge, which could explain the dominant role of this phylum in digestate samples from all bioreactors. The phylum *Firmicutes* is represented mainly by classes *Bacilli* (including the order *Lactobacillales*) and *Clostridia* (including the order *Clostridiales*) which participate in hydrolysis and acidogenesis, as well as by microbial genera that are involved in acetogenesis (*Acetobacterium* sp., *Syntrophomonas* sp.). The phylum *Proteobacteria* includes microorganisms that are active in the first three stages of methane fermentation, as well as genera that participate in the production of acetic acid, such as *Syntrophobacter* and *Syntrophus* (belonging to the order *Syntrophobacterales*) (Galperin, 2013; Czatzkowska et al., 2020).

Metronidazole present in the experimental bioreactor significantly decreased the number of reads characteristics of *Chloroflexi*, *Spirochaetes* and *Thermotogae*, and it completely eliminated *Synergistetes* and *Tenericutes* bacteria. The greatest decrease in the number of *Bacteroidetes*-specific reads was also noted in digestate samples from the bioreactor containing this antimicrobial substance, where a significant increase in the proportions of *Firmicutes* (29.3%) and *Proteobacteria* (17.8%) was also observed relative to control (6% and 6.8%, respectively) (Fig. S3). The increase in the proportions of the orders *Lactobacillales* and *Clostridiales*, which are responsible for the transformations in the first two stages of methane fermentation, could explain the accumulation of VFAs and the inhibition of methane production because the percentage of other microorganisms that participate in subsequent stages of fermentation was reduced (order *Syntrophobacteriales*) (Fig. 3). It should be noted that exposure to AMO and CIP also significantly decreased the proportions of *Syntrophobacteriales*. These antimicrobials could also inhibit acetogenesis.

Amoxicillin was the only antimicrobial which increased the number of reads characteristic of *Bacteroidetes*. In the human gut, the phylum *Bacteroidetes* is represented mainly by the genus *Bacteroides* (Panda et al., 2014); therefore, high *Bacteroides* counts can be expected in WWTPs and, consequently, in sewage sludge. According to Veloo et al. (2019), *Bacteroides* sp. harbor a mechanism that confers resistance

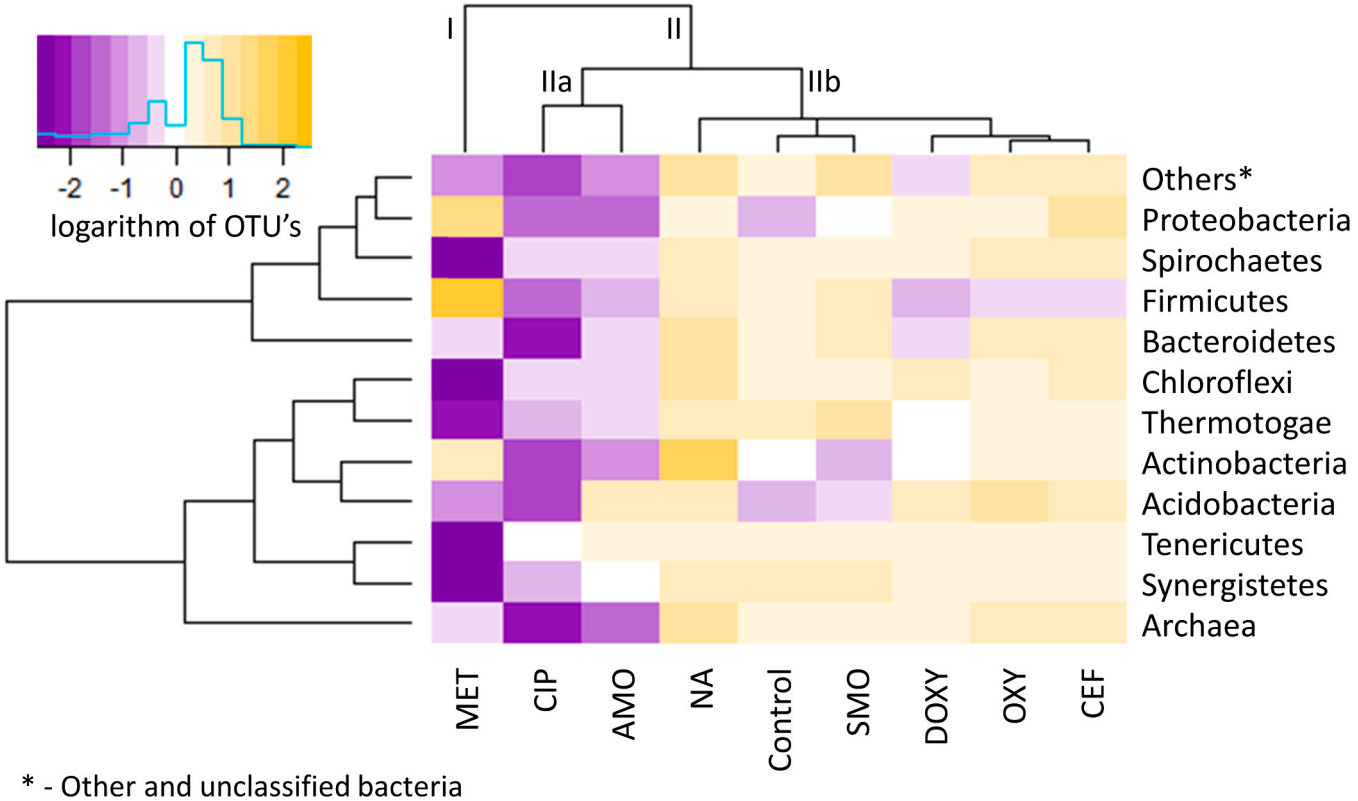

Fig. 2. Heatmap with the logarithmic number of OTU reads at phylum level in two groups (I-II) of digested sewage sludge samples in experimental and control bioreactors.

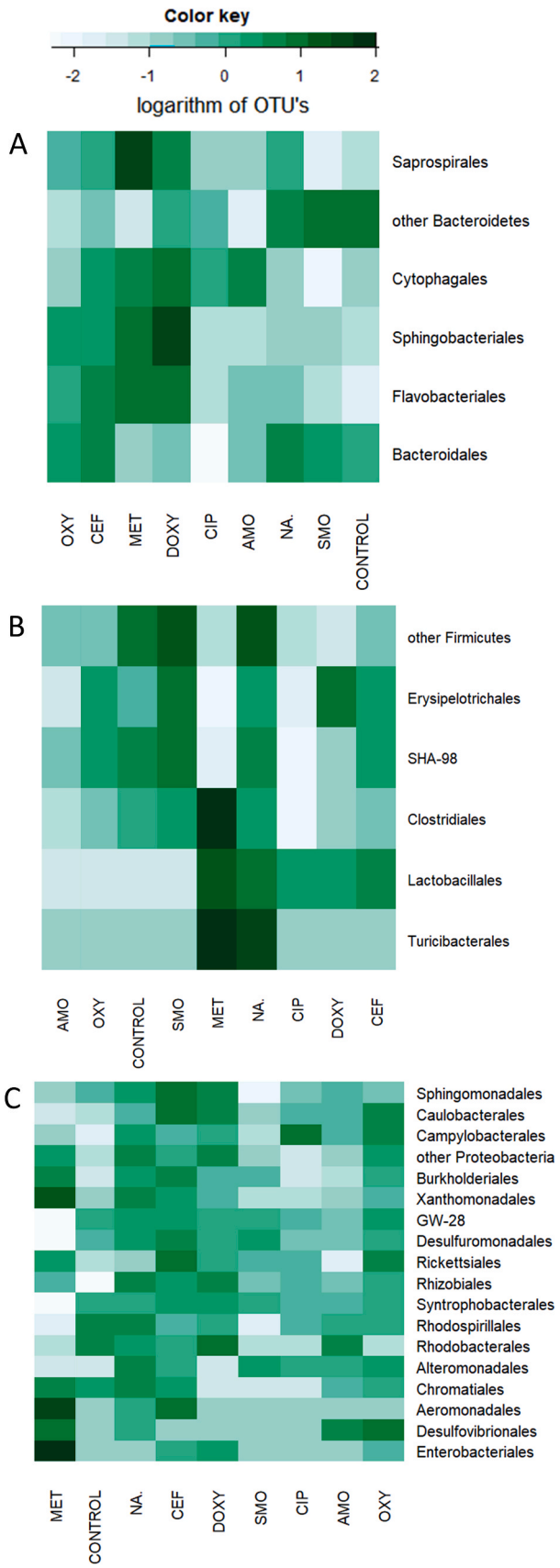

Fig. 3. Heatmap with the logarithmic number of OTU reads, presenting the changes in the structure of three dominant phyla (*Bacteroidetes* (A), *Firmicutes* (B) and *Proteobacteria* (C)) at the level of bacterial orders.

against β-lactam antibiotics. This observation could explain why the percentage of *Bacteroidetes* increased only in the presence of AMO, a β-lactam antibiotic.

Moreover, a considerable increase in the number of reads characteristic of *Proteobacteria* and *Spirochaetes* was noted in sewage sludge containing CEF, CIP and DOXY. The number of archaeal OTUs decreased significantly in sewage sludge exposed to AMO, CIP and MET (Fig. S3).

Sewage sludge microbiomes were grouped in hierarchical cluster analysis with the use of Ward's method (Fig. 2). Two main clusters were identified. The first cluster was composed of microorganisms present in sewage sludge exposed to MET (I), and the second cluster consisted of microorganisms that were identified in the remaining sewage sludge samples subjected to methane fermentation (II). The second cluster was further subdivided into two groups. The first group included microorganisms from bioreactors where AMO and CIP were added (IIa), whereas the second group consisted of sludge samples from the remaining bioreactors (IIb). Cluster IIb has been made up of samples characterizing by the lowest microbial biodiversity relative to the control sludge that was not exposed to antimicrobials. The above findings indicate that MET, AMO and CIP significantly influenced microbial biodiversity in the experimental bioreactors.

3.4. *Prevalence of genes characteristic of the families Methanosarcinaceae (MSC) and Methanosaetaceae (MST) of the domain Archaea and the methyl-coenzyme M reductase (mcrA) gene*

The concentrations of the genes specific to bacterial families of the domain *Archaea* in one gram of digestate (1 g_D^{-1}) remained at a high and stable level in the range of 10^5 (*MSC*) to 10^6–10^7 (*MST*), regardless of exposure to antimicrobial substances or their type (Table S4). These observations indicate that the methanogens of the families *MSC* and *MST* actively participate in methanogenesis and are not sensitive to antimicrobial substances. According to the literature, *Archaea* microorganisms are resistant to many drugs targeting Eubacteria, including antibiotics that inhibit the synthesis of murein (β-lactams) and nucleic acids (MLS antibiotics, tetracyclines, sulfonamides). However, these microorganisms are sensitive to drugs that inhibit the biosynthesis of cell wall polymers and proteins (chloramphenicol), substances that disrupt membrane function, and imidazole derivatives (Hilpert et al., 1981; Khelaifia and Drancourt, 2012). The concentration of the *mcr*A gene in 1 g_D^{-1} from bioreactors containing MET, CEF and NA was one order of magnitude lower than in control. The total concentration of genes specific to the families *Methanosarcinaceae* and *Methanosetaceae* ($\Sigma_{(MSC,\ MST)}$) decreased significantly in sewage sludge samples exposed to the above antimicrobials, which could suggest that methanogens are somewhat sensitive to these antimicrobials. In turn, DOXY induced a significant, nearly two-fold increase in the concentrations of *mcr*A and $\Sigma_{(MSC,\ MST)}$ (Fig. S4 Q, R). The efficiency of anaerobic decomposition of organic matter can be evaluated based on the expression of the *mcr*A gene which is characteristic of metabolic processes in methanogens. However, methane production should also be monitored to fully characterize the activity of methanogens (Aydin et al., 2015).

The statistical analysis revealed that the prevalence of the *mcr*A gene was significantly correlated with the concentrations of *MSC* and *MST* genes ($R^2 = 0.65$; 0.79; $p < 0.05$). All of the above genes were also correlated with *mef*A ($R^2 = 0.65$; 0.52; 0.68, respectively; $p < 0.05$), and *MSC* was additionally correlated with the *erm*F gene ($R^2 = 0.52$; $p < 0.05$) which encodes resistance to MLS antibiotics. Moderate correlations were observed between *MST* and *tet*A and between *mcr*A and *int*I1 ($R^2 = 0.41$; 0.5, respectively; $p < 0.05$) (Fig. 4, Fig. S5).

3.5. *The prevalence of antibiotic resistance genes and integrase genes in anaerobic bioreactors*

Sewage sludge from WWTPs is a significant reservoir of ARGs (Munir et al., 2011; Wang et al., 2019). Similar observations were made in the

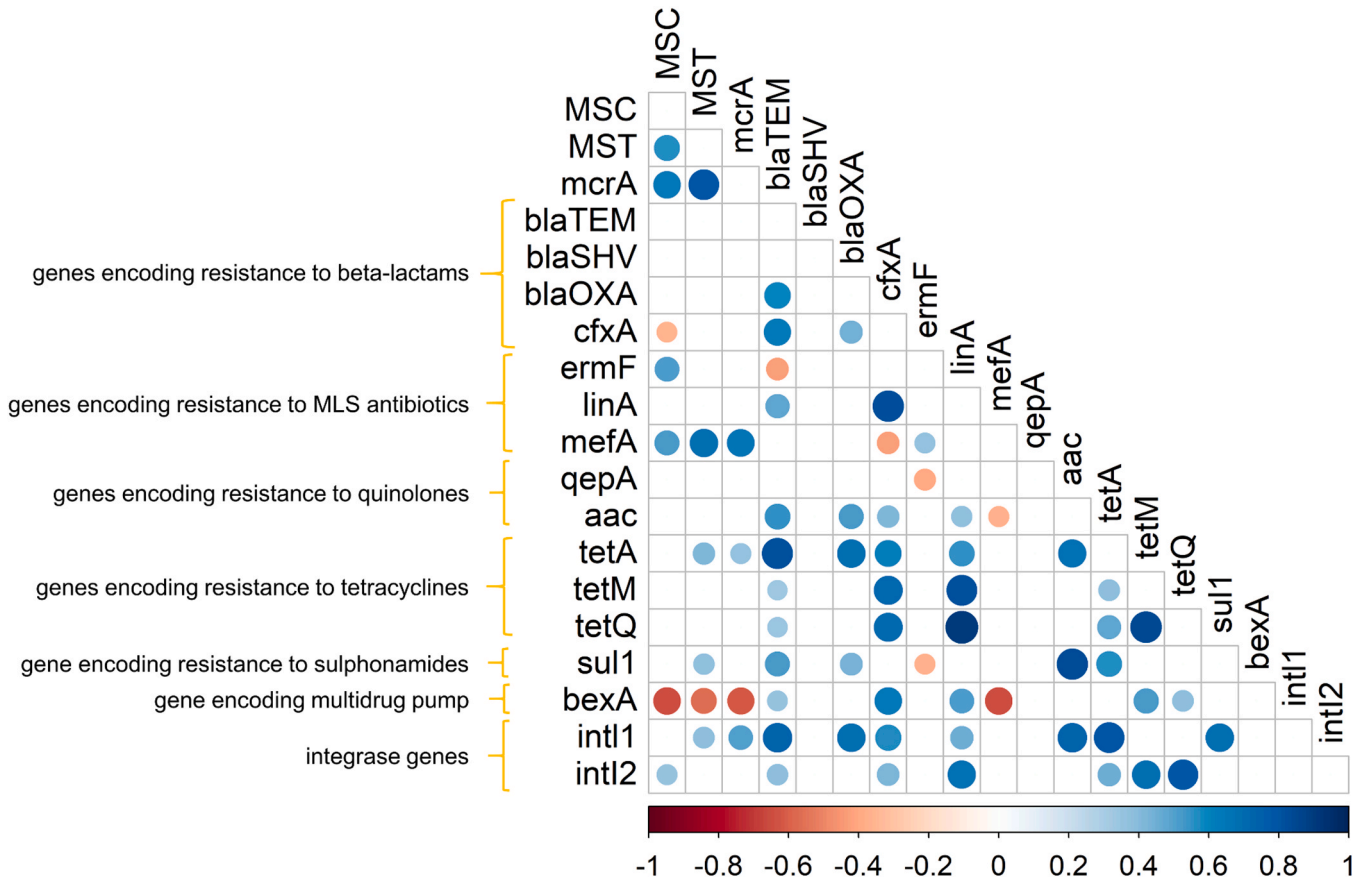

Fig. 4. Significant correlations between the concentrations of ARGs and other analyzed genes. Positive correlations are marked in blue and negative correlations are marked in red. Color intensity and the size of the circle are proportional to the correlation coefficients.

current study, where ARGs concentrations were high in both experimental and control digestate samples. Antimicrobial substances influenced the prevalence of the evaluated ARGs.

The copy numbers of genes encoding resistance to β-lactams in 1 g_D^{-1} were determined in the range of 10^4-10^7 (Table S4). The copy number of the bla_{TEM} gene in 1 g_D^{-1} increased significantly in most experimental bioreactors, in particularly under exposure to MET, relative to control. The group of genes encoding resistance to β-lactams, the largest and the most diverse group of antimicrobial substances (Niestępski et al., 2018), was dominated by the bla_{OXA} gene. Unlike bla_{TEM}, the concentration of the *cfx*A gene in 1 g_D^{-1} decreased by even two orders of magnitude under exposure to most antimicrobials, relative to control (Fig. S4 A, B, C). The bla_{SHV} gene was not identified in any samples (Table S4).

The bla_{TEM} gene is one of the most widespread ARGs in the environment, and it is presently used as an indicator of environmental contamination with ARB (Narciso-da-Rocha et al., 2014). The increased prevalence of bla_{TEM} in bacteria exposed to various antimicrobial substances can be attributed to the HGT process. The abundance of bla_{TEM} in environmental microorganisms can be maintained or increased regardless of the direction of bacterial selection in the presence of different antimicrobials. An increase in the frequency of the bla_{OXA} gene in process bioreactors can be similarly explained. Both bla_{OXA} and bla_{TEM} are frequently detected in WWTPs (Zieliński et al., 2019). These observations point to the presence of mutual relationships between antibiotic exposure and the concentrations of β-lactam ARGs.

The concentrations of tetracycline ARGs (*tet*A, *tet*M, *tet*Q) ranged from 10^3 to 10^7 in 1 g_D^{-1} (Table S4). The exposure of sewage sludge to MET and DOXY significantly increased (by up to two orders of magnitude) the concentrations of these genes in 1 g_D^{-1} relative to control. The copy numbers of the *tet*Q gene decreased significantly in sludge samples containing most of the remaining antibiotics (Fig. S4 E, F, G). Some authors observed a decrease in *tet* concentrations during anaerobic digestion (Diehl and Lapara, 2010; Zhang et al., 2015). In contrast, Liu et al. (2012) and Bai et al. (2019) reported an increase in the concentrations of *tet* genes in samples exposed to tetracyclines. An increase in ARGs concentrations in digested sewage sludge exposed to antimicrobials, compared with control sewage sludge, could indicate that microorganisms have acquired new ARGs (Aydin et al., 2015) or have gradually adapted to the new environment (Zhang et al., 2019).

The *sul*1 gene (10^7-10^8 in 1 g_D^{-1}) was the most prevalent ARG in this study (relative to the mean value) (Table S4). In comparison with control, its concentrations were significantly higher under exposure to all antibiotics, excluding SMO (Fig. S4 H). In turn, Gao et al. (2012) reported a strong positive correlation between total sulfonamide concentrations in wastewater and sewage sludge vs. the concentration of the *sul*1 gene. According to Ziembińska-Buczyńska et al. (2015), most bacterial strains isolated from activated sludge in WWTPs harbor only one of the three mechanisms of AR to SMO (*sul*1, *sul*2, *sul*3), and the minimum inhibitory concentration (MIC) of an antimicrobial substance does not increase the number of resistance mechanisms. Therefore, it can be assumed that in microorganisms present in digestate from the evaluated bioreactors, resistance to SMO was conditioned by not analyzed genes other than *sul*1.

The copy numbers of MLS ARGs (*erm*F, *lin*A, *mef*A) in 1 g_D^{-1} ranged from 10^4 to 10^8 (Table S4). The abundance of the *lin*A clearly decreased under exposure to most antimicrobial substances. The prevalence of *mef*A and *erm*F decreased only in response to MET and CIP. The copy numbers of the *lin*A gene increased significantly in response to tetracyclines and MET, relative to control (Fig. S4 I, J, K). Similar observations were made by Liu et al. (2014) who found that the concentrations of

MLS ARGs increased under exposure to MLS antibiotics.

