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Anaerobic Digestion of Cattle Manure Contaminated with an Antibiotic Mixture: A Nature-Based Solution for Environmental Management

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Abstract: Anaerobic digestion (AD) is a waste-to-energy strategy that leverages natural microbiological processes. It is increasingly used in farms to treat manure, resulting in biogas for energy production and digestate as fertiliser. However, animal manure often contains antibiotic (AB) residues, raising concerns about their impact on AD efficiency and their potential spread through digestate use. This multidisciplinary study evaluated the effects of an AB mixture (enrofloxacin, ciprofloxacin and sulfamethoxazole) on CH_4 production, microbial community (Fungi, Bacteria and Archaea) dynamics and antibiotic resistance gene (ARG) presence. The experiment used a cattle manure/digestate ratio of 1:35, typical of real digesters, with AB concentrations set at low (2.5 mg kg^{-1} each) and high (7.5 mg kg^{-1} each) levels. The ABs affected cumulative CH_4 production (ranging from 5939 to 6464 mL) only at the highest concentration. After 51 days, sulfamethoxazole reached residual levels, while enrofloxacin and ciprofloxacin were only partially degraded (<50%), but ARGs were significantly reduced. The microbial community, particularly prokaryotes, exhibited resilience, maintaining efficient CH_4 production. Overall findings strongly suggest that AD is an effective treatment for producing energy and good fertiliser, also reducing AB and ARG content as well as mitigating CH_4 emissions into the atmosphere.

Keywords: methane production; manure; digestate; antibiotic removal; microbial components; antibiotic resistance genes

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1. Introduction

The worldwide introduction of antibiotic (AB) therapies around 1950 greatly extended people's life expectancy, and ABs' use in veterinary practice made it possible to increase livestock production to meet the food needs of an ever-growing world population. However, livestock farming proved to be one of the most important sources of AB contamination [1–3]. Indeed, ABs are not fully metabolised by treated animals and can be released in faeces and urine in their original form (60–90%) or as active metabolites [4,5]. Thus, the common

practice of using cattle manure for soil fertilisation can be a source of ABs [6], as well as of antibiotic-resistant bacteria and antibiotic resistance genes (ARGs), which may impact agricultural ecosystems and human health through the consumption of contaminated fresh vegetables [5,7]. This possibility is of serious concern, especially considering the expected global growth of livestock farming in the near future, which may increase, along with the estimated consumption of ABs in developed countries' livestock farms, accounting for 50–80% of total AB production [1–3]. Currently, several cattle farms are employing anaerobic digestion (AD) biotechnology to address the challenge of managing the large amounts of manure and slurry produced daily. AD can be considered a nature-based solution because it relies on a natural process, spontaneous in anoxic environments. When applied in industrial digesters, it offers a dual benefit: energy generation from biogas and the production of an organic fertiliser in the form of digestate [8,9]. In particular, AD is a microbiological process of organic matter degradation under strictly anaerobic conditions, during which four main metabolic phases take place: hydrolysis, acidogenesis, acetogenesis and, only at the end, methanogenesis. All phases are essential for the degradation of organic materials. *Bacteria* carry out the first three steps, and *Archaea* have a role in the last one. The domain *Fungi* can also perform hydrolysis, which is considered the bottleneck of the AD process [10,11]. However, the role of *Fungi* in AD is still poorly understood, although their ability to effectively degrade the most resistant biomasses (i.e., lignocellulosic materials) has been recognised [11]. Despite being a fundamental component of the herbivore microbiome, they are often undervalued in studies on AD microbial communities.

The final product of AD trophic chain is biogas, consisting mainly of CH_4 and CO_2, 40–70% and 24–40% by volume, respectively. Biogas can be burnt in co-generation engines to generate electricity or refined into biomethane [12,13], finally releasing CO_2, which can be utilised by photosynthetic organisms. CH_4 production is an extremely advantageous process when controlled in anaerobic digesters. However, when produced from open manure-collecting ponds, it becomes an undesirable and harmful greenhouse gas (GHG) contributing to global warming [14]. This issue should encourage stakeholders and policy-makers to incentivise AD plant installation and discourage the storage of manure in open ponds, especially in the context of GHG emission and global climate change.

The digestate (the organic matter produced by the AD process) is commonly used as an organic fertiliser for agricultural soils, aligning with the European Green Deal goals of resource efficiency, the circular economy, biodiversity restoration and pollution reduction [15–18]. In this context, a decrease in the use of chemical fertilisers and an increase in organic ones, especially derived from livestock and poultry manure, is expected in the coming years [19,20]. Thus, it is of fundamental importance to evaluate, using an ecological approach, whether the cattle manure treatment in AD reactors can promote AB degradation and counteract ARGs' spread and whether it can produce a digestate free from antibiotic and ARG contamination. Indeed, AD reactors are necessarily stable and favourable environments for microorganisms, and there is concern about whether, under these conditions, ARGs may be spread among bacteria by horizontal [21] and vertical mechanisms [22].