Burch et al. (2016) reported a 99% decrease in the concentration of the *qnr*A gene (encoding resistance to fluoroquinolones) after anaerobic digestion of sewage sludge. In the current study, the *qep*A gene was detected only in the presence of MET, CIP and SMO. The copy numbers of *qep*A and *aac(6')-Ib-cr* ranged from 10^2 to 10^3 and 10^4 to 10^6 in 1 g_D^{-1}, respectively (Table S4). The concentration of *aac(6')-Ib-cr* increased significantly under exposure to CEF, CIP, and DOXY, relative to control (Fig. S4 L, M). Huang et al. (2016) demonstrated that the concentrations of fluoroquinolone ARGs increased at a much slower rate with low doses than with high doses of CIP. These observations indicate that ARGs transmission rates vary for different antimicrobials and doses. The *bex*A gene was identified only in control digestate and in sewage sludge digested in the presence of MET. Its concentration was higher in digestate exposed to MET than in the control bioreactor. The *bex*A gene encodes an efflux pump of the Multidrug and Toxic Compound Extrusion (MATE) family which removes toxins and drugs, including fluoroquinolones, from bacterial cells (Piddock, 2006; Eitel et al., 2013; Wiercińska et al., 2015; Niestępski et al., 2018).

Spearman's rank-order correlation analysis revealed a strong correlation between the prevalence of β-lactam ARGs and tetracycline ARGs, in particular *tet*A. The same groups of ARGs were correlated with *sul*1 and *aac(6')-Ib-cr* genes whose concentrations were also bound by mutual correlations. A significant correlation was noted between *lin*A genes, genes encoding resistance to β-lactams, tetracyclines, and the *bex*A gene (Figs. 4, S5).

The integrase gene (*int*I) is a structural element of integrons, namely genetic elements that acquire and express genes (Gillings et al., 2014). Integrons participate in HGT and are regarded as indicators of HGT (Di Conza and Gutkind, 2010; Gaze et al., 2011). Antibiotic resistance genes can also be spread between bacteria by HGT (Berglund, 2015; Wang et al., 2019). In the present study, the copy numbers of integrase genes (*int*I1, *int*I2) in 1 g_D^{-1} were high in the range of 10^6 to 10^8 (Table S4). The concentration of *int*I1 increased significantly in response to most of the analyzed antimicrobials. In turn, the concentration of *int*I2 increased only in sewage sludge exposed to MET (Fig. S4 O, P).

Spearman's rank-order correlation analysis revealed that the presence of *int*I1 was highly correlated (> 0.6) with bla_{TEM}, bla_{OXA}, *tet*A, *sul*1 and *aac(6')-Ib-cr* (R^2 = 0.73; 0.68; 0.79; 0.68; 0.73, respectively; $p < 0.05$). The presence of the *int*I2 gene was highly correlated with *lin*A, *tet*M and *tet*Q (R^2 = 0.67; 0.68; 0.78, respectively; $p < 0.05$) (Figs. 4, S5). The correlation analysis demonstrated that *int*I1 and *int*I2 play an important role in the transfer of ARGs during methane fermentation. *int*I1 is the most ubiquitous integrase gene in the environment, and, together with sulfonamide resistance genes, it has been recently recognized as an indicator of pollution caused by antibiotic-resistant bacteria (ARB), ARGs and other anthropogenic contaminants. Since integrase genes are linked with the HGT mechanism, integron-associated genes should be taken into account in analyses of antibiotic resistance in the environment (Gillings et al., 2014; Berendonk et al., 2015; Adelowo et al., 2018).

Studies investigating the fate of ARGs during methane fermentation often produce contradictory results. According to some authors, fermentation reduces the abundance of ARGs (Ma et al., 2011; Miller et al., 2013), whereas other researchers reported that anaerobic processes were not highly effective in removing ARGs from sewage sludge (Aydin et al., 2016). The results of this study revealed that methane fermentation can exert different effects on ARGs, but antimicrobials, especially at high concentrations, play an important role in the spread of AR. Moreover, exposure to the analyzed drugs does not always induce changes in the concentrations of genes encoding resistance to various antimicrobials. Selective pressure exerted by the analyzed compounds is not specific to genes encoding resistance to different classes of antimicrobial agents.

3.6. Correlations between methane fermentation parameters, microbial biodiversity and selected methanogen-specific genes, ARGs and integrase genes

The results of this study confirmed that sewage sludge from a WWTP is a significant reservoir of ARGs and integrase genes. Correlations were noted between the prevalence of ARGs and integrase genes, and antimicrobials were found to affect the concentrations of ARGs. Significant qualitative and quantitative changes in microbiota were also noted in sewage sludge exposed to antimicrobials. These findings indicate that the abundance of ARGs (identified in the qPCR assay, Fig. S4) was significantly correlated with the composition and structure of microbial communities (identified by metagenomic sequencing, Fig. S3).

Sewage sludge samples from bioreactors were grouped in hierarchical cluster analysis with the use of Ward's method based on gene concentrations determined in the qPCR assay (Fig. 5). Four main clusters were identified. The fourth cluster (IV) was composed of the genes identified in digestate samples exposed to CEF and MET (IVa) as well as AMO and CIP (IVb). Metronidazole, AMO and CIP influenced the formation of separate clusters, relative to control, based on changes in gene concentrations (Fig. 5) and the distribution of OTUs (Fig. 2). These antimicrobials induced the most significant changes in gene abundance and microbial biodiversity in the experimental bioreactors relative to control (Fig. 6). A significant decrease in the concentrations of *erm*F and *mef*A genes which encode resistance to MLS antibiotics and a significant increase in the concentrations of β-lactam, tetracycline and fluoroquinolone ARGs as well as integrase genes was also noted in sewage sludge containing the above antimicrobials (Fig. S4 A-G, I-M). According to Su et al. (2015), changes in bacterial communities considerably affect the profile of ARGs. The above observations indicate that the direction of selective changes in microbial structure altered gene profiles. Microorganisms harbored different ARGs that determined their AR.

The effect of MET on methane fermentation has not been investigated to date, and in this study, MET induced the most significant changes in gene concentrations (Fig. S4 A-N), in particular by increasing the concentrations of bla_{TEM}, *cfx*A, *tet*M, *bex*A and *int*I2 (Fig. 6). Metronidazole also induced the greatest changes in microbial biodiversity by significantly decreasing *Bacteroidetes* OTUs and significantly increasing *Firmicutes* and *Proteobacteria* OTUs (Figs. 6, S3). Some studies emphasized that the horizontal transfer of ARGs takes place mainly in the above bacterial phyla and that these phyla are a significant reservoir of mobile ARGs and integrons (Hu et al., 2016, Niestępski et al., 2020). These observations suggest that *Bacteroidetes*, *Firmicutes* and *Proteobacteria* harbored some of the analyzed ARGs. Metronidazole inhibited the growth of most ARB, but it also stimulated some ARB. Numerous bacterial species of the genus *Bacteroides* carry genes that confer resistance to MLS antibiotics, most notably the *erm* gene (Niestępski et al., 2017). This group of ARGs has been identified mainly in *Bacteroides* sp. and the related genera (Whittle et al., 2002). The concentration of the *erm*F gene was most significantly reduced in the presence of MET and CIP, which could be attributed to the decrease in the percentage of *Bacteroidetes* (Fig. S3), including bacteria of the order *Bacteroidales* (Fig. 3), induced by these antibiotics.

Metronidazole and AMO were the only antimicrobials that were fully degraded during anaerobic digestion. Despite the fact that MET was completely degraded in the solid fraction, its presence in sewage sludge resulted in the highest 6-fold decrease in methane production in the experimental bioreactor relative to the control bioreactor. Amoxicillin was also fully degraded during anaerobic digestion, and its significant inhibitory effect on methane production (1.3-fold) was comparable with that of CIP (1.6-fold decrease). Amoxicillin and CIP promoted the accumulation of VFAs. However, CIP was far less susceptible to degradation than AMO. Ciprofloxacin and NA were most resistant to degradation. Despite the above, NA induced no significant changes in methane fermentation parameters. These observations suggest that regardless of an antimicrobial's susceptibility to degradation during

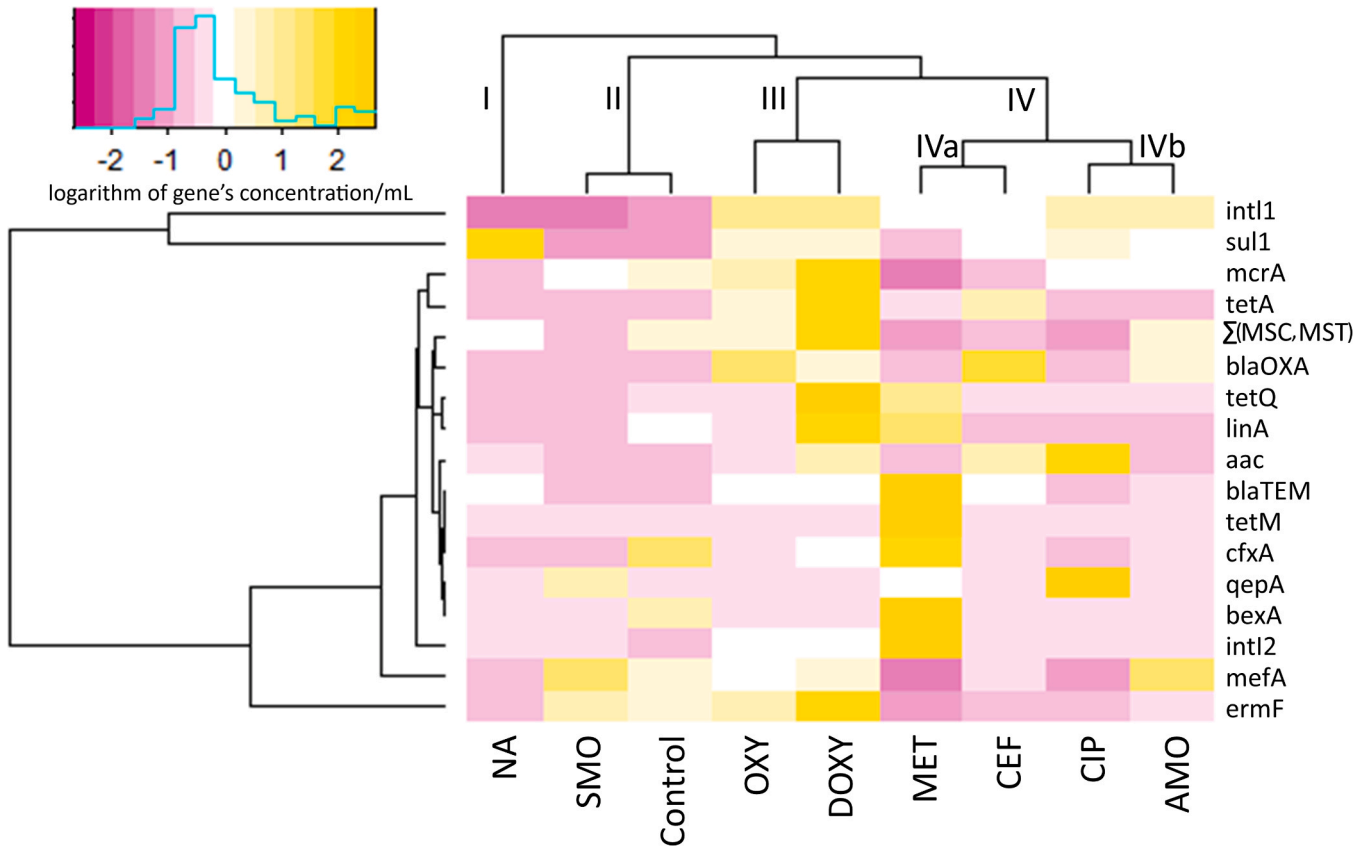

Fig. 5. Heatmap presenting the prevalence of genes (number of copies/1 mL) in samples of digested sewage sludge in experimental and control bioreactors (I-IV - group number).

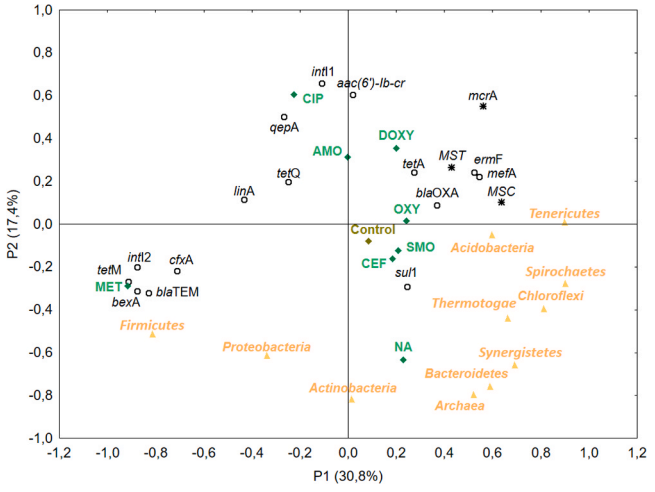

Fig. 6. Principal component analysis (PCA) based on the distribution of the analyzed ARGs, genes characteristic of methanogens, and bacterial phyla in bioreactors.

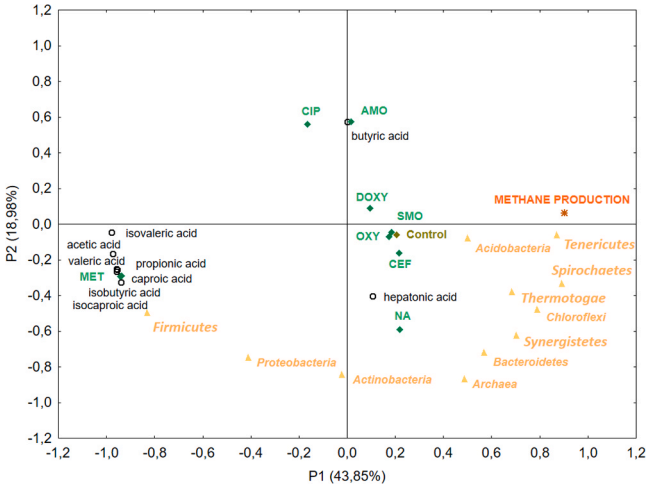

Fig. 7. Principal component analysis (PCA) based on the distribution of bacterial phyla and selected parameters of methane fermentation in bioreactors.

methane fermentation, the presence of antimicrobials in bioreactors in the initial stage of the process may considerably affect the composition of bacterial communities, ARGs concentrations, the efficiency of biogas production, and the methane content of biogas.

The analyzed antimicrobial substances present in sewage sludge also influenced the concentrations of VFAs produced during methane fermentation (Fig. 7). Significant accumulation of at least one of the identified VFAs was noted in the experimental bioreactors containing MET, AMO and CIP. The highest concentration of VFAs was noted in sewage sludge digested in the presence of MET. According to Jacob and Banerjee (2019), VFAs are accumulated during anaerobic treatment due to, among others, a higher content of inhibitory substances (such as antimicrobials), and they exert a considerable influence on microbial activity. The cited authors argued that under anaerobic conditions, the activity of methanogenic microorganisms is inhibited mainly by the accumulation of acetate and propionate. In the current study, these VFAs were accumulated in bioreactors containing MET and CIP. The concentrations of acetate and propionate were correlated ($R^2 = 0.9$, $p < 0.05$), and a strong negative correlation was noted between

methane production and acetate levels ($R^2 = -0.67$, $p < 0.05$). Methane production was also negatively correlated with seven out of the nine analyzed VFAs (Fig. S6). Therefore, the analysis of methane fermentation parameters suggests that the addition of antimicrobial substances to sewage sludge inhibited the activity of methanogens.

Somewhat different conclusions can be drawn from the results of the qPCR assay. The total concentration of genes *MSC* and *MST* ($\Sigma_{(MSC,\ MST)}$) and the abundance of the *mcr*A functional gene decreased significantly in most experimental bioreactors relative to control (Fig. S4 Q, R), but the observed differences did not exceed one order of magnitude. However, the importance of *mcr*A in the metabolism of methanogens was not reflected in the efficiency of methane fermentation. It should also be noted that the concentrations of genes specific to the domain *Archaea* increased significantly in sewage sludge exposed to tetracyclines (Fig. S4 Q, R) where methane production was significantly inhibited (Table 2). Therefore, the results of quantitative gene analyses do not corroborate the changes in methane fermentation parameters, and, consequently, do not reflect the real efficiency of the process.

Methane production has to be monitored, and changes in the populations of methanogenic microorganisms have to be determined at the molecular level to fully characterize the activity of methanogens. Ma et al. (2012) and Morris et al. (2014) demonstrated that the abundance of *mcr*A genes in fermented substrates differed significantly from the concentrations of *mcr*A transcripts. These discrepancies could result from reactions that exert a negative impact on methanogens and inhibit the transcription of *mcr*A genes. The present study revealed that a considerable decrease in methane production caused by antimicrobials is not always correlated with the abundance of the functional methanogen gene. In view of the above, the concentrations of the *mcr*A gene and its transcript should be taken into account in analyses to reliably assess the metabolic activity of methanogenic microorganisms.

4. Conclusions

The results of this study indicate that selected antimicrobial drugs, which are widely used in human medicine, influence the efficiency of methane fermentation in sewage sludge to a varied degree. Metronidazole, AMO and CIP exerted the most significant effect on the fermentation process. The presence of antimicrobials during methane fermentation affected the phylogenetic composition of bacteria and the profile of ARGs.

The tested antimicrobial substances differed in susceptibility to degradation during anaerobic digestion. However, the efficiency of their removal during methane fermentation was not correlated with the tested drugs' influence on microbial biodiversity, ARGs concentrations or the efficiency of the process.

This study demonstrated that the results of quantitative analyses of methanogen-specific genes (including the *mcr*A functional gene) are not consistent with changes in methane fermentation parameters and, therefore, do not reflect the actual efficiency of the process under exposure to antimicrobials.

CRediT authorship contribution statement

Małgorzata Czatzkowska: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Investigation, Visualisation. **Monika Harnisz**: Conceptualization, Methodology, Resources, Supervision, Project administration. **Ewa Korzeniewska**: Conceptualization, Methodology. **Paulina Rusanowska**: Methodology, Formal analysis, Writing - original draft. **Sylwia Bajkacz** and **Ewa Felis**: Methodology, Formal analysis, Writing - original draft. **Jan P. Jastrzębski:** Software, Data curation, Visualization. **Łukasz Paukszto**: Software, Data curation, Visualization. **Izabela Koniuszewska**: Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125773.

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OŚWIADCZENIE

Oświadczam, że w pracy:

Czatzkowska M., Harnisz M., Korzeniewska E., Rusanowska P., Bajkacz S., Felis E., Jastrzębski J. P., Paukszto Ł., Koniuszewska I. 2021. The impact of antimicrobials on the efficiency of methane fermentation of sewage sludge, changes in microbial biodiversity and the spread of antibiotic resistance. Journal of Hazardous Materials, 416, 125773, https://doi.org/10.1016/j.jhazmat.2021.12577

mój wkład polegał aktywnym przeprowadzeniu eksperymentów dotyczących analizy rozpowszechnienia genów oporności na antybiotyki. Współuczestniczyłam w planowaniu eksperymentów laboratoryjnych i prac bioinformatycznych. Brałam udział w konceptualizacji oraz interpretacji i opracowaniu wyników badań. Miałam wiodący udział w zaplanowaniu oraz przeprowadzeniu systematycznego przeglądu literatury. Przygotowałam pierwszą wersję manuskryptu oraz uczestniczyłam w jego korekcie po procesie recenzji. Co więcej, byłam odpowiedzialna za graficzne opracowanie rycin.

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OŚWIADCZENIE

Oświadczam, że w pracy:

Czatzkowska M., **Harnisz M.**, Korzeniewska E., Rusanowska P., Bajkacz S., Felis E., Jastrzębski J. P., Paukszto Ł., Koniuszewska I. 2021. The impact of antimicrobials on the efficiency of methane fermentation of sewage sludge, changes in microbial biodiversity and the spread of antibiotic resistance. Journal of Hazardous Materials, 416, 125773, https://doi.org/10.1016/j.jhazmat.2021.12577

mój wkład polegał na określeniu zadań badawczych i metod. Uczestniczyłam w planowaniu doświadczeń, analizie i interpretacji wyników. Nadzorowałam tworzenie manuskryptu, proces recenzji oraz korektę pracy przed złożeniem do druku. Ponadto, pełniłam funkcję kierownika projektu, z którego sfinansowano badania opisane w niniejszym manuskrypcie.

Monika Harnisz

Olsztyn, 12.07.2023

Prof. dr hab. Ewa Korzeniewska
Katedra Inżynierii Ochrony Wód
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Wydział Geoinżynierii
Uniwersytet Warmińsko-Mazurski w Olsztynie

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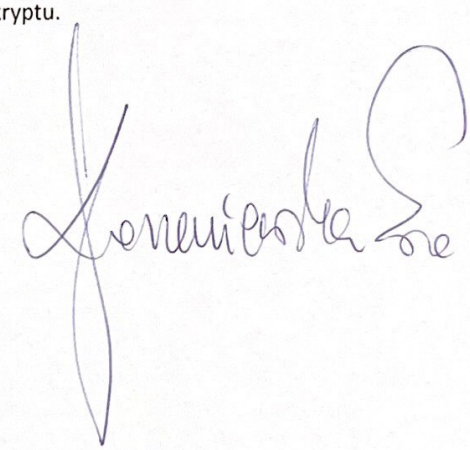
mój wkład polegał na udziale w opracowaniu metod i konceptualizacji. Dodatkowo, udzielałam konsultacji przy tworzeniu pierwszej wersji manuskryptu.



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dr Paulina Rusanowska
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Uniwersytet Warmińsko-Mazurski w Olsztynie

OŚWIADCZENIE

Oświadczam, że w pracy:

Czatzkowska M., Harnisz M., Korzeniewska E., **Rusanowska P.**, Bajkacz S., Felis E., Jastrzębski J. P., Paukszto Ł., Koniuszewska I. 2021. The impact of antimicrobials on the efficiency of methane fermentation of sewage sludge, changes in microbial biodiversity and the spread of antibiotic resistance. Journal of Hazardous Materials, 416, 125773, https://doi.org/10.1016/j.jhazmat.2021.12577

mój wkład polegał na nadzorowaniu technologicznej części eksperymentów, których wyniki przedstawiono w niniejszym artykule. Dodatkowo, uczestniczyłam w tworzeniu pierwszej wersji manuskryptu oraz w korekcie pracy przed złożeniem do druku.

Paulina Rusanowska

Olsztyn, 12.07.2023

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Uniwersytet Warmińsko-Mazurski w Olsztynie

OŚWIADCZENIE

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Czatzkowska M., Harnisz M., Korzeniewska E., Rusanowska P., Bajkacz S., Felis E., Jastrzębski J. P., Paukszto Ł., **Koniuszewska I.** 2021. The impact of antimicrobials on the efficiency of methane fermentation of sewage sludge, changes in microbial biodiversity and the spread of antibiotic resistance. Journal of Hazardous Materials, 416, 125773, https://doi.org/10.1016/j.jhazmat.2021.12577

mój wkład polegał na udziale w analizie rozpowszechnienia genów oporności na antybiotyki. Uczestniczyłam również w opracowaniu pierwszej wersji manuskryptu.

Izabela Wolak

Załącznik nr 3

Appendix 3

Czatzkowska M., Harnisz M., Korzeniewska E., Wolak I., Rusanowska P., Paukszto Ł., Jastrzębski J. P., Bajkacz S.

Long-term, simultaneous impact of antimicrobials on the efficiency of anaerobic digestion of sewage sludge and changes in the microbial community

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Article

Long-Term, Simultaneous Impact of Antimicrobials on the Efficiency of Anaerobic Digestion of Sewage Sludge and Changes in the Microbial Community

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Abstract: The aim of this study was to evaluate the influence of simultaneous, long-term exposure to increasing concentrations of three classes of antimicrobials (β-lactams, fluoroquinolones and nitroimidazoles) on: (1) the efficiency of anaerobic digestion of sewage sludge, (2) qualitative and quantitative changes in microbial consortia that participate in methane fermentation, and (3) fate of antibiotic resistance genes (ARGs). Long-term supplementation of sewage sludge with a combination of metronidazole, amoxicillin and ciprofloxacin applied at different doses did not induce significant changes in process parameters, including the concentrations of volatile fatty acids (VFAs), or the total abundance of ARGs. Exposure to antibiotics significantly decreased methane production and modified microbial composition. The sequencing analysis revealed that the abundance of OTUs characteristic of *Archaea* was not correlated with the biogas production efficiency. The study also demonstrated that the hydrogen-dependent pathway of methylotrophic methanogenesis could significantly contribute to the stability of anaerobic digestion in the presence of antimicrobials. The greatest changes in microbial biodiversity were noted in substrate samples exposed to the highest dose of the tested antibiotics, relative to control. The widespread use of antimicrobials increases antibiotic concentrations in sewage sludge, which may decrease the efficiency of anaerobic digestion, and contribute to the spread of antibiotic resistance (AR).

Keywords: sewage sludge; long-term anaerobic digestion; methane; antimicrobial influence; microbial community; antibiotic resistance

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1. Introduction

Due to their extensive use in recent years, antibiotics have emerged as a new type of pollutant that poses a threat to the environment and public health [1]. The environment is contaminated with antimicrobials from various sources, including pharmaceutical plants, agriculture, veterinary and human health care facilities. Direct human consumption of drugs is one of the sources of antimicrobials and their metabolites in wastewater [2]. Moreover, inappropriate disposal of unused and expired drugs increases the pool of antimicrobials in wastewater flowing into wastewater treatment plants (WWTPs). Each year, thousands of tons of antimicrobials and their metabolites are expelled and carried

with sewage to WWTPs [2,3]. Processes like wastewater treatment or anaerobic digestion allow antibiotic degradation, but there is notable variability in antibiotic removal rates as well as problems with their biologically active degradation products [4,5].

The technology involving activated sludge is an effective biological wastewater treatment method. This process generates sewage sludge, including excess activated sludge [6]. Antimicrobials are also detected in water because wastewater is being continuously enriched with these substances. Drugs are strongly absorbed by sewage sludge, and considerable research has been done to determine the influence of antimicrobials on both sewage sludge [7–10] and the environment [10–13].

Sewage sludge from WWTPs requires appropriate management. Anaerobic treatment is a technology that is applied mainly to stabilize sewage sludge [6]. In the process of methane fermentation, complex organic matter undergoes decomposition under anaerobic conditions, which results in the production of biogas composed mainly of methane and carbon dioxide. This technology offers an alternative approach to energy generation that consists of four interdependent stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [14,15]. Methane production involves a variety of microorganisms that determine the efficiency of the process. The efficiency of methane fermentation is influenced primarily by the presence of inhibitory compounds, including antibiotics, in sewage sludge. Antimicrobial substances can destabilize the entire process and decrease methane production. The accumulation of wastewater-borne antimicrobials in sewage sludge can influence the microbial community associated with various stages of methane fermentation. Methane production can be disrupted by the low activity of various microbial groups and variations in the quantitative and qualitative composition of microbiota [5,15–17]. Antimicrobial substances affect microorganisms in various ways, for example by inhibiting the replication of DNA (nitroimidazoles and fluoroquinolones), transcription of RNA, or generation of ATP. These inhibitors can also disturb the cell wall and nucleotide synthesis (β-lactams) and nucleotides, and they can compromise cell division and protein translation [18,19]. The presence of antimicrobial substances in anaerobic digesters can exert a direct influence on methane production [20]. In many cases, the efficiency of methane fermentation is often compromised by an increase in the concentration of volatile fatty acids (VFAs) which are generated during methanogenesis and which disrupt the equilibrium between microbial groups participating in the process [16]. A sound knowledge of microbial consortia that are involved in anaerobic processes can be useful in preventing and predicting system failures [21,22]. Despite significant progress in digester engineering and design, the participation of bacteria in methane fermentation continues to pose a challenge.

Antimicrobials that are organic inhibitors of methane fermentation also contribute to antibiotic resistance (AR). The widespread use and misuse of drugs leads to the emergence of microorganisms that carry more than one antibiotic resistance gene (ARG). Like antibiotics, ARGs belong to a new category of pollutants that can influence public safety and health [23–25]. The horizontal gene transfer (HGT) mechanism enables microorganisms to exchange ARGs. Wastewater and sewage sludge are characterized by subinhibitory levels of antimicrobial, and WWTPs are regarded as hotspots of HGT processes and ARG transmission between bacteria [26].

The impact of antimicrobials on anaerobic digestion [27,28] and the elimination of ARGs during methane fermentation [29,30] have been explored by many researchers. These problems were also addressed in our previous research [5,31]. Czatzkowska et al. [5] analyzed the effects of the most popular classes of antibiotics on sewage sludge fermentation. The study focused on the effects of individual antibiotics administered at high doses, and it demonstrated that exposure to single, high doses of antimicrobials during anaerobic digestion affected methane production, the structure of microorganisms and ARGs in sewage sludge. Fermentation, including the microbiological aspects of the process, was most exten-sively influenced by amoxicillin, ciprofloxacin and metronidazole. It should be noted that the cited research was the first study to investigate the effect of metronidazole on methane fermentation.

Metronidazole, one of the studied antibiotics, is used in human and veterinary medicine to treat infections caused by anaerobic and microaerophilic bacteria as well as protozoa. This antimicrobial drug is diffused across microbial cell membranes, and it is reduced by intracellular proteins, which explains the natural resistance of aerobic bacteria [32]. Metronidazole is active only in anaerobic conditions, and it is often used to treat infections caused by *Bacteroidetes*, which is one of the largest bacterial phyla involved in methane fermentation. The phylum *Bacteroidetes* includes β-lactamase–producing anaerobic bacteria that are often resistant to penicillin [33]. β-Lactams are the most diverse group of antibiotics that are widely applied in medicine. However, microorganisms have developed resistance to β-lactams, and the number of resistant strains continues to increase. According to estimates, annual β-lactam sales account for 65% of the global market of antibiotics, which raises serious concern in view of growing microbial resistance to this group of drugs. Amoxicillin, a β-lactamase-sensitive penicillin, is widely used in the treatment of upper respiratory tract infections. This broad-spectrum antimicrobial targets both Gram-negative and Gram-positive bacteria [34,35]. Ciprofloxacin is a broad-spectrum fluoroquinolone antimicrobial that is applied in the treatment of bacterial urinary tract infections and pneumonia [36]. Ciprofloxacin is also administered to prevent infections in cancer patients. Recent research has demonstrated growing levels of microbial resistance to this drug [37,38].

The present study was undertaken to evaluate the simultaneous, long-term influence of antimicrobials on sewage sludge fermentation. The experiment involved three classes of antimicrobials (β-lactams, fluoroquinolones and nitroimidazoles) that can potentially influence microorganisms participating in the entire anaerobic treatment process. The antimicrobial substances were selected based on our previous findings [5], as having the most significant, individual impact on biogas production and microbiota structure during anaerobic digestion of this substrate. This study is a continuation of our previous original research into metronidazole's effect on anaerobic digestion. Although the effects of several antibiotics on methane fermentation were previously investigated, these studies did not select the drugs classes currently most commonly used in human medicine [4,39]. Due to the continuous increase in the consumption of antibiotics and their release to wastewater and sewage sludge, we believe that the concept of research on the drug's effect on methane fermentation should include the most common antimicrobial substances consumed by humans. To the best of our knowledge, the concurrent impact of several widely used antimicrobials on methane fermentation of sewage sludge—included in the concept of this study—has not been investigated to date.

In view of the above, this study was undertaken to investigate the concurrent effect of selected antimicrobials on: (1) methane fermentation efficiency, (2) quantitative and qualitative changes in microbial consortia that participate in anaerobic digestion, (3) the fate of ARGs and the spread of AR. The study was designed to analyze the simultaneous impact of amoxicillin, ciprofloxacin and metronidazole on the anaerobic digestion of sewage sludge during long-term exposure to the examined antimicrobials. The presence of synergistic and antagonistic interactions between the studied antibiotics was determined. The results will provide new insights into antimicrobials' impact on the efficiency of methane fermentation of sewage sludge and changes in the structure of microbial consortia. Special emphasis was placed on the spread of AR. The results were analyzed with the use of high-throughput sequencing, a modern tool for typing microbial isolates in molecular microbiology.

2. Materials and Methods

2.1. Substrate and Inoculum

Sewage sludge was collected from the Łyna Municipal Wastewater Treatment Plant (WWTP) located in Olsztyn, Poland. The plant treat wastewater in an amount corresponding to approximately 177,000 PE. The average wastewater flow is 30,000 m^3/day. Fermentation chambers are fed with sludges produced during wastewater treatment process (from primary settling tanks and gravity thickeners) and operated at load of 0.055 $m^3 \cdot m^{-3} \cdot d^{-1}$,

sludge retention time of 18.5 days, and the temperature of 34–35 °C. Produced biogas is used to generate heat in WWTP buildings.

Sewage sludge had the following characteristics: total solids (TS)—52.1 ± 11.4 mg TS/g; volatile solids (VS)—39.9 ± 9.6 mg VS/g; pH—6.35 ± 0.3; total phosphorus (TP)—2.0 ± 0.5 mg TP/g; total nitrogen (TN)—4.3 ± 1.6 mg T/g. The substrate was stored at a temperature of 5 °C before analysis. The inoculum (anaerobic sludge) was obtained from a fermentation tank in the same WWTP. Anaerobic sludge had the following characteristics: 55.7 ± 1.5 g TS/L, 42.8± g VS/L, pH of 8.01 ± 0.4, 0.6 ± 0.2 mg TP/g, 2.1 ± 0.4 mg TN/g.

2.2. Methane Fermen Tation

Sewage sludge was treated by anaerobic digestion in semi-continuous flow digesters with a volume of 2 L (working volume of 1.8 L). The digesters were placed in the water bath with a temperature of 37 °C to maintain mesophilic conditions. The digesters were fed substrate containing a mixture of amoxicillin (AMO), ciprofloxacin (CIP) and metronidazole (MET) or sewage sludge only (control). The experiments were carried out in two replicates. The antibiotics were selected based on the results of our previous research [5]. The three antibiotics selected for this study, individually dosed into sewage sludge in anaerobic bioreactors, caused the most significant effect on methane production, as well as changes in the structure of the microbiota involved in the process and the ARGs profile. The present study consisted of six experimental series that differed in the concentrations of the tested antibiotics (Table 1). The initial concentration of each of the three antibiotics in the mixture dosed to sewage sludge during the first experimental series (D1) was similar to the concentration of these drugs in the wastewater flowing into the WWTP from which the sludge was obtained [40]. The concentrations of the antibiotics added to the digester were increased after doubling the hydraulic volume of each digester. Each of the experimental series lasted 45 days on average, and the entire study lasted 268 days.

Table 1. Concentrations of the antibiotics used in the experiment.

	Antibiotic Concentration (μg/mL)		
Dose ID	**AMO**	**CIP**	**MET**
D1	1	0.25	0.25
D2	2	0.5	0.5
D3	4	1	1
D4	8	4	4
D5	16	8	8
D6	36	16	16

The digesters were operated at an organic loading rate of 2.8 g VS·L^{-1}·d^{-1}. Hydraulic retention time was 22 days. The digesters were equipped with a mechanical stirrer and a feeding and discharge system, and they were connected to an automatic methane potential test system (AMPTS II) (Bioprocess Control, Lund, Sweden) which measured the amount of the produced methane. Biogas quality was analyzed in a gas chromatograph equipped with a thermal conductivity detector (GC-TCD, Agilent Technologies 7890 A, Irving, TX, USA). Gas was normalized for standard temperature (273.2 K) and pressure (1.01325 bar).

2.3. Sampling

During each experimental series, representative digestate samples were collected at weekly intervals from digester containing substrate supplemented with antibiotics (process digester) and sewage sludge only (control digester). To monitor the digestion process, digestate samples were analyzed for pH, FOS/TAC ratio (the TAC value denotes the estimated buffer capacity of the sample, and the FOS value denotes the content of volatile acids), and the content of TS, VS, TN and TP. A total of 56 samples, including 28 samples

from the control bioreactor and 28 samples from the process bioreactor, were collected. The nomenclature adopted for the examined samples is presented in Table 2. All samples collected from process and control bioreactors were analyzed for methane production and VFA content. Samples for DNA isolation and sequencing were collected from the process bioreactors at the beginning, in the middle and at the end of each experimental series, whereas one sample was collected from the control bioreactor during the first, fourth and last experimental series.

Table 2. Nomenclature for the samples used in the analyses of methane production, VFA (volatile fatty acid) content and sequencing analyses, including sample IDs.

		Methane and VFA Analyses		**Sequencing**	
Experimental series 1	Sample	Process Bioreactor	Control Bioreactor	Process Bioreactor	Control Bioreactor
		Sample ID			
	1	D1.1	C1.1	D1.1	C1.1
	2	D1.2	C1.2	D1.2	na
	3	D1.3	C1.3	na	na
	4	D1.4	C1.4	D1.4	na
Experimental series 2	Sample	Process bioreactor	Control bioreactor	Process bioreactor	Control bioreactor
		Sample ID			
	1	D2.1	C2.1	D2.1	na
	2	D2.2	C2.2	na	na
	3	D2.3	C2.3	D2.3	na
	4	D2.4	C2.4	na	na
	5	D2.5	C2.5	D2.5	na
Experimental series 3	Sample	Process bioreactor	Control bioreactor	Process bioreactor	Control bioreactor
		Sample ID			
	1	D3.1	C3.1	D3.1	na
	2	D3.2	C3.2	na	na
	3	D3.3	C3.3	D3.3	na
	4	D3.4	C3.4	D3.4	na
Experimental series 4	Sample	Process bioreactor	Control bioreactor	Process bioreactor	Control bioreactor
		Sample ID			
	1	D4.1	C4.1	D4.1	C4.1
	2	D4.2	C4.2	na	na
	3	D4.3	C4.3	na	na
	4	D4.4	C4.4	D4.4	na
	5	D4.5	C4.5	D4.5	na
Experimental series 5	Sample	Process bioreactor	Control bioreactor	Process bioreactor	Control bioreactor
		Sample ID			
	1	D5.1	C5.1	D5.1	na
	2	D5.2	C5.2	na	na
	3	D5.3	C5.3	D5.3	na
	4	D5.4	C5.4	na	na
	5	D5.5	C5.5	D5.5	na

Table 2. *Cont.*

		Methane and VFA Analyses		Sequencing	
	Sample	Process bioreactor	Control bioreactor	Process bioreactor	Control bioreactor
		Sample ID			
Experimental series 6	1	D6.1	C6.1	D6.1	na
	2	D6.2	C6.2	na	na
	3	D6.3	C6.3	D6.3	na
	4	D6.4	C6.4	na	na
	5	D6.5	C6.5	D6.5	C6.5

D-dose ID, C-control samples, na-not analyzed. For example, D1.1 is the first sample from the process bioreactor that was supplemented with the first dose, C1.4 is the fourth sample from the control bioreactor etc.

2.4. Analytical Procedures

The content of VFAs was determined based on a previously described method [41] with the use of a gas chromatograph (Brüker 450-GC, Billerica, MA, USA) with a flame ionization detector (FID) and a CP-FFAP CB capillary column (25 m × 0.53 mm). The FOS/TAC ratio was determined with the TitraLab AT1000 Series Titrator (Hach, Ames, IA, USA). The content of TS and vs. in biomass samples was determined in a gravimetric analysis [42]. The content of TN and TP in the samples was determined using the DR 5000 spectrophotometer with the HT 200 s mineralizer (Hach, Ames, IA, USA). All measurements were performed in duplicate.

2.5. Isolation of Genomic DNAs

One mL of each sample was transferred to sterile 2 mL Eppendorf tubes (Eppendorf, Germany) and centrifuged (5415R Centrifuge, Eppendorf, Germany) for 15 min at 9000 rpm at a temperature of 4 °C. The obtained supernatant was discarded. The procedure was repeated. The FastDNA™ Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA) was used for DNA isolation from the acquired pellet, according to the instructions supplied by the manufacturer. The quantity and quality of the obtained genetic material were measured using a spectrophotometer (Multiskan Sky, Thermo Scientific, Waltham, MA, USA). DNA was isolated in a total amount of 150 µL from two replicates and was stored until further analysis at −20 °C.

2.6. Library Preparation and Sequencing

Metagenomic sequencing was performed by Macrogen (Seoul, Korea) with the TruSeq DNAPCR-Free Kit (Illumina Inc., San Diego, CA, USA). Index sequences were attached to each sample during the preparation of the library. Barcoded libraries were sequenced by using a 2 × 151 bp paired-end protocol in the Illumina NovaSeq 64,000 system (Illumina Inc, San Diego, CA, USA). A total of 21 metagenomic samples were placed under accession No. PRJEB48473 in the European Nucleotide Archive (ENA) database.

2.7. Bioinformatics Analysis

Before downstream analysis, the quality of raw reads was estimated using Kneaddata v. 0.7.6 software (Huttenhower Lab, Harvard Chan Center for the Microbiome in Public Health, Boston, MA, USA) [43]. The raw data were trimmed by eliminating reads with a low PHRED score (<20) and removing reads with nonbacterial contamination. The human genome (hg37; GenBank ID—GCF_000001405.13) was used as contamination reference sequences for quality control. Ribosomal RNA reads were also recognized and eliminated with Kneaddata software. Filtered reads were used as the inputs for downstream analyses. Humann2 v.0.11.2 software (Huttenhower Lab) [44] and UniRef90 database were used to explore the gene family abundance. The bacterial community size was determined by

Metaphlan v. 2.0 (Huttenhower Lab) [45]. Kneaddata, Metaphlan, and Humann2 were used as part of the Biobakery v.0.15.1 workflow.

2.8. Statistical Analysis

The influence of antibiotics on methane and VFA production was analyzed by two-way ANOVA, and the results were presented in line graphs (GraphPad Software Inc., San Diego, CA, USA).