Recent works which have monitored the input and output of various full-scale AD plants have shown that AB concentrations and ARGs generally decreased at the end of an AD treatment [23,24]. However, the presence of ABs and ARGs can vary seasonally, with different combinations of the various chemical classes and abiotic and biotic factors, which also depend on the type of digester and type of waste used as feedstock [25].

Previous studies conducted in batch mode have reported different removal percentages of ABs after the AD process, indicating that some ABs, such as ciprofloxacin, are more recalcitrant than others, such as sulfamethoxazole [24]. However, most of these studies were carried out using batches fed only with manure or digestate, which did not

accurately represent the mixture of manure and digestate found in a real digester plant. Furthermore, many of these works did not assess degradation of antibiotic mixtures. Finally, very few studies in batch experiments have simulated a real anaerobic digester in a multidisciplinary context, evaluating at the same time the degradation of antibiotics, the reduction in antibiotic resistance genes (ARGs) and the impact of ABs on the anaerobic digestion process.

In this context, the aim of the present study was to assess whether enrofloxacin (a fluoroquinolone), alone or in combination with ciprofloxacin and the sulfamethoxazole sulfamide, could influence the efficiency of the anaerobic digestion (AD) process. From an ecological perspective, it was also investigated whether, during the process, the AB mixture negatively influenced the AD microbial community or whether the latter was able to counteract the impact of ABs by degrading them and reducing ARGs. The batch-mode experiment, which simulated a real AD plant, made it possible to determine whether the management of livestock residues with AD plants is the most effective way to handle this waste and obtain good fertiliser. The concentration of ABs and the occurrence of their corresponding ARGs (*sul1*, *sul2*, *qepA*, *qnrS aac-(6′)-Ib-cr* and the integron integrase *intI1*) were analysed at the start of the experiment and over the AD process (~200 days). The characteristics of the active microbiome in CH_4 production were also assessed.

2. Materials and Methods

2.1. Sampling of Manure and Digestate

Input material (cattle manure) and digestate were sampled at a full-scale biogas reactor (continuous stirred-tank reactor (CSTR) configuration) located on a beef and dairy cattle farm in Lazio (Central Italy) fed with livestock cattle manure.

In particular, the input material was collected at the plant's feed manifold, which, in turn, was connected to the cattle manure collection tank. Prior to sampling, the plant pump was switched on in the collection tank (15′) to stir and homogenise its contents. Subsequently, 2 L bottles (three replicates) were filled with input material. The digestate was collected from the discharge downstream from the reactor using 2 L bottles (three replicates). Both input material and digestate were transported to the laboratory under anaerobic and refrigerated conditions (4 °C) and were subsequently characterised in terms of pH and water content (%). Volatile and total solids (g L^{-1} VS and TS, respectively) were determined in triplicate according to the standard methods of the American Public Health Association [26]. In detail, samples (20 g) were thoroughly mixed for TS determination and dried in a pre-weighed capsule in an oven at 105 °C for 48 h. Then, the residues were incinerated at 550 °C for 4 h, and the weight loss was recorded as VS content.

Samples were characterised (in triplicate), with determination of the total solids (TS) and volatile solids (VS), according to standard methods [26]. In detail, samples (20 g) for TS determination were thoroughly mixed, evaporated in a pre-weighed capsule and dried in an oven at 105 °C for 48 h. Then, the residues were incinerated at 550 °C for 4 h, and the weight loss after this time was recorded as the VS content.

Background concentrations of ABs were determined in the mixture of cattle manure and digestate used to set up the experiment.

2.2. Set-Up of the Antibiotic-Spiked Tests

The experiment was carried out in batch mode using 120 mL glass reactors with 60 mL of working volume, under mesophilic conditions (37 °C ± 1 °C), using a mixture (1:35) of input material and digestate as the substrate. This ratio is within the range of substrate compositions used in full-scale reactors. Subsequently, the AB mixture was spiked in at a low (2.5 mg kg^{-1} of enrofloxacin + 2.5 mg kg^{-1} ciprofloxacin + 2.5 mg kg^{-1}

sulfamethoxazole) or high concentration (7.5 mg kg^{-1} of enrofloxacin + 7.5 mg kg^{-1} ciprofloxacin + 7.5 mg kg^{-1} sulfamethoxazole). Moreover, some batches were set up with only enrofloxacin.

The four experimental conditions were named as follows:

- Enrofloxacin + ciprofloxacin + sulfamethoxazole (2.5 mg kg^{-1} each): Mix_L.
- Enrofloxacin + ciprofloxacin + sulfamethoxazole (7.5 mg kg^{-1} each): Mix_H.
- Enrofloxacin (7.5 mg kg^{-1}): ENR.
- Control batches (not spiked with ABs): Control.

For each condition, 27 replicates were performed.

Destructive samplings were performed at fixed times chosen on the basis of the CH_4 production trend (i.e., 0, 10, 15, 51 and 197 days). Day 0 corresponded to the sampling carried out after AB addition. The end of the experiment was initially fixed at 51 days (based on the range of 30–50 days used in real plants). However, because the CH_4 production trend was in a "growing phase" at this time, it was maintained for a longer time (about 200 days) in order to verify the efficiency of the microbial community in respect to the received substrate mixture and conditions.

2.3. Methane Production Determination and Analysis of Process Intermediates

Biogas volumetric measurements were conducted using the water displacement technique [27], with measurements taken every three days during the first nine days and thereafter weekly until day 197. The composition of the biogas in terms of CH_4, CO_2 and H_2 content was analysed using a gas chromatograph (GC, Focus GC, Thermo Fisher Scientific, Waltham, MA, USA), equipped with a thermal conductivity detector (TCD) and a 3 m stainless steel column packed with Hayesep Q (800/100 mesh). Nitrogen was used as the gaseous eluent, with a flow rate of 35 mL min^{-1}. The temperature of the column and injector was 120 °C, and that of the TCD was 200 °C. The cumulative CH_4 production was calculated using the Logan equation [28], considering the headspace volume of the reactors, the total volume of biogas produced in each sampling time interval and the concentration (%) of CH_4 in the biogas.

The main process intermediates produced by microorganism metabolism were determined by high-performance liquid chromatography, or HPLC (Thermo Spectra System P4000, Waltham, MA, USA), using the isocratic analysis method at 75 °C with a 300 × 7.8 mm Rezek ROA-Organic Acid H+ (8%) column (Phenomenex, Torrance, CA, USA) and a pre-column with 4 × 30 mm Carbo-H cartridges (Phenomenex, Torrance, CA, USA). The mobile phase used was H_2SO_4, 5 mM, with a flow rate of 0.6 mL min^{-1}. The detectors used, placed in series, were a UV spectrophotometer (l1/4210 nm) for the measurement of organic acids and sugars and a refractive index detector for the measurement of alcohols. Prior to column injection, the samples were centrifuged at 13,000 rpm for 10 min; the supernatant was then diluted 1:10 with 5 mM H_2SO_4 and centrifuged again under the same conditions. At this point, approximately 700 µL of the supernatant was aliquoted into the glass vials of the instrument. Volatile fatty acids (VFAs) such as acetic, butyric, propionic, succinic, formic, valeric, isobutyric and isovaleric acids were analysed, as was lactic acid.

2.4. Analytical Determination of Antibiotics

Enrofloxacin (99%), sulfamethoxazole (99%) and ciprofloxacin (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibiotics were extracted from the input material and digestate mixture used in the batch experiments through pressurised liquid extraction (PLE, E-916 Speed Extractor, Büchi, Italy), as described by Rauseo et al. [29]. The resulting extracts were purified using solid-phase extraction (SPE) with Oasis HLB cartridges, following the procedure

outlined by Visca et al. [24]. Analytical determination was then carried out using an HPLC system (micro–Pump Series 200, Perkin Elmer, Hopkinton, MA, USA) coupled with a triple quadrupole mass spectrometer (MS/MS, API 3000, AB Sciex, Darmstadt, Germany) equipped with an electrospray ionisation source, as reported in Spataro et al. [30]. A Gemini (150 × 4.6 mm, 5 μm RP C 18, Phenomenex, Le Pecq, France) was used as chromatographic column, maintained at 25 °C (column oven mod. LC-100, Perkin Elmer, Hopkinton, MA, USA). The injection volume was 20 μL. MeOH and an aqueous formic acid solution (0.1%) were used as mobile phases A and B, respectively, flowing into the chromatographic column at 0.3 mL min^{-1}. The gradient elution of the mobile phase was set at 10% phase A at 0 min, increasing to 90% over 10 min and returning to the initial condition within 15 min. The main MS/MS parameters for analysing SMX, CIP and enrofloxacin were previously described by Visca et al. [24]. The MS/MS system operated in multiple reaction monitoring mode, with nitrogen (purity > 99.999%) used as the collision and drying gas. The nebuliser and curtain gases were set at 14 and 12 units, respectively, with a source temperature of 400 °C and an ion-spray voltage of +5 kV. The HPLC-MS/MS system was controlled and data were acquired using Analyst® 1.6 Software (AB Sciex, Concord, ON, Canada). The three antibiotics were identified based on m/z ion ratios, ion transition intensity ratios and retention times (RTs, with a criterion of ±0.2 min). Linearity for SMX, CIP and enrofloxacin was established over a concentration range of 0.25–5.0 μg L^{-1} (0.25, 0.5, 1.0, 2.5 and 5.0 μg L^{-1}), with correlation coefficients (R^2 values) consistently exceeding 0.98 and relative standard deviations (three replicates) below 15%. Internal standard calibration, using deuterated standards (sulfamethoxazole-d4 and ciprofloxacin-d8 hydrochloride hydrate), minimised potential matrix effects. Additional details, including recovery percentages, limits of detection and limits of quantification, are available in a previous work [24].