The structure of microbial consortia was visualized by phyloseq (San Francisco, CA, USA) [46], circlize 0.4.13 (Heidelberg, Germany) [47] R packages and graphlan 1.1.3 (Huttenhower Lab) [48] software. Trimmed sequences were used to analyze ARG abundance and diversity in the ARG-OAP v.2.0 pipeline (University of Hong Kong, Hong Kong, China) [49]. Antibiotic resistance genes were annotated, and gene type, subtype, and abundance were determined for each gene. Heatmaps showing microbial community structure and ARG abundance were constructed using hclust2 python script (Segata Lab, University of Trento, Trento, Italy) [50]. Pearson's correlation coefficients were calculated with the custom R function in github [51] to determine the strength of the relationships between the abundance of ARGs and the microbial communities.

Diagrams were generated and the remaining calculations were ran in Microsoft Excel 2013 (Redmond, WA, USA) and GraphPad Software Inc. The abundance of selected microbial groups in samples from process digesters was compared by Spearman's rank-order correlation analysis and Kruskal-Wallis non-parametric test for several independent samples (Statistica 13.1, Statsoft, Krakow, Poland). The relationships between microbial biodiversity in bioreactors, the abundance of ARGs and methane production were examined by principal component analysis (PCA, Statistica 13.1).

3. Results and Discussion

3.1. The Impact of Increasing Antibiotic Concentrations on the Efficiency of Anaerobic Digestion

The key stages of anaerobic digestion involve hydrolysis, acidogenesis, acetogenesis and methanogenesis. The products of each stage act as substrates for successive stages, and methane and VFA concentrations indicate which stage was inhibited or compromised. To determine the influence of antibiotics on the methane fermentation of sewage sludge, the production of methane and VFAs was monitored in the control bioreactor and in the process bioreactor where sewage sludge was exposed to a mixture of metronidazole, amoxicillin and ciprofloxacin applied at increasing concentrations over a long period of time. The results of the standard technological analyzes (pH, FOS/TAC, TS, VS, TP and TN) are included in Table S1.

Similar trends in methane production were observed in the control bioreactor and in the process bioreactor where sewage sludge was exposed to a combination of the tested antibiotics (Figure 1). However, the efficiency of methane production was lower in the process bioreactor than in the control bioreactor, and the observed differences were significant ($p < 0.05$) (Table S2). An analysis of average methane production in each experimental series demonstrated the greatest decrease in methane production efficiency in the bioreactor supplemented with the first (D1) and the second (D2) dose (by 8.5 and 11.1 $NL \cdot kgVS^{-1}$, respectively), relative to control. Average methane production in the process bioreactor decreased with a rise in antibiotic concentrations (D3–D6) in the supplemented substrate relative to control. These findings suggest that prolonged exposure to a combination of antimicrobials influenced microbial acclimation inside the bioreactor, and similar observations were made by other authors [52,53]. Acclimation promotes microbial adaptation and selective growth of microbial groups that play a key role in methane fermentation [54].

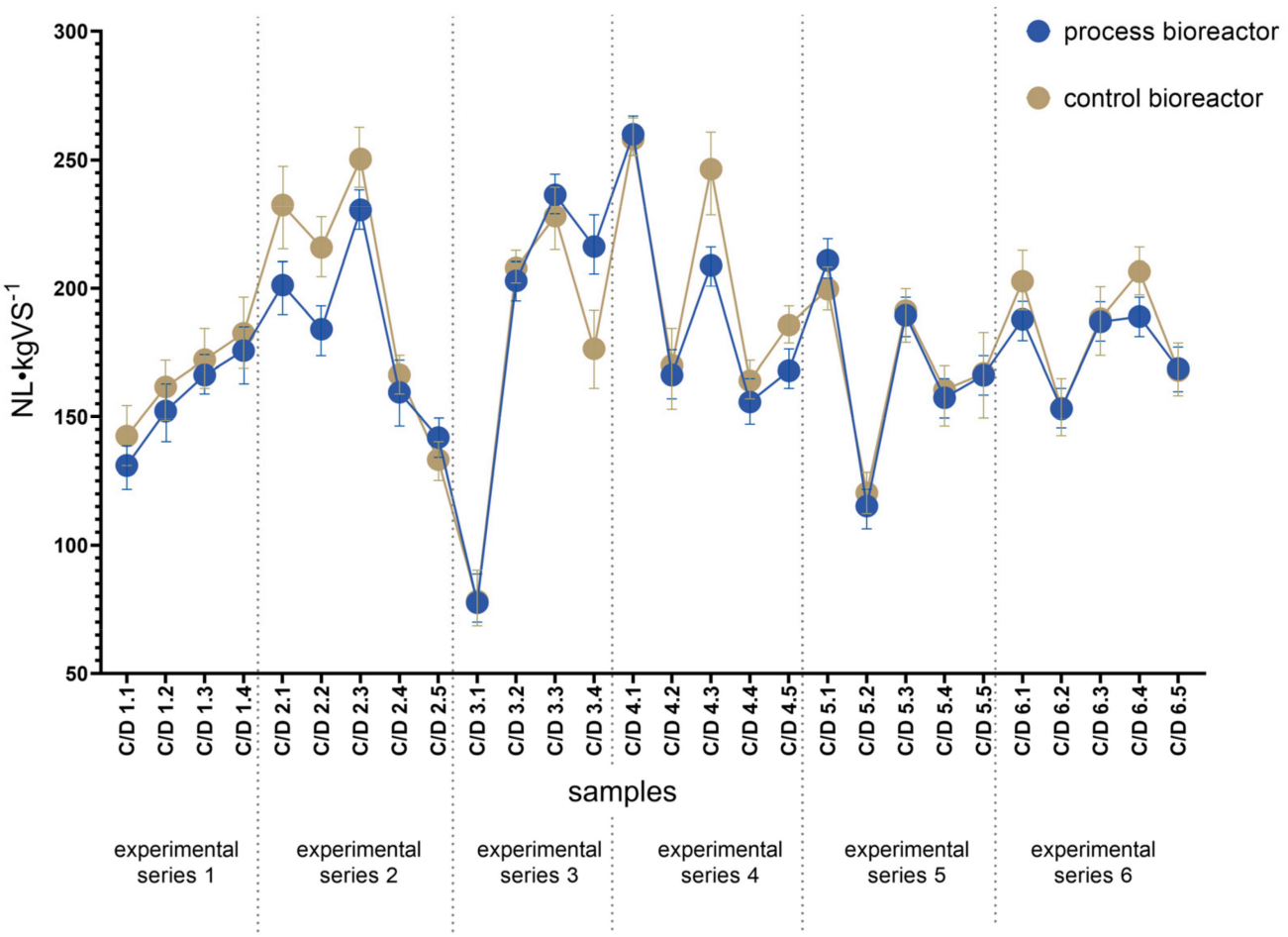

Figure 1. Methane production (NL·kgVS^{-1}) in the process and control bioreactors. C-control samples, D-dose ID. For example, "C/D 1.1" indicates samples C1.1 and D1.1 (green and blue points on the line graph, respectively).

In our previous study [5], high individual doses of metronidazole (512 µg/g), amoxicillin (1024 µg/g) and ciprofloxacin (512 µg/g) significantly decreased the efficiency of methane production (by 228.5, 74.7 and 108.4 NL·kgVS^{-1}, respectively) during the anaerobic digestion of sewage sludge relative to control. In the present study, sewage sludge was exposed to a combination of three antimicrobials applied at low concentrations, and the resulting decrease in biogas production was less pronounced than in our previous research. The observed differences in methane production can be probably attributed to different antibiotic concentrations. Moreover, synergistic interactions between metronidazole, amoxicillin and ciprofloxacin can be ruled out within the range of concentrations tested in this study. According to the literature [55,56], low concentrations of selected antimicrobials do not always disrupt anerobic digestion, and methane production decreases only when a certain threshold concentration of the drug is achieved. Other authors reported that in environments characterized by low antibiotic concentrations, such as municipal wastewater and sewage sludge, antimicrobials exert a minimal impact on the overall efficiency of methane production [54,57].

Changes in the content of VFAs, the main products of protein and carbohydrate hydrolysis during anaerobic digestion, are an important determinant of the efficiency of methane fermentation [58]. The accumulation of VFAs in anaerobic bioreactors points to the inhibition of acetogenesis or methanogenesis. In the present study, no significant differences in the concentrations of individual VFAs ($p > 0.05$) were found between sewage sludge samples collected from process and control bioreactors (Table S3). However, a

sudden increase in the concentrations of butyric acid (D1, D2) and isovaleric acid (D3) was noted in samples from the process bioreactor (Figure S1). The observed differences did not exceed 1–2 g/L and were not statistically significant, but they could have resulted from a decrease in the efficiency of acetogenesis. Acetogenic bacteria convert butyric and isovaleric acids to acetic acid which is utilized by acetoclastic methanogens in methane production. It should be noted that acetogenesis plays an important role in biogas production, and around 70% of methane is produced during acetate reduction [59]. Acetate concentration decreased rapidly after the substrate was supplemented with antimicrobial doses D1–D3 (Figure S1). As previously mentioned, the greatest decrease in methane production relative to control was also observed after the application of the lowest drug doses (Figure 1). The influence of the tested combination of antibiotics on microbial consortia, including methanogens, is discussed in detail in Section 3.2.

3.2. Diversity of Microbial Consortia

The sequencing analysis supported the identification of nine dominant bacterial phyla in sewage sludge subjected to anaerobic digestion: *Firmicutes*, *Nitrospirae*, *Chloroflexi*, *Tenericutes*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Candidatus Cloacimonetes* (Figure 2).

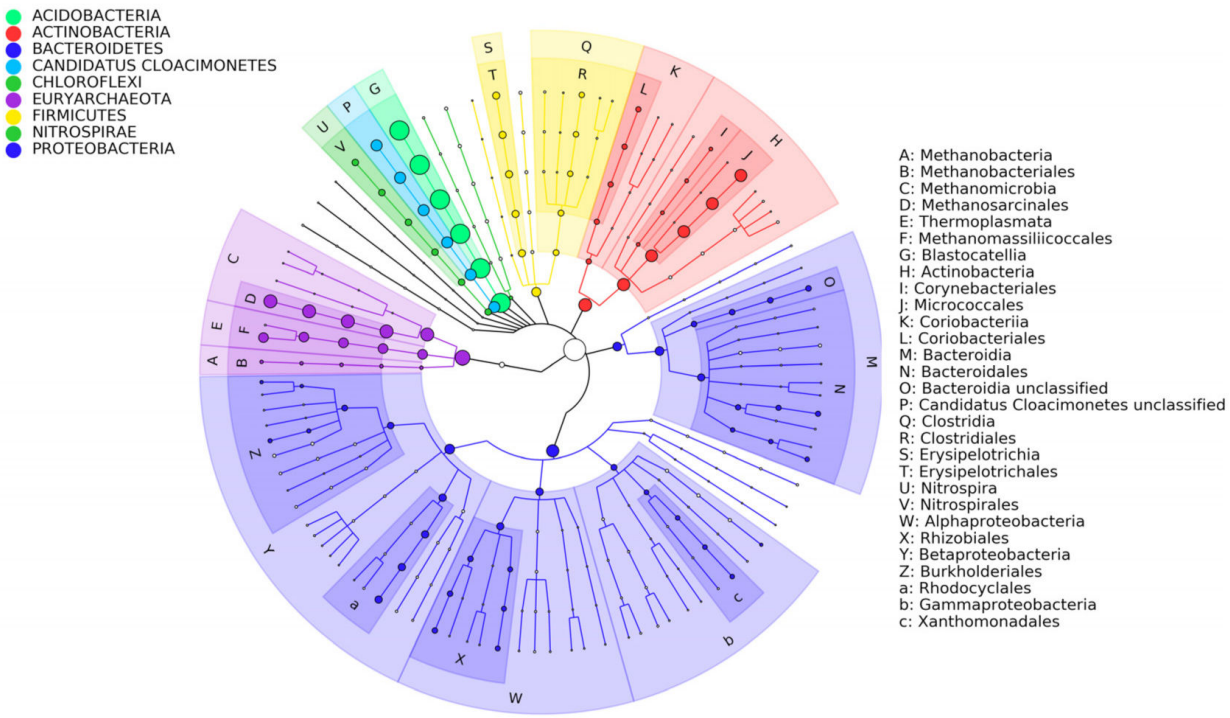

Figure 2. The structure of all microbial consortia in the sequenced samples of sewage sludge. Phylum nodes are colored, and branch areas are shaded. The first two outer tracks (with letters) indicate Classes and Orders. The next seven tracks (Species, Genera, Family, Order, Class, Phylum, Kingdom) describe relative bacterial community abundance calculated by Metaphlan software. The size of the node is relative to the abundance of each taxonomic level.

Bacteria of the phylum *Acidobacteria* were predominant in sewage sludge samples collected from both the control and process bioreactors, and the abundance of *Acidobacteria* differed significantly between substrates exposed to antibiotics and the control substrate. *Acidobacteria* are ubiquitous in soil ecosystems, and they are capable of decomposing various carbon sources, from simple sugars to complex substrates [60]. *Acidobacteria* are also highly prevalent in WWTPs [61]. The number of OTUs characteristic of *Acidobacteria* decreased gradually in the control and process bioreactors in the first three experimental series. A

comparison of the corresponding OTU values revealed a decrease of 10 ppm (parts per million) (D4.1 vs. D1.1) in process reactor and a decrease of 20 ppm (C4.1 vs. C1.1) in the control bioreactor (Figure 3A,B, Table S4).

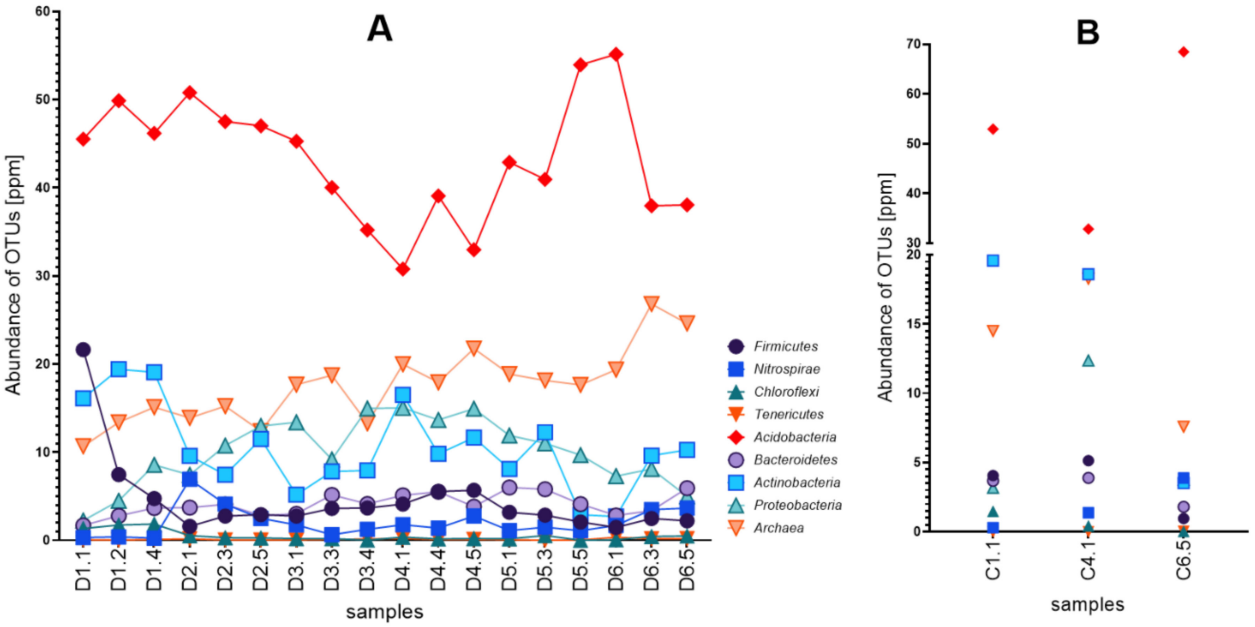

Figure 3. Abundance of OTUs [ppm] characteristic of the dominant microbial phyla in the process (**A**) and control bioreactors (**B**). D-dose ID, C-control samples.

During the fourth experimental series, the number of OTUs characteristic of *Acidobacteria* increased in samples collected from both process and control bioreactors. At the end of long-term anaerobic digestion, the abundance of *Acidobacteria* differed significantly in digestate samples from process and the control bioreactors and was almost twice higher in the control (38 and 68 ppm, respectively). The first five antibiotic doses (D1–D5) did not induce significant changes in the proportions of *Acidobacteria* during prolonged anaerobic digestion, whereas the last and highest dose (D6) significantly reduced the number of OTUs characteristic of *Acidobacteria*. These observations suggest that combined exposure to amoxicillin (36 µg/mL), ciprofloxacin (16 µg/mL) and metronidazole (16 µg/mL) significantly inhibited the growth of *Acidobacteria*. It should be noted that *Acidobacteria* were not identified as the predominant bacterial phylum in other studies analyzing microbial communities in sewage sludge subjected to methane fermentation [62–64]. This is the first study to demonstrate that *Acidobacteria*, including bacteria of the class *Blastocatellia* that are ubiquitous in activated sludge flocs [61], are predominant in sewage sludge during anaerobic digestion. These results suggest that sewage sludge from the process bioreactor contained surplus activated sludge from aeration chambers.

Bacterial phyla *Firmicutes* and *Actinobacteria* were also ubiquitous at the beginning of anaerobic digestion (D1/C1), and their abundance was determined at 21 and 16 ppm in sewage sludge samples from the process bioreactor and at 4 and 19 ppm in the control bioreactor, respectively. The high initial abundance of *Firmicutes* in the process bioreactor decreased under exposure to the tested antibiotics. The number of OTUs characteristic of *Firmicutes* decreased three-fold after the application of the second antibiotic dose (D2), and it decreased ten-fold at the end of the process (D6) relative to initial values (Figure 3A). A similar, but less pronounced trend was observed in control samples. In digestate samples collected at the end of the process (C6.5), the abundance of *Firmicutes* decreased four-fold relative to initial values (C1.1) (Figure 4B). The phylum *Firmicutes* was represented mainly by the orders *Erysipelotrichales* and *Clostridiales* which contain numerous bacterial species with hydrolytic capabilities [65].

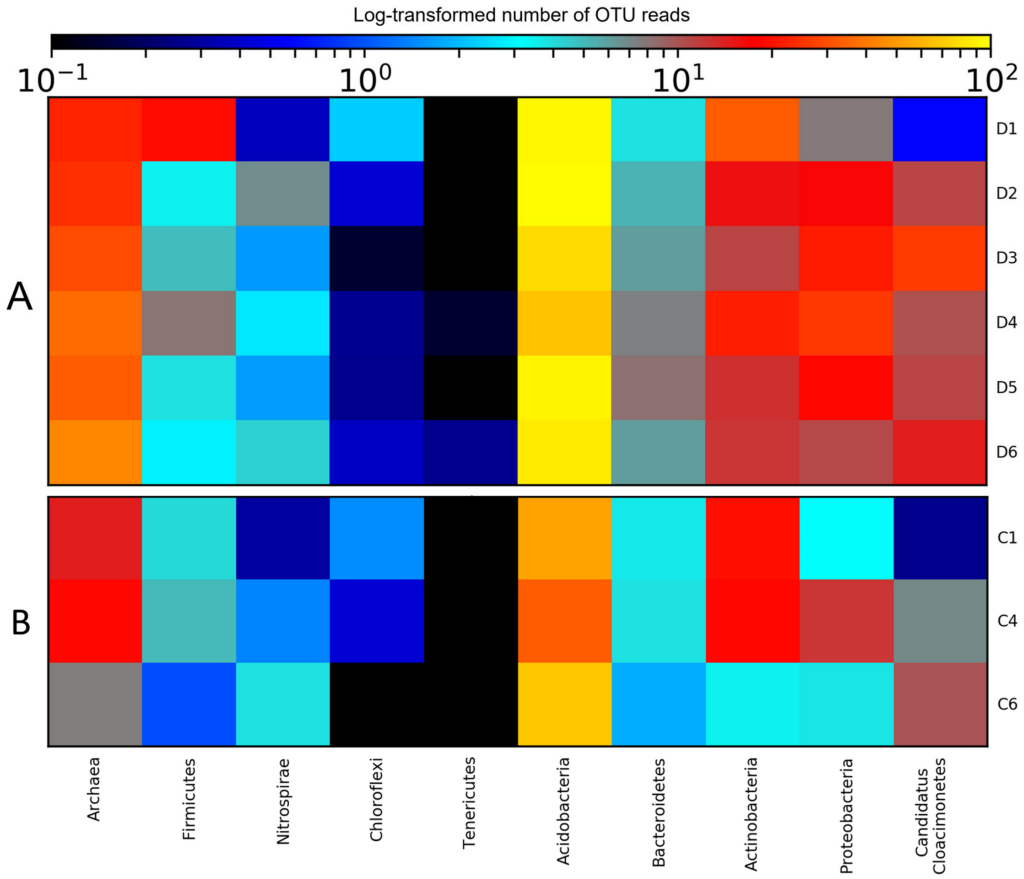

Figure 4. Heatmap with log-transformed number of OTU reads [ppm] at phylum level, presenting changes in microbial abundance in sewage sludge samples from process (**A**) and control bioreactors (**B**). The mean values noted in each experimental series used in the analysis. D—dose ID, C—control samples.