2.5. Microbial Abundance and Fluorescence In Situ Hybridisation

An aliquot (1 mL) of each batch sample was fixed with 4% formaldehyde for 3 h at 4 °C; the samples were then centrifuged for 5 min at 15,000 rpm, and the supernatant was washed twice with 1× PBS (phosphate-buffered saline). Finally, the pellets were suspended in 1 mL of 1× PBS to establish the initial volume of the samples. Further dilutions were performed to obtain the appropriate volumes to analyse, as described in detail in a previous work [31]. Then, the microbial abundance (number of cells mL^{-1}) was determined by the direct epifluorescence count method after staining fixed samples with DAPI (4′-6-diamino-2-phenylindole), a DNA intercalant. For each sample, 100 μL of DAPI (1 μg mL^{-1}) was added to 1 mL of sample volume, after which the samples were stirred and placed in the dark for 15 min. Then, each sample was filtered through a black polycarbonate filter (0.22 μm porosity and 25 mm diameter, Millipore, Billerica, MA, USA) using a vacuum pump filtration apparatus (Sartorius) under dark conditions to preserve fluorescence. Three replicates were made for each batch. The filters were mounted on microscope slides and covered by cover slips with immersion microscopy oil (Zeiss). The cell count was carried out using an epifluorescence microscope (AXIOSKOP 40, Carl Zeiss, Oberkochen, Germany) equipped with an HXP illuminator with optical fibre and microscope collector (Zeiss HXP 120 V lamp), with an immersion objective of 100× magnification. For each filter, the cells in at least 15 randomly selected grid fields on the filter were counted. The total number of cells on the entire filter was obtained using Franklin's formula [32].

The fluorescence in situ hybridisation (FISH) technique was performed, as reported in previous works [31,33]. Prior to FISH analysis, a cell extraction procedure was performed to separate cells from inorganic particles, as previously described [33,34]. Briefly, 1 mL of Optiprep (Sentinel Diagnostics, Milan, Italy, density of about 1.3 g mL^{-1}) was carefully added to 1 mL of sample using a syringe and put on the tube base. After centrifugation

(14,000× g, 90 min, 4 °C), stratification was achieved, and an aliquot (100–300 µL) was taken from the cell layer; the aliquot was diluted and filtered through a polycarbonate filter (0.22 µm porosity, 25 mm diameter, Millipore, Billerica, MA, USA).

Oligonucleotide probes with specific 16S rRNA targets at different phylogenetic levels were used for phylogenetic identification, such as EUB338, II and III for *Bacteria* and ARCH915 for *Archaea*. Probe details are available on the ProbeBase website (http://probebase.csb.univie.ac.at (accessed on 7 June 2023) [35]). Fluorescent cells on the filters were observed with the epifluorescence microscope described above. The number of cells bound to the fluorescent probes was calculated from the number of positive cells vs. the number of DAPI-stained cells.

Fungal abundance, expressed as the total number of single fungal structures detected per mL, was estimated using the Calcofluor White (CFW) fluorochrome, which binds to β-1,3 and β-1,4 polysaccharides [36]. CFW (20 µL) was added to each suitably diluted sample. After shaking, the samples were placed in the dark for 15 min. The obtained slides were analysed under the epifluorescence microscope.

2.6. DNA Extraction and qPCR Analysis (ARGs)

The total DNA was extracted from each replicate of input material and digestate mixture used in the batch experiment using the DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA), following the manufacturer's instructions. DNA-free water was used as a negative control throughout the workflow. DNA extraction yield and quality were evaluated using a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All extracts were stored at −20 °C until use.

The targeted qPCR assays used in this study are reported in Visca et al. [5]. Briefly, 25 ng of genomic DNA was used as the template to investigate the genes *sul1*, *sul2*, *intI1*, *qnrS*, *qepA* and *aac-(6′)-Ib-cr*. Results are reported as relative abundances normalised to the 16S rRNA gene. The primers used are listed in Table 1.

Table 1. Primer names and sequences for antibiotic resistance genes, mobile genetic elements and bacterial 16S rRNA gene; fw: forward; rv: reverse.

Primer Name	*Target Gene*	*Primer Sequence (5′->3′)*	*Reference*
Sul1 fw	*sul1*	*CGCACCGGAAACATCGCTGCAC*	[37]
Sul1 rv		*TGAAGTTCCGCCGCAAGGCTCG*	
Sul2 fw	*sul2*	*GCGCTCAAGGCAGATGGCATT*	[38]
Sul2 rv		*GCGTTTGATACCGGCACCCGT*	
IntI1 fw	*intI1*	*TCGTGCGTCGCCATACA*	[39]
IntI1 rv		*GCTTGTTCTACGGCCGTTTGA*	
16S fw	*16S rRNA*	*CGGTGAATACGTTCYCGG*	[40]
16S rv		*TACCTTGTTACGACTT*	
qnrS fw	*qnrS*	*GACGTGCTAACTTGCGTGAT*	[41]
qnrS rv		*TGGCATTGTTGGAAACTT*	
qepA fw	*qepA*	*GCAGGTCCAGCAGCGGGTAG*	[42]
qepA rv		*CTTCCTGCCCGAGTATCGTG*	
Aac-(6′)-Ib cr fw	*aac-(6′)-Ib-cr*	*TGCATCACAACTGGGCAAAGGCT*	[43]

2.7. Statistical Analysis

All data manipulation and statistical analysis were performed using the software R (ver. 4.3) and a significance threshold of 0.05 was considered.

A one-way ANOVA (aov function) with the Tukey HSD test (TukeyHSD function) as a post hoc test was performed to found significant differences in microbial abundance, AB removal percentages, CH_4 production and gene quantities among experimental conditions.

To assess the differences in CH_4 cumulative production among the conditions, a distance matrix was calculated using an appropriate dissimilarity test tailored for time series (TSDatabaseDistances function from the package TSdist [44]). Differences among productions were then validated using a multivariate ANOVA with permutations (PERMANOVA) using the adonis2 function from the vegan package (https://CRAN.R-project.org/package=vegan, accessed on 3 July 2023).

3. Results and Discussion

3.1. Biogas Production

Figure 1 shows the cumulative CH_4 production under the different experimental conditions compared to the Control. In all cases, CH_4 production started immediately and increased over time, with the maximum rate in the first 51 days. An AB mixture effect was found only at the highest concentration of ABs (Mix_H) compared to the Control (Tukey test: $p < 0.003$ at day 23 and $p < 0.02$ at day 30).

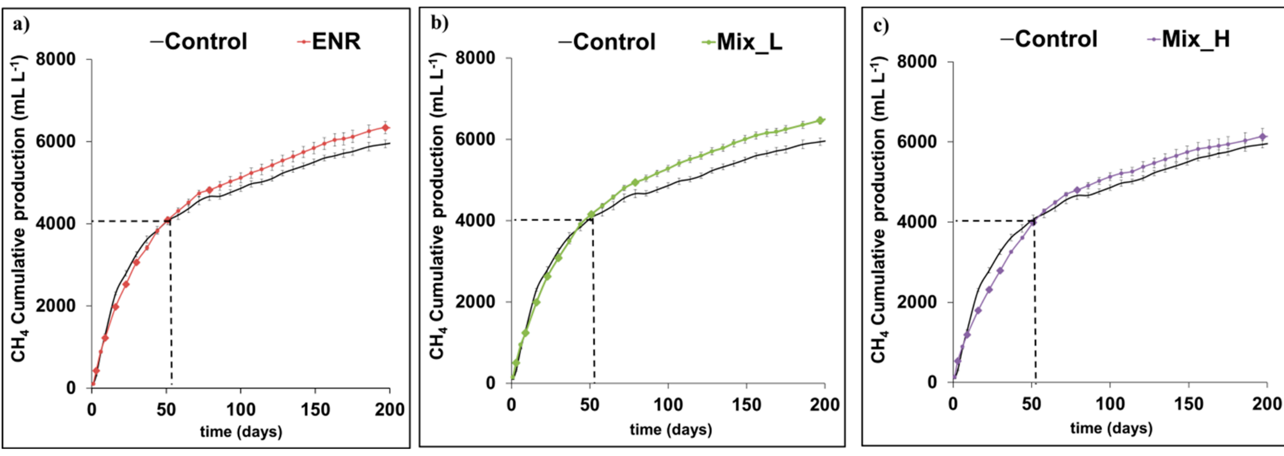

Figure 1. Cumulative CH_4 production in the three experimental conditions; (**a**) ENR, (**b**) Mix_L and (**c**) Mix_H. In each graph, the condition of addition of ABs is compared with the Control. Vertical bars represent the standard errors.

At day 51, all the experimental conditions, including the Control, reached a cumulative CH_4 production of approximately 4000 mL CH_4 L^{-1}. This value corresponded to more than two-thirds of the final CH_4 production.

From day 51, changes in CH_4 production trends were observed in all spiked conditions. The effect was particularly evident in Mix_L, which, from day 93, showed higher cumulative CH_4 production than the Control ($p < 0.01$). The latter result suggests a sort of "positive effect" of fluoroquinolones on CH_4 production, as reported by other authors. For example, Zhi et al. [45], investigating the effects on AD of seven commonly used ABs (comprising enrofloxacin and ciprofloxacin) over a wide range of single doses (0–500 mg L^{-1}), found that an AB concentration up to 100 mg L^{-1} could have a stimulating effect on CH_4 yield (presumably favouring fluoroquinolone-resistant microorganisms). In another work, Zhao et al. [46] reported that CIP at 0.76, 7.6 and 37.6 mg L^{-1} reduced only the CH_4 production rate and not the final amount obtained.

A positive effect of CIP on CH_4 production also occurred in a recent study by Mazzurco Miritana et al. [23], in which the presence of CIP alone or in combination with SMX, tested up to 10 mg L^{-1}, promoted the AD of cattle manure by providing cumulative production up to 10 times higher than the unspiked condition. It has to be considered that only cattle manure was used as the substrate. In another work, Tang et al. [47], analysing the effects of CIP on AD, found that a concentration of 0.5 mg L^{-1} enhanced CH_4 production, but as the CIP dose increased (up to 2 mg L^{-1}), CH_4 production showed lower values than the control.