A comparison of sewage sludge samples collected from the process bioreactor at the beginning and end of long-term anaerobic digestion revealed that the initially predominant *Firmicutes* were gradually replaced by bacteria of the unclassified phylum *Candidatus Cloacimonetes* and phyla *Bacteroidetes* (order *Bacteroidales*) and *Proteobacteria* (orders *Burkholderiales*, *Rhizobiales*, *Nitrosomonadales* and *Myxococcales*) (Figure 4A). The phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* are characteristic of biomass in anaerobic digesters, and they contain many species of acidogenic bacteria [66]. Although *Candidatus Cloacimonetes* is a novel phylum, several papers indicated the importance of its presence in anaerobic digesters. This phylum has been implicated in extracellular hydrolysis and syntrophic propionate-oxidation [67–71]. As reported by Poirier et al. [70], the prevalence of *Cloacimonetes* is positively correlated with methane production efficiency. An analysis of the abundance of this phylum in sewage sludge samples at the beginning and at the end of long-term anaerobic digestion revealed an increase in the frequency of readings (from 0.42 to 9.6 and from 0.26 to 9.9 in the process and control bioreactors, respectively) (Figure 3A,B). During the entire process, the prevalence of *Candidatus Cloacimonetes* and *Proteobacteria* were similar in substrates sampled from process and control bioreactors, whereas the abundance of OTUs characteristic of *Bacteroidetes* differed significantly between samples collected from process and control bioreactors. At the end of anaerobic digestion, the abundance of *Bacteroidetes* was three times lower in samples of control digestate (C6.5) than in samples of experimental digestate (D6.5), whereas the number of OTUs characteristic of *Nitrospirae* increased (Figure 4B). According to Veloo et al. [72], bacteria of the order *Bacteroidales* harbor genetic determinants of resistance to β-lactam antibiotics. In our previous study [5], the abundance of *Bacteroidetes* OTUs in sewage sludge decreased in response to high concentra-

tions of metronidazole and ciprofloxacin, but increased under exposure to amoxicillin. In the present study, metronidazole and ciprofloxacin concentrations were two and four times lower, respectively, than amoxicillin concentrations, which could explain the observed increase in the proportion of *Bacteroidales* in sewage sludge from the process bioreactor.

The abundance of *Actinobacteria* was similar at the beginning of anerobic digestion in supplemented (D1.1) and control (C1.1) digestate, but it at the end of the process, the prevalence of *Actinobacteria* decreased significantly—more than five-fold in control samples (C6.5), but only 1.5-fold in supplemented samples. In sewage sludge, *Actinobacteria* were represented mainly by members of the order *Bifidobacteriales*, which are characteristic of gut microbiota and constitute a potential reservoir of ARGs [73,74], as well as members of the order *Corynebacteriales* which are less susceptible to antimicrobials due to the lower permeability of their cell walls [75,76]. The abundance of *Actinobacteria* was higher in digestate samples from the process bioreactor than in control samples, which could be probably attributed to selective pressure exerted by a mixture of the tested antibiotics as well as the lower susceptibility of *Actinobacteria* to these antimicrobials.

Methanogenic bacteria of the phylum *Euryarchaeota* are responsible for methanogenesis, the last stage of anaerobic digestion. These bacteria can be divided into three groups based on their methanogenesis pathways and the substrates utilized in methane production: (I) acetoclastic methanogens that use acetate to produce methane; (II) hydrogenotrophic methanogens that utilize formate and hydrogen, and (III) methylotrophic methanogens that rely on methylated compounds such as methanol, methyl sulfides and methylamines as substrates for methane production [66]. In the group of methanogenic archaea, only bacteria of the order *Methanosarcinales* are capable of metabolizing all three substrates. *Methanosarcinales* are also the only acetoclastic methanogens to have been identified to date. The group of methylotrophic methanogens includes *Methanosarcinales* (hydrogen-independent), *Methanomassiliicoccales*, and one species of the order *Methanobacteriales* (hydrogen-dependent) [77–79].

The sequencing analysis of sewage sludge samples from process and control bioreactors supported the identification of four orders of methanogens: *Methanosarcinales* and *Methanomicrobiales* of the class *Methanomicrobia*, *Methanomassiliicoccales* of the class *Thermoplasmata*, and *Methanobacteriales* of the class *Methanobacteria*. The abundance of OTUs characteristic of methanogens was similar in process and control bioreactors at the beginning of anaerobic digestion. Bacteria of the order *Methanosarcinales* were predominant in the first experimental series (D1/C1) in samples of supplemented (10 ppm) and control digestate (14 ppm) (Figure 5A,B, Table S5). In other studies, acetoclastic methanogens were also predominant in anaerobic digesters, or acetoclastic methanogenesis was the main pathway during the methane production process [80,81]. The number of OTUs characteristic of the orders *Methanobacteriales* and *Methanomicrobiales* was low in all analyzed samples. These bacterial orders are associated with the hydrogenotrophic pathway of methane production [82], which suggests that this methanogenic pathway was not predominant in the experiment. Significant differences ($p < 0.05$) in the abundance of *Methanosarcinales* and *Methanomassiliicoccales* were noted in samples of process and control digestate over time. In control samples, the proportion of *Methanosarcinales* decreased significantly from 14 ppm at C1 to <2 ppm at C6 (Figure 5B), whereas the abundance of OTUs characteristic of *Methanomassiliicoccales* increased from <1 ppm at C1 to >5 ppm at C6. However, the total abundance of *Archaea* in control digestate was relatively low (<10 ppm), and prolonged anaerobic digestion led to a significant decrease in the number of OTUs characteristic of this microbial kingdom (Figures 3B and 4B).

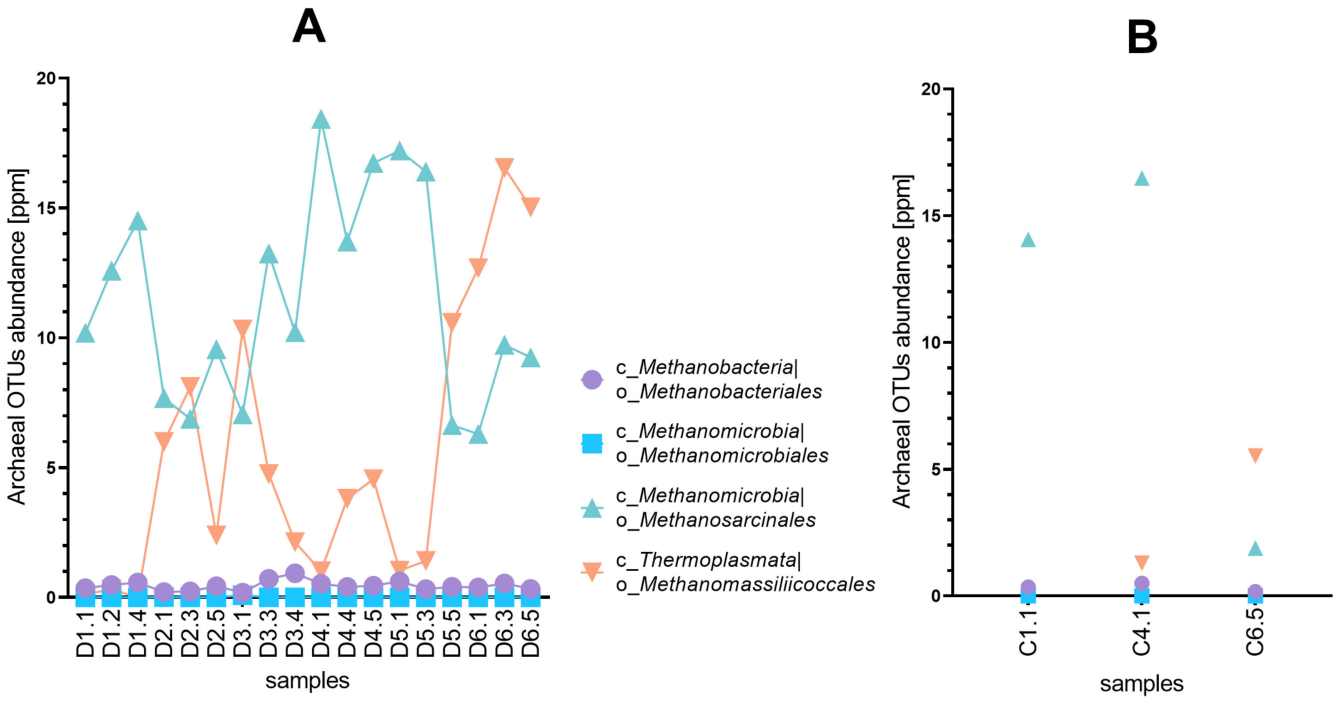

Figure 5. Abundance of OTUs [ppm] characteristic of different orders of the kingdom *Archaea* in the process (**A**) and control bioreactors (**B**). D—dose ID, C—control samples.

The analysis of genome sequences in samples collected from the process bioreactor revealed that the second antibiotic decreased the abundance of *Methanosarcinales* by 50% and significantly increased the proportion of *Methanomassiliicoccales* (from <1 ppm at D1 to 8 ppm at D2) (Figure 5A). The third antibiotic dose (D3) increased the abundance of *Methanosarcinales*, but significantly decreased the prevalence of *Methanomassiliicoccales*. The fifth antibiotic dose (D5) significantly decreased the abundance of OTUs characteristic of *Methanosarcinales* and increased the proportion of *Methanomassiliicoccales*, and this trend was maintained until the end of anaerobic digestion. The abundance of *Methanosarcinales* and *Methanomassiliicoccales* was bound by a significant negative correlation ($R2 = -0.68$; $p < 0.05$) (Table S6). Other researchers have observed that dynamic changes in microbial communities and microbial adaptation to changing environmental conditions are essential for the stable operation of anaerobic digesters [64,83].

At the end of the anaerobic digestion process, the abundance of *Methanosarcinales* (9 ppm) and *Methanomassiliicoccales* (15 ppm) was considerably higher in digestate samples collected from the process bioreactor than from the control bioreactor (<2 and >5 ppm, respectively). The total abundance of *Archaea* was significantly higher in the last sample (D6.5) of sewage sludge supplemented with antibiotics (>24 ppm) in comparison with the first sample (D1.1). Moreover, the abundance of *Archaea* was nearly 2.5 times higher in process digestates than in control digestates (<10 ppm) sampled at the end of the anaerobic digestion process (Figure 3A,B). These results indicate that the prevalence of *Archaea* in the process digester increased steadily in response to growing concentrations of the tested antibiotics (Figure 4A). Therefore, antibiotic exposure increased the proportion of *Archaea* in the process bioreactor relative to control, whereas methane production efficiency decreased significantly during the anaerobic digestion process.

An analysis of the prevalence of the predominant methanogens in sewage sludge supplemented with antibiotics revealed that various methanogenic pathways predominated during the anaerobic digestion process. Every decrease in the proportion of *Methanosarcinales* was accompanied by an increase in the prevalence of *Methanomassiliicoccales*. According to the literature [81,84], the presence of specific methanogenic inhibitors can increase the activity of other *Archaea* that utilize different substrates to produce methane. There-

fore, it can be assumed that hydrogenotrophic, acetoclastic or methylotrophic (hydrogen-independent) pathways were the dominant methanogenic pathways when the structure of archaeal communities was dominated by *Methanosarcinales*. In turn, in periods characterized by the highest abundance of *Methanomassiliicoccales*, methane was produced mainly via the hydrogen-dependent methylotrophic pathway. Zhang et al. [85] reported on the high activity of the order *Methanomassiliicoccales* and its significant role in efficient methane generation. It should also be noted that *Methanomassiliicoccales* clearly dominated over *Methanosarcinales* under exposure to the highest antibiotic doses (D5 and D6), and this trend was maintained until the end of the anaerobic digestion process. Moreover, a comparison of *Methanomassiliicoccales* abundance in the last samples of experimental and control digestates (D6.5 and C6.5) demonstrated that this bacterial order was three times more prevalent in supplemented digestate. The analysis of changes in the structure of microbial communities indicates that the hydrogen-dependent methylotrophic pathway could significantly contribute to the stability of methane generation in the presence of antimicrobial substances.

The anaerobic digestion process is characterized by dynamic changes in the populations of various microbial groups that are directly or indirectly involved in methane production [66]. The sequencing analysis revealed significant differences in the structure of microbiota between samples of control and supplemented sewage sludge during the experiment. Antibiotic exposure induced changes in the structure of microbial populations; therefore, the correlations between dominant bacterial phyla in supplemented samples were analyzed statistically. A correlation analysis was not performed in the control digestate because only a small number of control samples were subjected to sequencing analysis. In sewage sludge exposed to antibiotics, significant positive correlations were observed between the abundance of *Archaea* and the prevalence of hydrolytic *Bacteroidetes* and acetogenic *Tenericutes* (Figure 6, Table S7). The proportions of *Bacteroidetes* and *Tenericutes* increased steadily in successive experimental series of anaerobic digestion. The abundance of *Acidobacteria* decreased in response to higher antibiotic doses, and it was negatively correlated with *Archaea* and with *Bacteroidetes* and *Proteobacteria* whose prevalence increased over time. A significant negative correlation was observed between the abundance of *Candidatus Cloacimonetes*, which increased steadily during the first three experimental series of anaerobic digestion, and the abundance of *Actinobacteria*, *Firmicutes* and *Chloroflexi*, which decreased over this time. Moreover, a significant positive correlation was noted between *Actinobacteria* and *Firmicutes* whose prevalence decreased steadily under exposure to antibiotics.

3.3. The Prevalence of Antibiotic Resistance Genes in Anaerobic Digesters

The influence of anaerobic digestion on ARG profiles was examined by several authors [26,27]. A number of studies also evaluated the impact of antimicrobials on the spread of ARGs in bioreactors [86,87]. The observed variations in the profile and abundance of ARGs under exposure to antibiotics can be attributed to changes in the structure of microbial communities inside the bioreactor as well as HGT processes. It should be noted that both intracellular and extracellular ARGs can participate in HGT. Zou et al. (2020) [88] reported a decrease in the abundance of some ARGs during the methane fermentation of sewage sludge. In turn, other authors [86,87] concluded that the diversity and abundance of ARGs are enhanced in the presence of selected antibiotics during anaerobic fermentation. Our previous study demonstrated that high individual doses of metronidazole, amoxicillin and ciprofloxacin significantly modified the overall ARG profile during the methane fermentation of sewage sludge [5].

The sequencing analysis supported the identification of ten classes of ARGs encoding resistance to aminoglycosides, bacitracin, β-lactams, macrolide-lincosamide-streptogramin (MLS) antibiotics, polymyxins, rifamycins, sulfonamides, tetracyclines and vancomycin, as well as genes encoding multidrug efflux pumps. Sewage sludge samples collected from both process and control bioreactors (Figure 7A,B, Table S8) were characterized by a pre-

dominance of genes conferring resistance to multiple drugs and MLS antibiotics (>20 ppm). Tetracycline and bacitracin resistance genes were also abundant (5–15 ppm). The antibiotics tested in this study, which were selected based on our previous research [5], belonged to the classes of β-lactams, fluoroquinolones and nitroimidazoles. However, fluoroquinolone and nitroimidazole resistance genes were not highly prevalent in samples of process and control digestate. Moreover, the abundance of genes encoding resistance to β-lactams, including amoxicillin, was very low (Figure 8A,B). Selective pressure exerted by antibiotics did not specifically target genes encoding resistance to various classes of antimicrobials.

The analysis of changes in the abundance of different ARGs in process and control digestate revealed significant changes in the ARG profile during long-term anaerobic digestion. However, a comparison of the first and last samples of experimental and control digestates (D1.1 vs. C1.1 and D6.5 vs. C6.5, respectively) demonstrated that exposure to the tested combination of antibiotics applied at relatively low and increasing concentrations did not induce significant changes in the abundance of specific ARG groups. Total ARG abundance decreased in both process and control (Table S8) digestates at the end of fermentation. In both bioreactors, average ARG abundance was similar at the beginning and end of anaerobic digestion (91 ppm at D1 and C1; 86 and 87 ppm at D6 and C6, respectively) (Figure 8A,B, Table S8). However, it should be noted that the prevalence of multidrug resistance genes increased in samples of supplemented sewage sludge, whereas a reverse trend was observed in control samples (Table S8) during long-term methane fermentation. The transfer of multidrug resistance genes between bacteria poses a serious public health threat around the world [89].

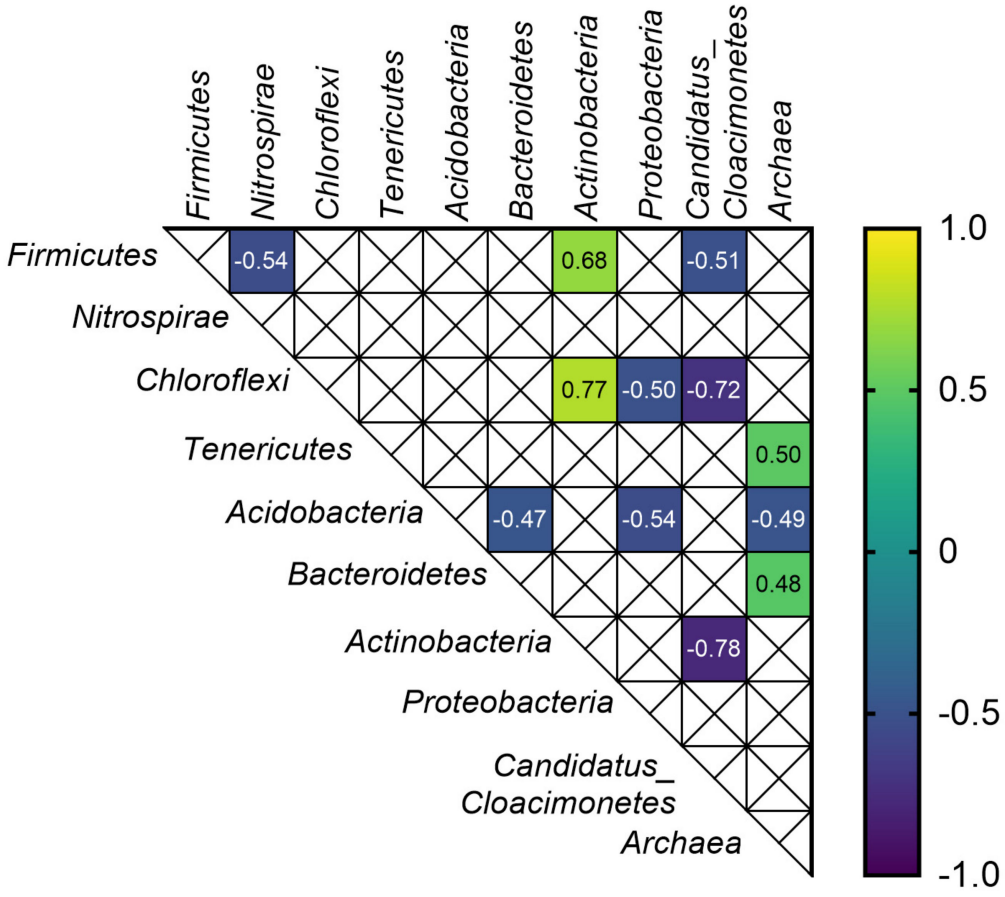

Figure 6. Significant correlations between dominant microorganisms in the process bioreactor. Positive correlations are marked in green, and negative correlations are marked in blue. Color intensity is proportional to the correlation coefficients.