Interestingly, in another work, SMX alone (1 and 10 mg L^{-1}) did not negatively influence biogas production in AD systems [48]. On the other hand, the present study was performed simultaneously using SMX (characterised by a bacteriostatic action) and two fluoroquinolones (bactericides), and this AB combination, especially at high concentration, did not favour CH_4 production. Our results show how both the concentration of each AB and their synergic interactions can influence microorganisms involved in the AD process.

The CH_4 content of the produced biogas also provided interesting information on the effects of ABs on AD. In fact, although in all conditions the highest CH_4 concentration was reached at day 15, the highest value was recorded for the highest concentration of ABs, i.e., in Mix_H with 66.0 ± 1.4%. Mix_L, ENR and the Control showed lower CH_4 content, with 62.8 ± 0.6%, 61.2 ± 1.1% and 61.7 ± 0.4%, respectively. These results suggest that AB promoted a change in the microbial community composition and favoured resistant bacteria (see below).

Interestingly, no accumulation of H_2 was detected during the experiment, indicating that the entire chain of metabolic reactions occurring during the AD process maintained its functionality and stability even in the presence of ABs. On the contrary, an accumulation of H_2 would have revealed an imbalance between the hydrolysis/acidogenesis and methanogenesis phases of DA, as occurred in a previous study performed by Mazzurco Miritana et al. [23] where only cattle manure (sampled from the collecting ponds) was used as the substrate. Further confirmation that the entire process was not significantly inhibited by the AB addition arose from the analysis of the process intermediates. In this study, no significant accumulation of VFAs was detected, confirming a balanced course of the AD process in all its functional phases. Only acetic acid, which can be directly utilised by acetogenic *Archaea*, was detected, although at low concentrations (no more than 120 mg L^{-1} and only during the first 15 days). On the contrary, Zhi et al. [45] observed that sulphonamides promoted the accumulation of VFAs, increasing the concentrations of acetic and propionic acids. Nevertheless, in our experiment, the AD process was able to handle the effects of ABs, as shown by the absence of VFAs and accumulation of H_2.

Surprisingly, CH_4 production was maintained for about six months (197 days). At the end of the experiment, the highest cumulative CH_4 production was obtained from Mix_L, which produced 6464 ± 94 mL CH_4 L^{-1}. The other conditions showed slightly lower production, with values of 6337 ± 142 mL CH_4 L^{-1} and 6133 ± 203 mL CH_4 L^{-1} for ENR and Mix_H, respectively. Mix_L production showed a significant increase of 8.8% ($p < 0.01$) compared with the Control, which produced 5939 ± 93 mL CH_4 L^{-1}. The values obtained were up to three times higher than those obtained in the abovementioned study by Mazzurco Miritana et al. [23].

3.2. Antibiotic Removal

The analyses of the input material and digestate mixture used for the batch experiments showed residual concentrations of the three ABs: CIP (4.5 mg kg^{-1}) > ENR (0.724 ± 0.045 mg kg^{-1}) > SMX (0.023 ± 0.008 mg kg^{-1}). These data are in line with those

found previously in the same AD plant [24] and show how bacteria can presumably be adapted to them.

Figure 2 shows the residual concentrations (%) enrofloxacin, sulfamethoxazole and ciprofloxacin measured in the ENR, Mix_L and Mix_H conditions at different sampling times (0, 51, 79 and 197 days). The highest removal rates were found for SMX, which had a very low residual concentration at 51 days (ranging from 0.007–0.009 mg kg^{-1}), in line with the results of other works showing lower persistence of this AB compared to fluoroquinolones [24,49], confirming that it is substantially removed during the AD process.

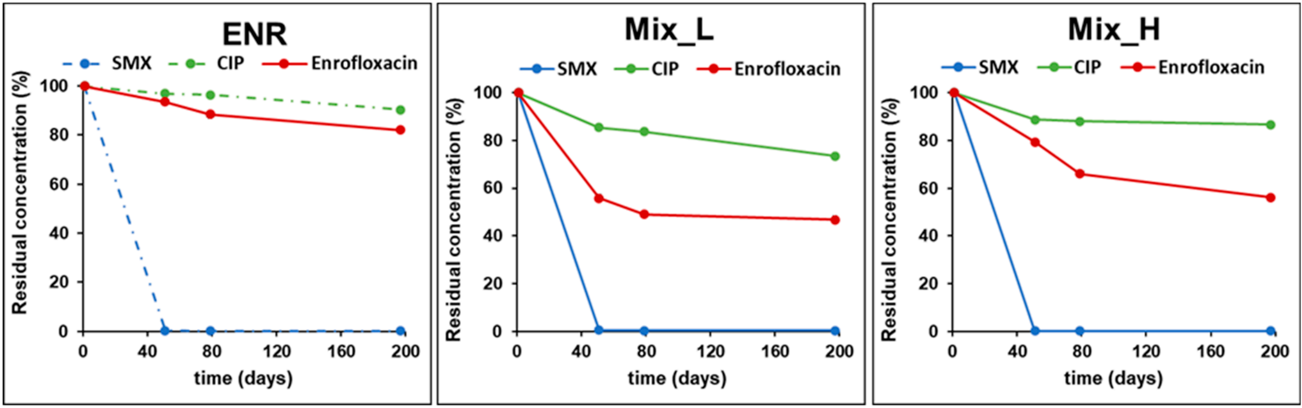

Figure 2. Antibiotic residual concentrations (expressed as percentages) in the ENR, Mix_L and Mix_H conditions at different sampling times. The dotted lines in the ENR condition represent the residual concentrations of ABs already existing in the cattle manure and digestate mixture used to set up the experiment (not added by the spiking process).

In contrast to SMX, ciprofloxacin was found to be the most persistent AB ($p < 0.01$), with the lowest removal percentages, from 9.8 ± 0.3% (ENR condition) to 26.6 ± 0.1% (in the Mix_L condition). The co-presence of SMX (Mix_L and Mix_H) seems to have favoured CIP removal, as found in other works [50,51]. The low CIP degradation found in the present study can be ascribed not only to its intrinsic high persistence but also to its possible formation from enrofloxacin [52] as reported in other works [24,49].

Enrofloxacin was found to be less persistent than CIP, and it was partially removed (17.9%) in the ENR condition. In a similar way to ciprofloxacin, the highest removal percentages ($p < 0.01$) were observed in the co-presence of SMX, with the highest values in Mix_L (53.1 ± 0.2%), followed by Mix_H (43.8 ± 0.1%).

The overall results suggest that the AD process favoured a decrease in the quantity of ABs, especially in the Mix_L condition, where they were present in lower amounts. The higher concentration of ABs (Mix_H) may have partially inhibited some microbial populations involved in fluoroquinolone degradation.

In a previous AD study conducted for 90 days in batch mode using only fresh manure as a substrate and with the addition of SMX and CIP, ABs were removed at lower rates than in this experiment [49].

3.3. Microbial Community Structure

The microbial community abundance as determined at 0, 10, 15, 51 and 197 days is reported in Figure 3.

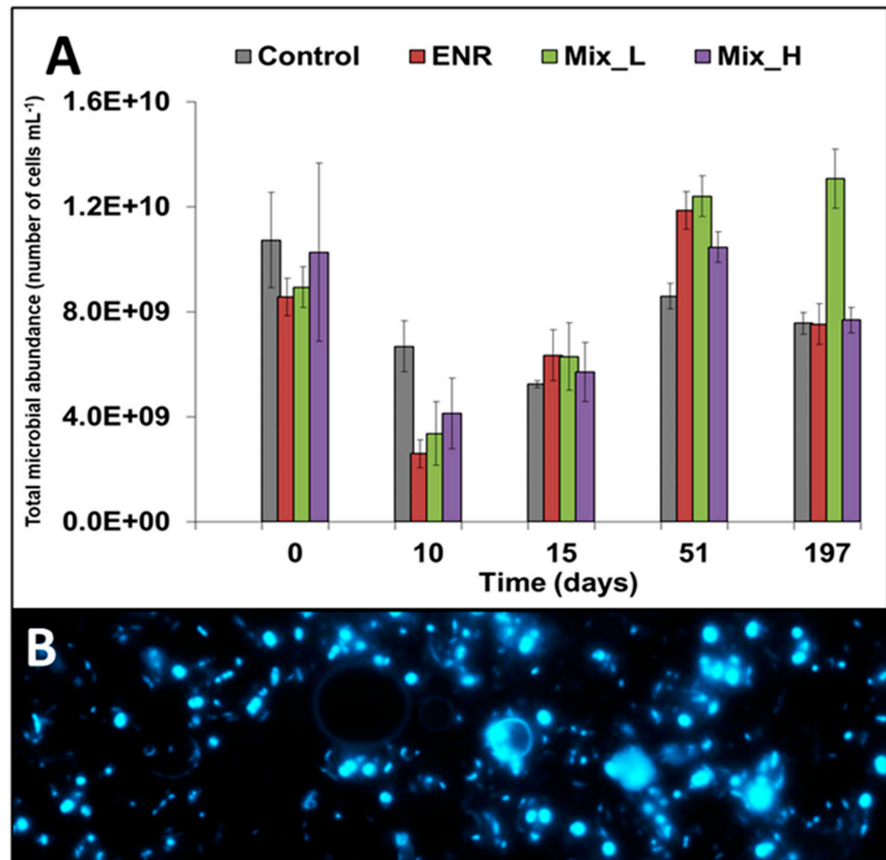

Figure 3. (**A**) Total microbial abundance determined by the direct count method using DAPI staining. The vertical bars represent the standard deviations. (**B**) Micrograph of the microbial community visualised under an epifluorescence microscope (Axioskop 40, Carl Zeiss).