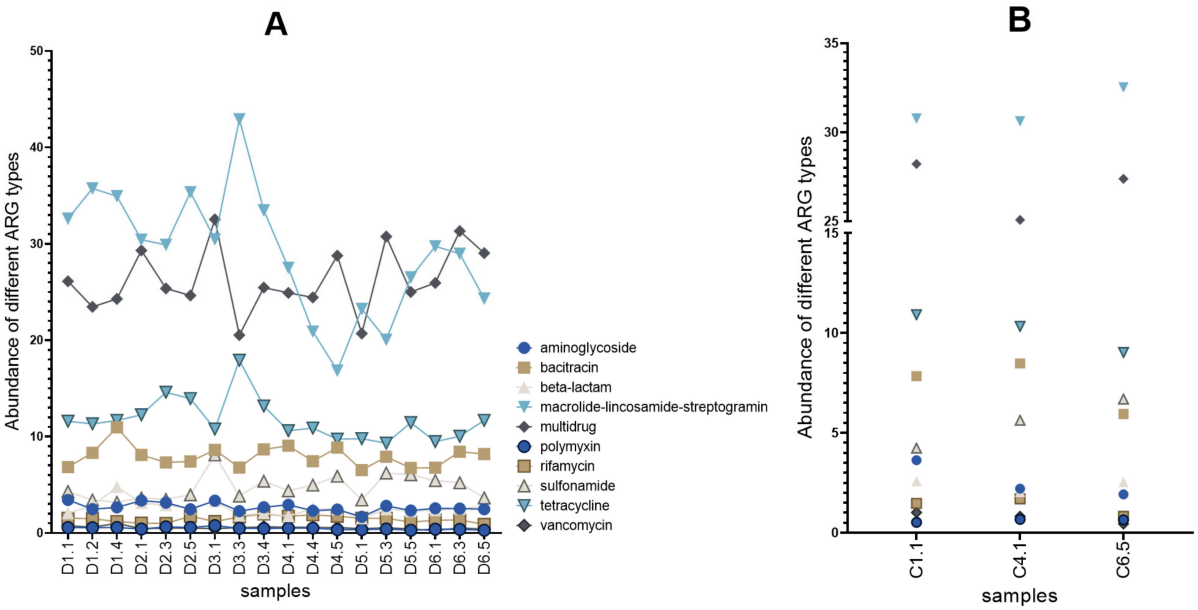

Figure 7. Abundance of ARGs [ppm] in process (**A**) and control bioreactors (**B**). The presented scale differs from that shown in Figure 3. D—dose ID, C—control samples.

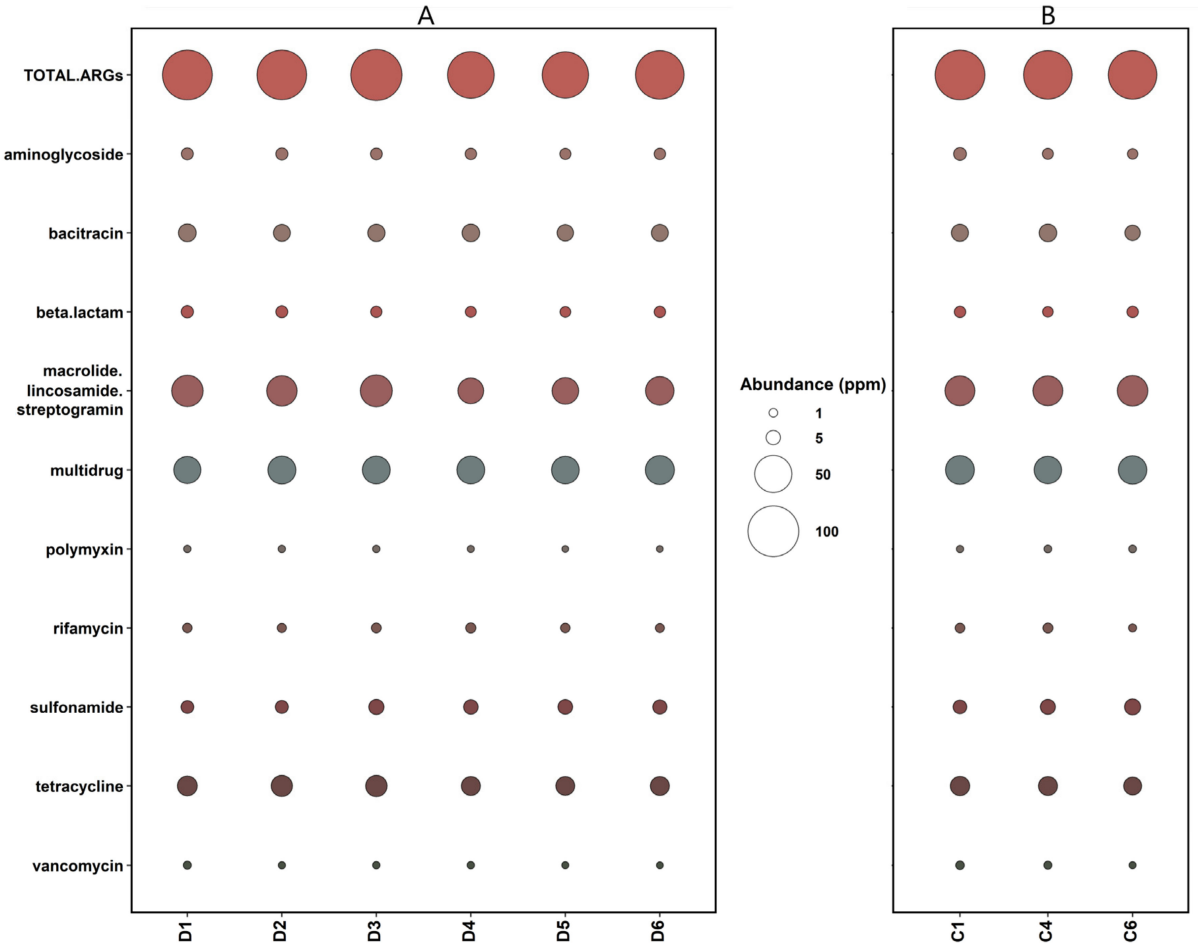

Figure 8. A bubble plot presenting changes in ARG abundance [ppm] in sewage sludge samples from process (**A**) and control bioreactors (**B**). The mean values noted in each experiment were used in the analysis. D-dose ID, C-control samples, ARGs-antibiotic resistance genes.

A number of studies analyzed antibiotic concentrations in wastewater flowing into WWTPs as well as in sewage sludge which is utilized as a substrate for methane fermentation [40]. Metronidazole, amoxicillin and ciprofloxacin were detected in wastewater entering WWTPs at concentrations of 69–250, 120–280 and 184–1260 ng/L, respectively [40,90]. These values exceed the predicted no-effect concentrations (PNEC) (125, 250 and 64 ng/L, respectively) suggested by Bengtsson-Palme and Larsson [91]. The predicted no-effect concentration is a concentration of an antibiotic which, when exceeded, can promote the spread of antibiotic resistance in the environment. Bacteria present in the process bioreactor could have adapted to metronidazole, amoxicillin and ciprofloxacin within the concentration ranges analyzed in this study. These observations also point to the absence of synergistic reactions between the tested antimicrobial substances.

3.4. Correlations between Anaerobic Digestion Parameters, Microbial Biodiversity and Selected ARGs

The data relating to methane production and the abundance of different ARGs and microbial groups in the process bioreactor during long-term anaerobic digestion were processed statistically by principal component analysis (PCA) (Figure 9). The first two principal components (PC1 and PC2) explained 40.3% and 19.7% of the variance, respectively. Three distinct clusters were identified. The first cluster contained OTUs characteristic of *Bacteroidetes*, *Proteobacteria*, *Candidatus Cloacimonetes* and genes encoding resistance to sulfonamides and rifamycin. The phyla *Bacteroidetes* and *Proteobacteria* represented by hydrolytic and acidogenic bacteria, and *Candidatus Cloacimonetes* as a potential acetogenic microorganism were grouped together with methane production. This cluster also contained the results obtained in experimental series D3, D4 and D5, which were characterized by an increase in methane production and the highest average abundance of sulfonamide and rifamycin resistance genes (Figure 7A,B). The results obtained in the first experimental series (D1) formed a separate cluster containing the phyla *Firmicutes*, *Actinobacteria* and *Chloroflexi*, as well as genes encoding resistance to polymyxins, bacitracin and vancomycin. The abundance of the above phyla (Figure 4A) and ARGs (Table S8) was highest during exposure to the lowest concentrations of the tested antibiotics (D1), and it decreased in successive experimental series of long-term anaerobic digestion. Moreover, a correlation between the prevalence of the phyla *Actinobacteria* and *Chloroflexi*, and the abundance of ARGs encoding resistance to vancomycin was visualized in Circos (Figure S2). The last cluster contained the results obtained in the final experimental series (D6), the abundance of multidrug resistance genes, and the prevalence of the phyla *Tenericutes* and *Nitrospirae* and the kingdom *Archaea* which increased in response to the highest doses of the tested antibiotics. It indicates that the share of a particular phylum of microorganisms varied depending on the experimental series and the dose of the mixture of antibiotics. These changes resulted in various methane production and changes in the ARG pool.

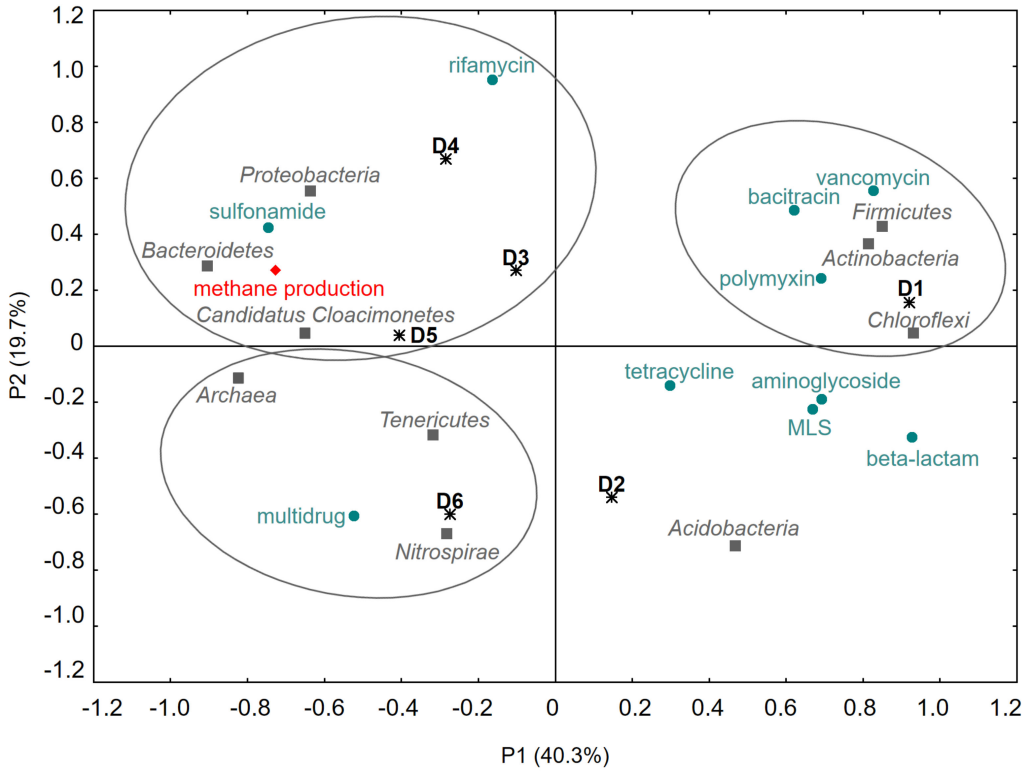

Figure 9. Ordination analysis of samples from the process bioreactors (D1–D6). Principal component analysis (PCA) of microbial abundance, ARG abundance and methane production. D-dose ID.

4. Conclusions

Long-term supplementation of sewage sludge with a combination of metronidazole, amoxicillin and ciprofloxacin applied at different doses did not induce significant changes in the concentrations of VFAs, or the total abundance of ARGs. However, the tested antimicrobials significantly decreased methane production and modified the structure of microbial populations. Moreover, exposure to antibiotics significantly increased the number of OTUs characteristic of *Archaea* in sewage sludge, but these changes did not affect the efficiency of biogas production. The observed changes in the composition of methanogenic consortia suggest that the hydrogen-dependent methylotrophic pathway of methanogenesis may significantly contribute to the stability of anaerobic digestion in the presence of antimicrobial substances.

The predominance of the phylum *Acidobacteria* in bioreactors also indicates that the effect of antimicrobials on the anaerobic digestion of sewage sludge is determined not only by the presence of antibiotics, but also by substrate and the substrate-specific composition of microbiota. Moreover, this study provides further evidence to support the importance of *Candidatus Cloacimonetes* in anaerobic digestion, which has been reported in the literature.

The greatest changes in microbial diversity were noted in supplemented sewage sludge exposed to the highest dose of the tested antibiotics, relative to control. These findings suggest that higher concentrations of antimicrobials in sewage sludge can substantially compromise the efficiency of anaerobic digestion. Antibiotic consumption is growing steadily worldwide, which increases antimicrobial concentrations in wastewater flowing into WWTPs and in sewage sludge. Exposure to antibiotics may compromise the efficiency of methane fermentation of this substrate.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en15051826/s1, Table S1: The mean values of pH, FOS/TAC ratio, TS, VS, TP and TN in each of the experimental series in the control (CB) and process (PB) bioreactors. TS—total solids, vs.—volatile solids, TP—total phosphorus, TN—total nitrogen; Table S2: Differences

in methane production between samples from process and control bioreactors (two-way ANOVA; $p < 0.05$); Table S3: Differences in VFA (volatile fatty acid) content between samples from process and control bioreactors (two-way ANOVA; $p < 0.05$); Table S4: Sequencing analysis data presenting the abundance of dominant bacterial phyla in all sequenced samples, expressed in reads per million reads [ppm]; Table S5: Sequencing analysis data presenting the abundance of four orders of methanogens in all sequenced samples, expressed in reads per million reads [ppm]; Table S6: Correlations between the occurrence of *Archaea* in the process bioreactor (Spearman's rank correlation coefficient; significant results are marked in red, $p < 0.05$); Table S7: Correlations between the occurrence of bacterial phyla in the process bioreactor (Spearman's rank correlation coefficient; significant results are marked in red, $p < 0.05$); Table S8: Sequencing analysis data presenting the abundance of ARG classes in all sequenced samples, expressed in reads per million reads [ppm]; Figure S1: Content of VFAs (g/L) in bioreactors containing antimicrobials and in the control bioreactor. C—control samples, D—dose ID, VFAs—volatile fatty acids. For example, "C/D 1.1" indicates samples C1.1 and D1.1 (green and blue points on the line graph, respectively); Figure S2: The taxonomic distribution of microbial diversity and ARG abundance in all sequenced samples of sewage sludge. The links describe the Pearson correlation (>0.7, $p < 0.05$) between the abundance of ARGs and the relative abundance of the bacterial community on different taxonomic levels.

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Oświadczam, że w pracy:

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mój wkład polegał współudziale w planowaniu eksperymentów laboratoryjnych i prac bioinformatycznych. Byłam odpowiedzialna za przeprowadzenie eksperymentów dotyczących analizy rozpowszechnienia genów oporności na antybiotyki. Współuczestniczyłam w analizie i interpretacji wszystkich wyników badań, przedstawionych w niniejszej pracy. Miałam wiodący udział w zaplanowaniu oraz przeprowadzeniu systematycznego przeglądu literatury. Byłam odpowiedzialna za wizualizację wyników oraz opracowanie tabel i rycin. Przygotowałam pierwszą wersję manuskryptu oraz uczestniczyłam w jego korekcie po procesie recenzji.

Małgorzata Czatzkowska

Olsztyn, 12.07.2023

Prof. dr hab. inż. Monika Harnisz
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i Mikrobiologii Środowiskowej
Wydział Geoinżynierii
Uniwersytet Warmińsko-Mazurski w Olsztynie

OŚWIADCZENIE

Oświadczam, że w pracy:

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mój wkład polegał na określeniu zadań bawaczych i metod. Uczestniczyłam w planowaniu doświadczeń, analizie i interpretacji wyników. Nadzorowałam tworzenie manuskryptu, proces recenzji oraz korektę pracy przed złożeniem do druku. Ponadto, pełniłam funkcję kierownika projektu, z którego sfinansowano badania opisane w niniejszym manuskrypcie.

Monika Harnisz

Olsztyn, 12.07.2023

Prof. dr hab. Ewa Korzeniewska
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Wydział Geoinżynierii
Uniwersytet Warmińsko-Mazurski w Olsztynie

OŚWIADCZENIE

Oświadczam, że w pracy:

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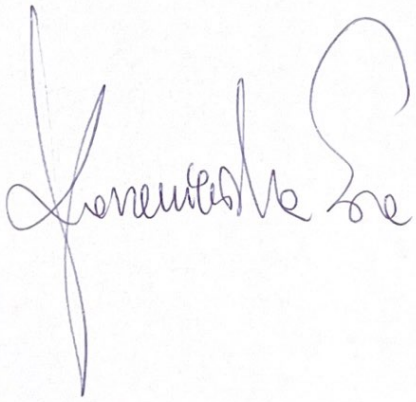
mój wkład polegał na udziale w opracowaniu metod i konceptualizacji. Dodatkowo, udzielałam konsultacji przy tworzeniu pierwszej wersji manuskryptu.



Olsztyn, 12.07.2023

Mgr Izabela Wolak
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Uniwersytet Warmińsko-Mazurski w Olsztynie

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mój wkład polegał na udziale w analizie rozpowszechnienia genów oporności na antybiotyki. Uczestniczyłam również w opracowaniu pierwszej wersji manuskryptu.

Izabela Wolak

Olsztyn, 12.07.2023

dr Paulina Rusanowska
Katedra Inżynierii Środowiska
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Uniwersytet Warmińsko-Mazurski w Olsztynie

OŚWIADCZENIE

Oświadczam, że w pracy:

Czatzkowska M., Harnisz M., Korzeniewska E., Wolak I., **Rusanowska P.**, Paukszto Ł., Jastrzębski J. P., Bajkacz S. 2022. Long-Term, Simultaneous Impact of Antimicrobials on the Efficiency of Anaerobic Digestion of Sewage Sludge and Changes in the Microbial Community. Energies, 15, 5, 1826, https://doi.org/10.3390/en15051826

mój wkład polegał na nadzorowaniu technologicznej części eksperymentów, których wyniki przedstawiono w niniejszym artykule. Dodatkowo, uczestniczyłam w tworzeniu pierwszej wersji manuskryptu oraz w korekcie pracy przed złożeniem do druku.

Paulina Rusanowska

Załącznik nr 4

Appendix 4

Czatzkowska M., Wolak I., Korzeniewska E., Harnisz M.

Anaerobic Digestion in the Presence of Antimicrobials—Characteristics of Its Parameters and the Structure of Methanogens

Applied Sciences

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Anaerobic Digestion in the Presence of Antimicrobials—Characteristics of Its Parameters and the Structure of Methanogens

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Abstract: Antibiotics are widely used in human and veterinary medicine, and they are accumulated in various types of waste, including sewage sludge (SS) and cattle slurry (CS), processed by anaerobic digestion (AD). Anaerobic treatment is a method enabling the stabilization of these substrates before transferring to the environment. The presence of contaminants, such as antimicrobials, in organic substrates processed by AD is not regulated by law. The accumulation of antimicrobials in SS and CS is a crucial issue because it may reduce the effectiveness of their stabilization. This study aimed to evaluate the long-term impact of growing concentrations of a mixture of antibiotics on the AD of SS and CS. Methane (CH_4) yield, which is the main indicator of the efficiency of AD, was determined. Antibiotic exposure significantly decreased CH_4 production only in SS (by 5–8% relative to control; $p < 0.05$). The copy numbers of the *mcr*A gene, a functional marker of methanogenesis, were not reliable indicators of CH_4 yields in either substrate. During long-term AD, the average concentrations of the *mcr*A gene were determined at 10^8 in 1 g of SS digestate and from 10^8 to 10^9 in 1 g of CS digestate samples. At the end of long-term AD, methanogens belonging to the family *Methanosarcinaceae* were more prevalent than methanogens of the family *Methanosaetaceae* both in SS and CS samples (10^7 and 10^8–10^9 gene copies in 1 g of digestate, respectively).

Keywords: anaerobic digestion; antibiotics; methane production; sewage sludge; cattle slurry

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1. Introduction

Recycling waste is becoming a relevant challenge, combined with an important aspect of the environment. The reuse of organic waste has been supported by the approval of Directive 2018/851/EC of the European Parliament, which regulates waste utilization management by supporting the principles of the circular economy and promoting the minimization of waste production [1,2]. Anaerobic digestion (AD) is a popular method of stabilizing organic matter such as sewage sludge (SS) and cattle slurry (CS), enabling receive mainly biomethane and fertilizers [3,4] which leads to the production of methane (CH_4). Methanogens, mostly microorganisms belonging to the families of *Methanosaetaceae* and *Methanosarcinaceae*, directly participate in CH_4 synthesis [5,6]. *Methanosarcinaceae* rely on H_2, CO_2, or acetate to produce CH_4, and they easily adapt to changing environmental conditions [7]. In turn, methanogens of the family *Methanosaetaceae* are resistant to elevated concentrations of acetate [8]. However, the presence of antibiotics and their metabolites can compromise the efficiency of AD [9,10]. Drugs and their transformation products can inhibit AD by altering the structure of microbial communities and decreasing CH_4 yields. The *mcr*A gene encoding methyl-coenzyme M reductase is specific to methanogens, and it can be used as a functional marker to evaluate the efficiency of AD. The efficiency of the analyzed process can also be indirectly determined based on methanogen counts, mostly microorganisms belonging to the families of *Methanosarcinaceae* and *Methanosaetaceae* [11,12].

The overuse of antibiotics in human and veterinary medicine, as well as in livestock and crop farming, increases the concentrations of antibiotics and their metabolites in municipal sewage and CS that are discharged to the environment [13]. The stability of these substances in biomass and the environment poses a significant problem. Municipal sewage containing antibiotics is processed in wastewater treatment plants (WWTPs). Pharmaceuticals are accumulated in SS, which becomes a reservoir of antibiotics [14,15]. In turn, excessive antibiotic use in veterinary medicine promotes the spread of antimicrobials in animal wastes such as CS. The medicated premixes are the main source of contamination with antibiotic residues. Antimicrobials are used both in the treatment and prevention of infectious diseases [16]. Substrates, such as SS and CS, which contain antibiotics and their metabolites, are processed by AD and often reused in agronomy [16,17]. Although there are European legal limits for contamination of stabilized organic matter before its disposal in the environment (Directive 86/278/EEC), these do not include antibiotics. The presence of antimicrobial substances in SS and CS before stabilization is also not legally controlled. Importantly, due to the growth of the human population and the intensification of animal production, higher production of SS and CS is predicted [2]. Since the worldwide production of antibiotics is still rising [18], the accumulation of antimicrobials in these substrates may reduce the effectiveness of their stabilization.

Research has demonstrated that raw wastewater often contains amoxicillin, ciprofloxacin, and metronidazole [19], whereas amoxicillin, metronidazole, and enrofloxacin have been detected in CS [20]. These antibiotics are widely used in human and veterinary medicine, but they are not always effectively absorbed in the intestines. As a result, 30–90% of the parent compound may be excreted from the body in the unmodified form [21] and transferred to the environment with SS and CS. The presence of antibiotics in these substrates may affect the efficiency of their anaerobic treatment. Worrying is that the scope and consequences of antibiotic pollution have not yet been fully elucidated, especially concerning substrates particularly liable to the accumulation of antimicrobial substances. Considering the above, we resolved to fill the gap in scientific knowledge concerning the evaluation of the synergistic action of commonly used antibiotics in the anaerobic treatment of two commonly stabilized organic substrates.

This study aimed to evaluate the long-term impact of exposure to growing concentrations of a mixture of antibiotics on the AD of SS and CS. The influence of antibiotics present in SS and CS on CH_4 yields was analyzed. The copy numbers of genes specific to methanogens (*mcr*A) were determined to evaluate their effect on the efficiency of AD. Moreover, the prevalence of families of the order *Methanosarcinales*, *Methanosaetaceae*, and *Methanosarcinaceae* was detected using group-specific methanogenic primers. The study result expands our knowledge about the AD of various substrates and the extent to which antimicrobials contribute to decreasing the effectiveness of this process. Thereby the study provides new information supporting the optimization of anaerobic treatment to obtain the highest possible efficiency at low financial and environmental costs.

2. Materials and Methods

2.1. Substrates and Inoculum

Sewage sludge from the WWTP in Olsztyn (Poland) and CS from a farm in Bałdy (Poland) were the substrates for AD. On the farm, 90% and 10% pose dairy cattle and horses, respectively. In the WWTP, 80% of the incoming sewage is domestic and household sewage and 20% is industrial sewage. The average daily flow in the sewage treatment plant is 60,000 cubic meters per day. Sewage sludge from the WWTP was used as the inoculum in the AD of both SS and CS. Fermentation in the WWTP is carried out by populations of saprophytic and methane bacteria under stable, optimal conditions in the chambers, which include: the temperature within 33–35 °C, pH between 6.8–7.5, load with organic substances 10–25 days, hydration, and intensive agitation of the sediment. The characteristics of substrates were variable during the long-term experiment. The initial characteristics of SS, CS, and the inoculum are presented in Table 1.

Table 1. Characteristics of the substrates and the inoculum used in the AD process.

	TS [a] g_D^{-1} [b] (mg)	VS [c] g_D^{-1} (mg)	pH	TP [d] g_{TS}^{-1} [e] (mg)	TN [f] g_{TS}^{-1} (mg)
SS [g]	52.0 ± 11.37	39.92 ± 9.59	6.35 ± 0.3	2.02 ± 0.48	4.32 ± 1.61
CS [h]	107.46 ± 29.01	84.23 ± 22.17	7.75 ± 0.42	0.95 ± 0.26	4.10 ± 1.64
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}—the value of parameter per one gram of digestate samples; [c] VS—volatile solids; [d] TP—total phosphorus; [e] g_{TS}^{-1}—the value of parameter per one gram of TS; [f] TN—total nitrogen, [g] SS—sewage sludge, [h] CS—cattle slurry.

2.2. Anaerobic Digestion Process

The AD of SS and CS was conducted in 2 L bioreactors operating under semi-continuous, dynamic conditions. The initial substrate to inoculum ratio was 0.05 (VS basis). The substrates (CS or SS) fed to the process bioreactors (PB) were supplemented with a combination of antimicrobials. Control substrates without antibiotics were fed to the control bioreactors (CB). The experiments were conducted in two replications. Antibiotics were selected based on the results of our previous studies [19,20] investigating the effects of individual doses of different antimicrobials on AD. The cited studies demonstrated that in SS supplemented with only one antibiotic, amoxicillin (AMO), ciprofloxacin (CIP), and metronidazole (MET) exerted the greatest effect on CH_4 yields and the structure of microbial communities, whereas, in bioreactors filled with CS, the most potent antimicrobials were AMO, enrofloxacin (ENR) and MET. Therefore, in the present study, PB containing SS were supplemented with a mixture of AMO, CIP, and MET, and those containing CS with a mixture of AMO, ENR, and MET in gradually increasing doses (Table 2). The study was divided into experimental series with different antibiotic concentrations: six series in bioreactors containing SS and seven series in bioreactors containing CS. Antibiotic doses were increased after the digester's hydraulic volume had doubled. Each experimental series lasted 45 and 59 days on average, and the entire experiment lasted 268 and 417 days for SS and CS, respectively.

Table 2. Concentrations of antimicrobials (Dose ID) added to SS and CS in successive experimental series.

			Antibiotic Concentrations (μg mL^{-1})			
Substrate	**Series**	**Dose ID**	**AMO**	**CIP**	**MET**	**ENR**
SS	1	D1	1	0.25	0.25	X
	2	D2	2	0.5	0.5	
	3	D3	4	1	1	
	4	D4	8	4	4	
	5	D5	16	8	8	
	6	D6	36	16	16	
CS	1	D1	1	X	0.25	0.25
	2	D2	2		0.5	0.5
	3	D3	2.5		0.75	0.75
	4	D4	5		1.5	1.5
	5	D5	10		3	3
	6	D6	16		4	4
	7	D7	32		8	8

The bioreactors were operated at an organic loading rate of 2.8 g vs. $L \cdot d^{-1}$. Hydraulic retention time (HRT) was 22 and 28 days for SS and CS, respectively. The digesters were equipped with a mechanical stirrer and a feeding and discharge system, and they were connected to an automatic methane potential test system (AMPTS II) (Bioprocess Control, Lund, Sweden) which measured the amount of the produced CH_4. Gas was normalized for standard pressure and temperature (1.01325 bar and 273.2 K). Methane quality was

analyzed in a gas chromatograph equipped with a thermal conductivity detector (GC-TCD, Agilent Technologies 7890 A, Irving, TX, USA). The bioreactors were placed in a water bath to simulate mesophilic conditions (37 °C).

The pH of digestate samples, the FOS/TAC ratio (where FOS denotes the content of volatile fatty acids (VFAs), and TAC is the estimated buffer capacity of the sample), and the content of total solids (TS), volatile solids (VS), total nitrogen (TN) and total phosphorus (TP) were determined to monitor the AD process. The content of VFAs and other process parameters were analyzed with the use of a previously described method [22].

2.3. Sampling

In all experimental series, digestate samples were sampled at approximately weekly intervals from both PB and CB containing SS and CS. A total of 68 SS samples and 96 CS samples were collected. The ID numbers assigned to the samples in each experimental series are presented in Table 3.

Table 3. Identification numbers (Samples No. 1–No. 9) assigned to SS and CS samples collected from process (PB) and control (CB) bioreactors during the AD process in each experimental series (Series 1–7).

Series	Samples	SS		CS	
		PB [a]	CB [b]	PB	CB
		Sample ID			
1	1	SS D1.1	SS C1.1	CS D1.1	CS C1.1
	2	SS D1.2	SS C1.2	CS D1.2	CS C1.2
	3	SS D1.3	SS C1.3	CS D1.3	CS C1.3
	4	SS D1.4	SS C1.4	CS D1.4	CS C1.4
	5			CS D1.5	CS C1.5
	6		x	CS D1.6	CS C1.6
2	1	SS D2.1	SS C2.1	CS D2.1	CS C2.1
	2	SS D2.2	SS C2.2	CS D2.2	CS C2.2
	3	SS D2.3	SS C2.3	CS D2.3	CS C2.3
	4	SS D2.4	SS C2.4	CS D2.4	CS C2.4
	5	SS D2.5	SS C2.5	CS D2.5	CS C2.5
	6		x	CS D2.6	CS C2.6
3	1	SS D3.1	SS C3.1	CS D3.1	CS C3.1
	2	SS D3.2	SS C3.2	CS D3.2	CS C3.2
	3	SS D3.3	SS C3.3	CS D3.3	CS C3.3
	4	SS D3.4	SS C3.4	CS D3.4	CS C3.4
	5			CS D3.5	CS C3.5
	6		x	CS D3.6	CS C3.6
4	1	SS D4.1	SS C4.1	CS D4.1	CS C4.1
	2	SS D4.2	SS C4.2	CS D4.2	CS C4.2
	3	SS D4.3	SS C4.3	CS D4.3	CS C4.3
	4	SS D4.4	SS C4.4	CS D4.4	CS C4.4
	5	SS D4.5	SS C4.5	CS D4.5	CS C4.5
	6		x	CS D4.6	CS C4.6
5	1	SS D5.1	SS C5.1	CS D5.1	CS C5.1
	2	SS D5.2	SS C5.2	CS D5.2	CS C5.2
	3	SS D5.3	SS C5.3	CS D5.3	CS C5.3
	4	SS D5.4	SS C5.4	CS D5.4	CS C5.4
	5	SS D5.5	SS C5.5	CS D5.5	CS C5.5
	6		x	CS D5.6	CS. C5.6

Table 3. *Cont.*

Series	Samples	SS		CS	
		PB [a]	CB [b]	PB	CB
		Sample ID			
6	1	SS D6.1	SS C6.1	CS D6.1	CS C6.1
	2	SS D6.2	SS C6.2	CS D6.2	CS C6.2
	3	SS D6.3	SS C6.3	CS D6.3	CS C6.3
	4	SS D6.4	SS C6.4	CS D6.4	CS C6.4
	5	SS D6.5	SS C6.5	CS D6.5	CS C6.5
	6	x		CS D6.6	CS C6.6
7	1	x		CS D7.1	CS C7.1
	2			CS D7.2	CS C7.2
	3			CS D7.3	CS C7.3
	4			CS D7.4	CS C7.4
	5			CS D7.5	CS C7.5
	6			CS D7.6	CS C7.6

[a] PB—process bioreactor, [b] CB—control bioreactor.

2.4. Genomic DNA Isolation from Digestate Samples

Digestate samples of 2 g each were transferred to 2 mL Eppendorf centrifuge tubes (Eppendorf, Germany) and were 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 the Fast DNA Spin Kit for Soil ® (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. The concentration and quality of the extracted genetic material were determined in the Multiskan SkyHigh microplate spectrophotometer (Thermo Scientific™, Waltham, MA, USA). gDNA from digestate samples was stored in a freezer (−20 °C) for qPCR analysis.

2.5. Analysis of Gene Characteristics for Methanogens

The counts and activity of methanogenic microorganisms were determined by Real-Time PCR (qPCR). This method was used to determine the prevalence of two methanogen families of the order *Methanosarcinales*: *Methanosaetaceae* and *Methanosarcinaceae*, both responsible for CH_4 production. The group-specific methanogenic primers have been used [23]. A meaningful share of other genera in the domain *Archaea*, including *Methanobacteriales*, *Methanococcales*, and *Methanomicrobiales*, were eliminated in a preliminary analysis (data not shown). The activity of methanogenic microorganisms was evaluated by estimating the concentration of the gene encoding methyl-coenzyme M reductase (*mcrA*), which catalyzes methanogenesis, the last step of AD. Standard curves were plotted before gene quantification based on serial dilutions of samples with known copy numbers of the examined genes. Amplicons were cloned from positive controls in vector pCR2.1-TOPO (Invitrogen, Waltham, MA, USA). The abundance of genes characteristic for methanogenic *Archaea* (*mcr*A gene, *Methanosaetaceae*- and *Methanosarcinaceae*-specific genes) during the AD of SS and CS was determined with the LightCycler® instrument (Roche Diagnostics, Mannheim, Germany) with LightCycler ® software (version 1.5.0). The concentrations were expressed by the copy number in 1 g of digestate ($g_D{}^{-1}$). All qPCR reactions were performed according to the methodology described in our previous study [20]. Reaction conditions and primer sequences [23,24] are presented in Table S1 in the Supplementary Materials.

2.6. Data Analysis

Data were processed statistically in Statistica 13.1 (Statsoft, Krakow, Poland). Differences in CH_4 production, VFA content, and target gene copy numbers were determined by two-way ANOVA. The results were regarded as statistically significant at $p < 0.05$. The correlations between gene concentration and CH_4 production in PB and CB containing SS

and CS were visualized in principal component analysis (PCA). The distribution of genes in the analyzed substrates was visualized in charts and heatmaps developed with the use of GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. The Long-Term Impact of Antibiotics on CH4 Production and mcrA Gene Abundance

In PB containing SS, exposure to increasing concentrations of AMO, CIP, and MET significantly decreased the efficiency of CH_4 production (by 5–8%) relative to control ($p < 0.05$) during long-term AD (Table S2). However, CH_4 production trends were similar in both experimental and control samples of SS (Figure S1). The amount of CH_4 produced from SS during AD was 176 and 182 L kg VS^{-1} on average in PB and CB, respectively. Average CH_4 yields were significantly higher in PB than in CB (by 6%; 10 L kg VS^{-1}) only in the third experimental series. The greatest differences in the efficiency of biogas production between PB and CB were noted in SS supplemented with AMO, CIP, and MET doses of 2, 0.5, and 0.5 μg mL^{-1} (series 2) and 8, 4, and 4 μg mL^{-1} (series 4), respectively (Figure 1).

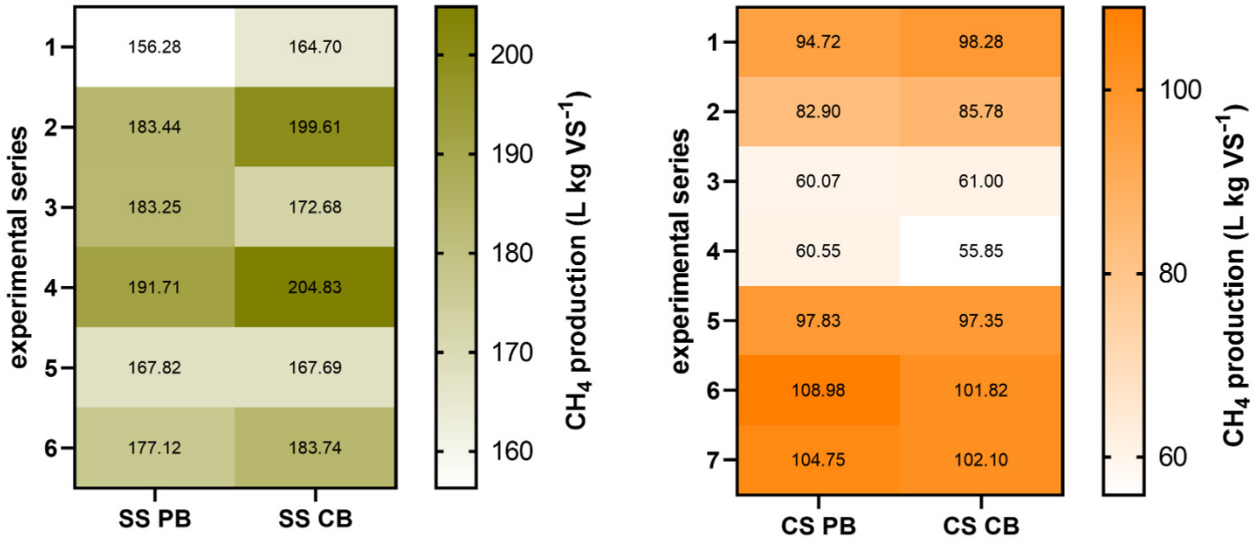

Figure 1. Heatmap presenting average methane production (L kg VS^{-1}) in each experimental series in process (PB) and control bioreactors (CB) containing sewage sludge (SS) and cattle slurry (CS).

No significant differences in CH_4 production during AD were found between PB and CB containing CS ($p > 0.05$) (Figure 1 and Figure S1, Table S2). In all seven experimental series, the average CH_4 yields were more than twice lower in PB and CB containing CS (87 and 86 L kg VS^{-1}, respectively) than in PB and CB containing SS. These results indicate that the tested antibiotics did not significantly affect the AD of CS and that CS fermentation was significantly less effective than SS fermentation in terms of CH_4 yields.

The organic matter in CS is more difficult to digest than the organic matter in SS [25]. Importantly, in this study, the substrate such as CS was characterized by a particular variability of individual parameters depending on the sampling time during a long-term experiment. The values of vs. and TS were variable, depending on the intensity of precipitation and the season of the year, which resulted in high standard deviations of these values (Table 1). Moreover, both SS and CS represent different environments characterized above all by diverse communities of microbes. Large amounts of bacterial and archaeal populations are involved in the complex microbiological process, such as AD. There are numerous studies indicating the benefits of anaerobic co-digestion of various types of organic waste, among others SS and manure [25–27]. The literature shows that co-digestion can enable the rise of organic matter levels, improve the activity of individual microorganisms in the anaerobic system and the stability of anaerobic biomass, and finally increase the efficiency of methane production.

In a previous study analyzing the influence of high individual antibiotic doses on the AD of SS and CS [5], biogas yields in both tested substrates were significantly lower under exposure to the tested drugs. The accumulation of VFAs in PB also testified to the inhibitory effects exerted by antibiotics on various stages of AD. In the present study, no significant differences in VFA concentrations ($p > 0.05$) were observed between SS and CS samples collected from PB and CB (Table S3). These results indicate that the mixtures of AMO, CIP, and MET as well as AMO, ENRO, and MET did not exert synergistic effects within the tested range of drug concentrations. According to other authors [28], the AD process is inhibited, and its efficiency declines rapidly only when a threshold concentration of antibiotics has been reached. Moreover, the fact that the differences between the average CH_4 yields in PB and CB containing SS decreased during long-term AD could be attributed to the gradual acclimation of methanogenic microorganisms to antimicrobials [29].

The efficiency of AD was monitored not only by measuring CH_4 and VFA production but was also evaluated at the molecular level. The *mcr*A gene encoding methyl-coenzyme M reductase is specific to methanogens. Changes in the concentration of the *mcr*A gene could point to fluctuations in methanogen activity which is crucial for the AD process. Therefore, the *mcr*A gene is regarded as a specific molecular marker of AD, and its abundance testifies to the presence and activity of methanogens in samples from various environments [30]. In the current study, *mcr*A concentrations in SS and CS samples collected from PB and CB differed across the experimental series. During long-term AD, the average concentrations of the *mcr*A gene were determined at 4.5×10^8 and 1.3×10^8 gene copies in 1 g_D^{-1} of SS samples collected from PB and CB, respectively, and at 7.6×10^8 and 1.0×10^9 gene copies in 1 g_D^{-1} of CS samples collected from PB and CB, respectively (Figure 2).

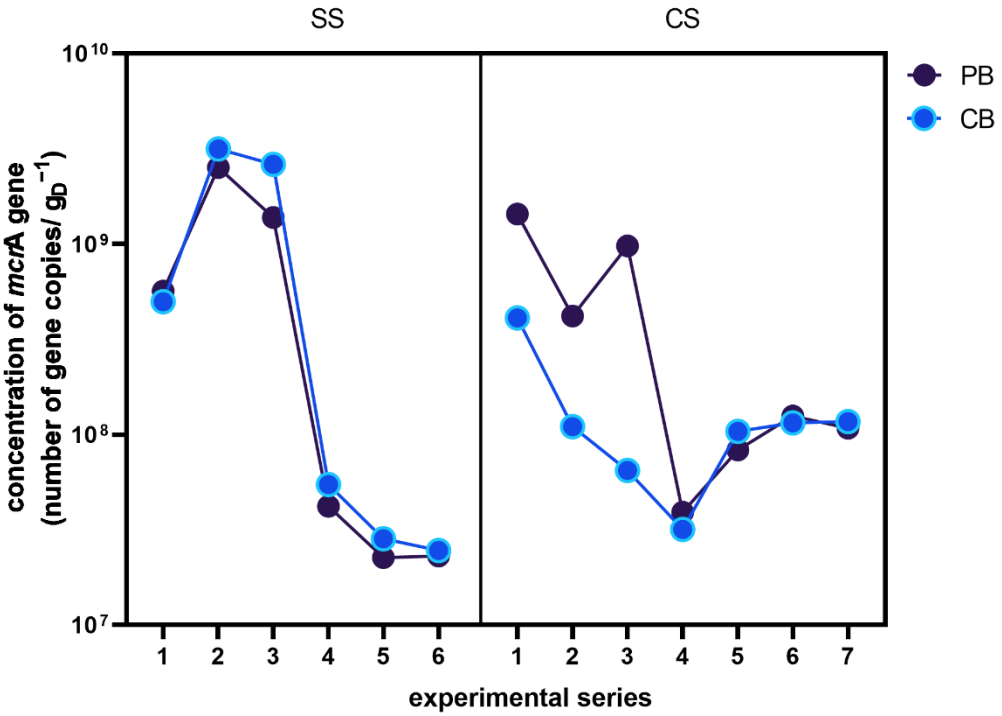

Figure 2. Prevalence of the *mcr*A gene in sewage sludge (SS) and cattle slurry (CS) samples from process (PB) and control bioreactors (CB). The mean values noted in each experimental series were used.

Similar trends in *mcr*A abundance were observed in SS samples collected from PB and CB in successive experimental series (Figure 2). The concentration of *mcr*A increased in the first experimental series, but it declined gradually in the following series (2–6). Significant differences in *mcr*A levels were observed between CS samples collected from PB and CB in the first three experimental series. In these series, *mcr*A concentrations decreased over

time and were significantly higher in PB than in CB. It should be noted that CH_4 yields were comparable in all experimental series in both PB and CB. In CS samples collected from PB and CB, *mcr*A abundance followed a similar increasing trend from the beginning of the fourth experimental series until the end of the experiment. In both substrates, significant differences in *mcr*A concentrations were observed between PB and CB (Table S2). In SS samples, the concentration of the *mcr*A gene and CH_4 yields were higher in control samples. In turn, the efficiency of AD was comparable in CS samples regardless of drug supplementation, and average *mcr*A abundance was significantly higher in CS samples from PB. The scatter plot generated in the principal component analysis (PCA) indicates that CH_4 production in bioreactors containing SS and CS was not correlated with the prevalence of *mcr*A during long-term AD (Figure 3).

This long-term experiment confirmed that changes in CH_4 yields could not be reliably measured based on the abundance of the *mcr*A gene, which is characteristic of methanogens. Despite the fact that this gene has been proposed as a bioindicator for monitoring methanogen activity [31], some authors have reported significant differences between *mcr*A abundance and the concentrations of *mcr*A transcripts in fermented substrates [32,33]. Therefore, the abundance of *mcr*A transcripts should be taken into consideration to improve the reliability of analyses examining AD efficiency at the molecular level.

3.2. Real-Time PCR Quantification of Genes Specific to Methanogens

Methanogenic microorganisms are responsible for the last stage of AD-methanogenesis. Methanogenic members of *Archaea* can be divided based on their methanogenesis pathways; acetoclastic, hydrogenotrophic, and methylotrophic. Among the methanogens, only the order *Methanosarcinales* is capable of methane production through all three pathways. Moreover, *Methanosarcinales* are also the only acetoclastic methanogens that have been identified to date [34–36]. Bacteria belonging to both *Methanosarcinaceae* and *Methanosaetaceae* families can perform acetoclastic methanogenesis. In this pathway, acetate is activated to acetyl-CoA, either by the combined action of transacetylase and acetate kinase or by the activity of acetyl-CoA synthetase, in the case of *Methanosarcinaceae* and *Methanosaetaceae*, respectively [37]. The abundance of methanogens can be indicative of AD efficiency [4]. The prevalence of the above methanogens are affected by the composition of the fermented substrate and the presence of inhibitors such as antibiotics [5]. The presence of specific methanogenic inhibitors can promote the activity of some methanogenic *Archaea* that can perform different pathways to produce methane. In this study, the structure of archaeal communities was dominated by families belonging to the order *Methanosarcinales*, so it can be assumed that acetoclastic, hydrogenotrophic, or methylotrophic pathways could be dominant in CH_4 production.

In SS samples from PB and CB, the copy numbers of gene characteristics for *Methanosarcinaceae* remained stable at 10^4 $g_D{}^{-1}$ in most experimental series (Figure 4a). In the last sixth experimental series, *Methanosarcinaceae*-specific genes increased to 10^6 in SS samples from both PB and CB. No significant changes ($p > 0.05$) in the copy numbers of gene characteristics for *Methanosarcinaceae* were found during the AD of SS (Table S2). The copy numbers of that gene point to a stable increase in *Methanosarcinaceae* counts and activity during SS fermentation regardless of antibiotic supplementation [23].

Cattle slurry samples were characterized by significantly higher ($p < 0.05$) variations in the copy numbers of the gene characteristic for *Methanosarcinaceae* relative to SS samples (Figure 4b; Table S2). Interestingly, in samples collected in the third, sixth, and seventh experimental series, copy numbers of *Methanosarcinaceae*-specific genes were one order of magnitude higher in samples exposed to antibiotics (10^5, 10^6, and 10^6 $g_D{}^{-1}$, respectively) than in control samples (10^4, 10^5, and 10^5 $g_D{}^{-1}$, respectively). The presence of higher doses of antibiotics disrupted the structure of methanogens, promoting the growth of microorganisms exhibiting the greatest adaptation. According to the literature, microorganisms belonging to the family *Methanosarcinaceae* have a high level of metabolic capability [6,38]. In comparison to *Methanosaetaceae*, *Methanosarcinaceae* are characterized

by faster growth and more effective CH_4 production, also under unfavorable conditions inside bioreactors [11].

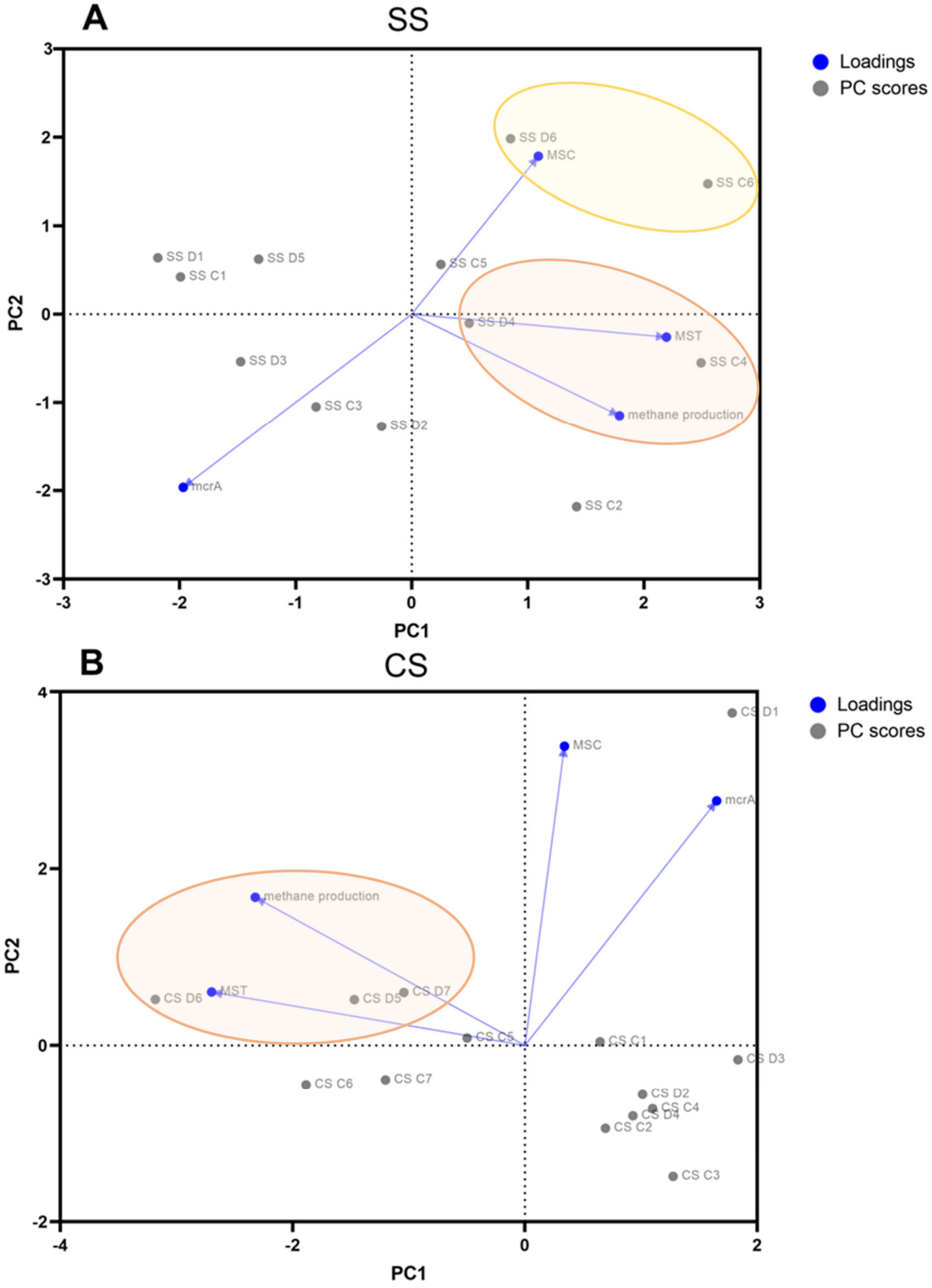

Figure 3. Ordination analysis of sewage sludge ((**A**) SS) and cattle slurry ((**B**) CS) samples from process and control bioreactors. Principal component analysis (PCA) of the abundance of *mcr*A gene, genes characteristics for *Methanosarcinaceae* (MSC), *Methanosaetaceae* (MST), and methane production. D—dose ID, C—control.

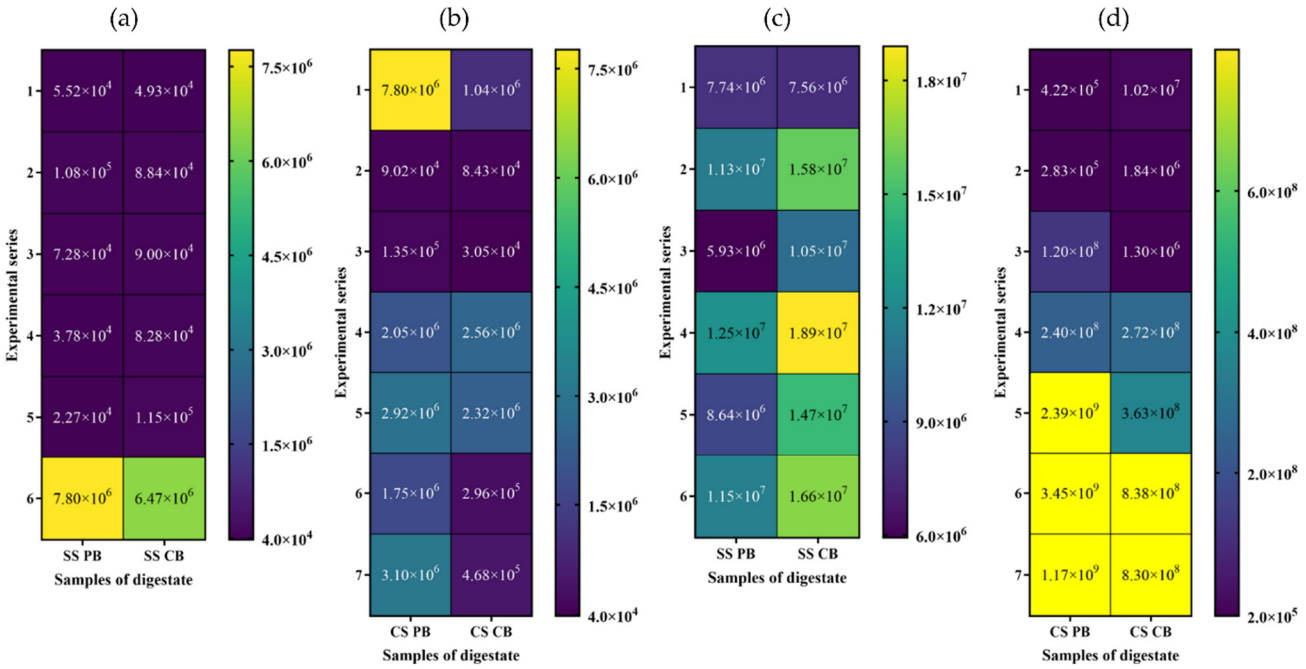

Figure 4. Average abundance of specific genes in digestate samples exposed to a mixture of antibiotics (PB) and in control samples (CB): (**a**) gene characteristic for *Methanosarcinaceae* in SS samples, (**b**) gene characteristic for *Methanosarcinaceae* in CS samples, (**c**) gene characteristic for *Methanosaetaceae* in SS samples, and (**d**) gene characteristic for *Methanosaetaceae* in CS digestate samples.

In SS samples collected from both PB and CB during six experimental series, the copy numbers of gene characteristics for *Methanosaetaceae* remained fairly stable at around 10^6–10^7 g_D^{-1}. The average copy number of *Methanosaetaceae*-specific genes decreased significantly ($p > 0.05$) by one order of magnitude only in the third and fifth experimental series relative to control samples (10^7 g_D^{-1}) (Figure 4c; Table S2). This observation could imply that increasing antibiotic doses added to the SS bioreactor in successive experimental series led to sudden disruptions in the AD process. However, the copy numbers of gene characteristics for *Methanosaetaceae* were similar in SS samples collected from PB and CB (10^7 g_D^{-1}) in the last experimental series, which suggests that microbial consortia adapted to growing drug concentrations [39].

Significant changes in gene copy numbers were observed in CS samples collected from PB in successive experimental series ($p > 0.05$) (Table S2). In the first series, the number of gene copies characteristic of *Methanosaetaceae* was lower in experimental samples (10^5 g_D^{-1}) than in control samples (10^7 g_D^{-1}) (Figure 4d). In the second series, the number of that gene copies was also one order of magnitude lower in experimental samples than in control samples, where it reached 10^6 g_D^{-1}. In the fourth series, the number of *Methanosaetaceae*-specific genes in CS samples exposed to 5 μg mL^{-1} of AMO and 1.5 μg mL^{-1} of ENR and MET was similar to that noted in control samples (10^8 g_D^{-1}), and it was one order of magnitude higher (10^9 g_D^{-1}) in the remaining series (3, 5–7). The last experimental series of CS treatment was characterized by the predominance of genes characteristic of *Methanosarcinaceae* and *Methanosaetaceae* in samples exposed to drugs related to the control. It proves the adaptation of methanogens belonging to the order *Methanosarcinales* to the presence of inhibitors. However, the copy number of *Methanosaetaceae*-specific genes was three orders of magnitude higher than that of *Methanosarcinaceae*-specific genes.

The scatter plot generated in the PCA shows a correlation between the last experimental series and the presence of *Methanosaetaceae* in SS samples and *Methanosarcinaceae* in CS samples (Figure 3). At the end of the experiment, both SS and CS samples were characterized by a predominance of *Methanosaetaceae*, which were probably the most involved in methane production (orange clusters). However, the prevalence of *Methanosarcinaceae*

in the last experimental series of SS treatments increased intensely (yellow cluster). The predominance of a given methanogenic family in the substrate is determined by the origin of organic matter [40], the ability to metabolize various substrates by methanogenesis (Lackner et al., 2016), and environmental factors such as the presence of inhibitors [41], which may ultimately affect the efficiency of treatment. According to the literature, some authors [42] observed the predominance of methanogens belonging to *Methanosarcinaceae*, while other authors [43] noted that *Methanosaetaceae* was the dominant methanogenic family in fermented organic matter.

Microbial balance is also essential for efficient CH_4 production [44]. The increase in CH_4 yields with a rise in antibiotic concentrations can be attributed to the stable growth of *Methanosarcinaceae* and *Methanosaetaceae* populations. Traversi et al. (2011) [45] reported a positive correlation between *Methanosaetaceae* counts and biogas production during the AD of the organic fraction of solid municipal waste and SS, which confirms that this methanogen family plays a key role in CH_4 production. To sum up, we noted the variability in the dominance of individual families of methanogens in SS and CS and showed that the methanogen community and CH_4 production might be closely related to the type of substrate or the presence and concentrations of inhibitors such as drugs.

4. Conclusions

This experiment demonstrated that the extent to which long-term exposure to antibiotics influences the effectiveness of AD is dependent on the type of processed substrate. The conditions inside the bioreactor and the type of substrate also determine the structure of microbial communities, including methanogens. The dominance of individual methanogens in bioreactors influences the variety of principal methanogenesis pathways, which affects the efficiency of treatment. Due to the still increasing consumption of antibiotics and their accumulation in organic matter, it is advisable to monitor the substrates processed by AD for antibiotic concentration. The study also revealed that assessments of methanogen activity based on the abundance of the *mcr*A gene at the molecular level should also involve analyses of *mcr*A transcriptomes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12178422/s1, Table S1: Oligonucleotide primers and PCR reaction profile; Table S2: Differences in methane production (a), the concentration of the mcrA gene (b), Methanosarcinaceae- (c) and Methanosaetaceae-specific genes (d) between samples of sewage sludge and cattle slurry from process and control bioreactors (two-way ANOVA; $p < 0.05$); Table S3: Mean content of volatile fatty acids (VFAs) (gL^{-1}) in process bioreactors (PB) containing antimicrobials and in the control bioreactor (CB). The table presents the results of two-way ANOVA (differences in VFA concentration between samples of sewage sludge and cattle slurry from PB and CB); Figure S1: Methane production (L kg VS^{-1}) in process (PB) and control bioreactors (CB) containing sewage sludge (SS) and cattle slurry (CS). Refs [23,24] are cited in the Supplementary Materials.

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

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Oświadczam, że w pracy:

Czatzkowska M., Wolak I., Korzeniewska E., Harnisz M. 2022. Anaerobic Digestion in the Presence of Antimicrobials—Characteristics of Its Parameters and the Structure of Methanogens. Applied Sciences, 12, 17, 8422, https://doi.org/10.3390/app12178422

mój wkład polegał współudziale w planowaniu eksperymentów laboratoryjnych. Byłam odpowiedzialna za przeprowadzenie eksperymentów dotyczących analizy rozpowszechnienia genów oporności na antybiotyki w osadach ściekowych. Współuczestniczyłam w analizie i interpretacji wyników badań. Miałam wiodący udział w zaplanowaniu oraz przeprowadzeniu systematycznego przeglądu literatury. Byłam odpowiedzialna za wizualizację wyników podczas opracowywania rycin. Uczestniczyłam w przygotowaniu pierwszej wersji manuskryptu oraz jego korekcie po procesie recenzji.

Małgorzata Czatzkowska

Olsztyn, 12.07.2023

Mgr Izabela Wolak
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Uniwersytet Warmińsko-Mazurski w Olsztynie

OŚWIADCZENIE

Oświadczam, że w pracy:

Czatzkowska M., **Wolak I.**, Korzeniewska E., Harnisz M. 2022. Anaerobic Digestion in the Presence of Antimicrobials—Characteristics of Its Parameters and the Structure of Methanogens. Applied Sciences, 12, 17, 8422, https://doi.org/10.3390/app12178422

mój wkład polegał na udziale w analizie i interpretacji wyników badań oraz opracowaniu pierwszej wersji manuskryptu. Dodatkowo, współuczestniczyłam w opracowaniu tabel i rycin.

Olsztyn, 12.07.2023

Prof. dr hab. Ewa Korzeniewska
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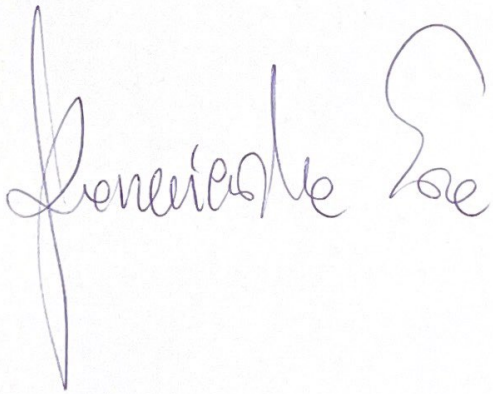
mój wkład polegał na udziale w opracowaniu metod i konceptualizacji. Dodatkowo, udzielałam konsultacji przy tworzeniu pierwszej wersji manuskryptu.



Olsztyn, 12.07.2023

Prof. dr hab. inż. Monika Harnisz
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mój wkład polegał na określeniu zadań bawaczych i metod. Uczestniczyłam w planowaniu doświadczeń, analizie i interpretacji wyników. Nadzorowałam tworzenie manuskryptu, proces recenzji oraz korektę pracy przed złożeniem do druku. Ponadto, pełniłam funkcję kierownika projektu, z którego sfinansowano badania opisane w niniejszym manuskrypcie.

Monika Harnisz