At the start of the experiment, no differences were found in the total microbial abundances among the different conditions (average value of 9.6×10^9 cells mL L^{-1}). Thereafter, a general decrease was observed at day 10, with the lowest values recorded in the spiked conditions (ENR, Mix_L and Mix_H). This fact can be ascribed not only to the initial effect of biocide AB on some microbial populations, as found in another work [53], but also to the general disturbance caused by the experimental set-up, as described by Demirel and Yenigün [54]. Indeed, a partial reduction in total microbial abundance was also observed in the Control, although to a lesser extent.

From day 15, only spiked conditions showed an increase in total microbial abundance, and from day 51 in ENR and Mix_L the values exceeded those recorded at the start of the experiment. The negative impact of ABs on the microbial community was transient, and, interestingly, in the case of Mix_L (from day 51, in concomitance with SMX degradation), the abundance values remained high, in line with the significant increase in cumulative CH_4 production.

Direct fluorescence microscope analysis of the overall microbial community (*Fungi*, *Bacteria* and *Archaea*) involved in AD is reported in Figure 4. *Fungi* are generally involved in the first phase of the process (the predominantly hydrolytic one), *Bacteria* in the acidogenesis and acetogenesis (fermentation) phases and *Archaea* in the final phase of CH_4 production (methanogenesis).

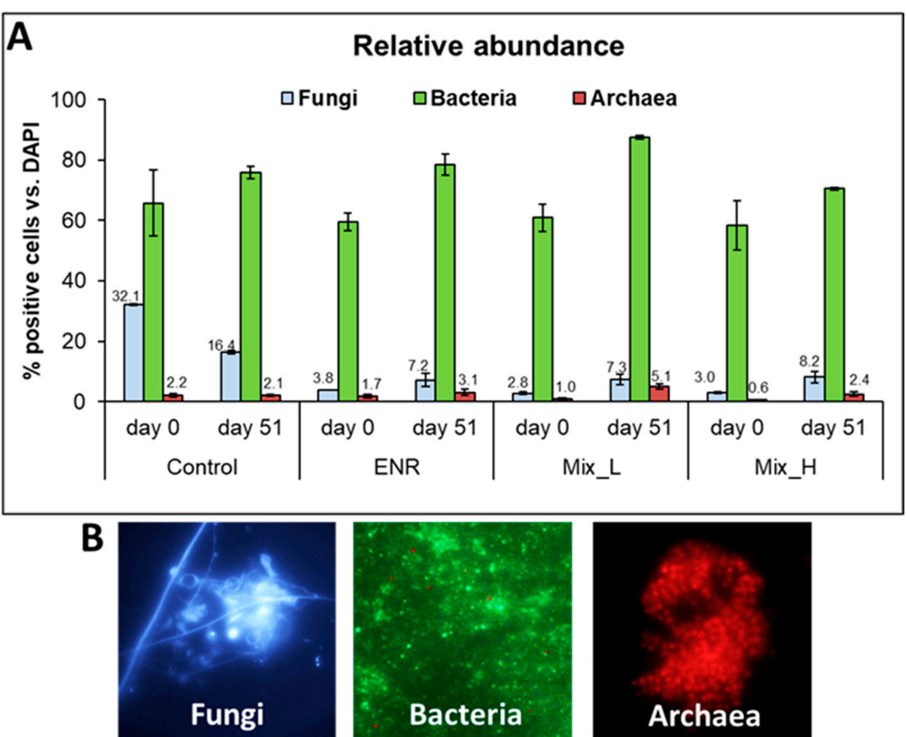

Figure 4. (**A**) Microbial community composition (%) at the start of the experiment and day 51 using fluorescence in situ hybridisation. *Fungi* (blue bars), *Bacteria* (green bars) and *Archaea* (red bars). The vertical bars represent the standard deviations. (**B**) Micrographs visualised by epifluorescence microscopy: *Fungi* (Calcofluor staining), *Bacteria* and *Archaea* detected using different probes with the FISH technique (FAM-labelled Eub, II and III and Cy3-labeled ARCH915 probes, respectively).

Overall, *Fungi* and *Archaea* were found to be hampered by ABs. This effect was transient for *Archaea* but not for *Fungi*. In fact, at day 51, an increase in all spiked conditions was recorded, attributable to SMX degradation and consequent reduced selective pressure. However, a reduction in *Fungi* of approximately 50% compared to the initial percentage was also observed in the Control condition. This result may be due to the batch configuration of the AD experiment, which promoted a succession peculiarity, with q predominance of hydrolytic microorganisms (i.e., *Fungi*) at the start of the experiment. In fact, they are necessary for breaking the intermolecular hydrogen bonds of lignocellulosic fibres [11,55,56] occurring in manure, which is rich in straw residues.

In any case, fungal presence is still poorly analysed compared with *Bacteria* and *Archaea* in the context of microbial community structure and the efficiency of the trophic chain of ADs, especially in the presence of ABs. Recently, fungal enzymatic hydrolysis has been found to promote AB removal. Cui et al. [57] reported that enzymatic hydrolysis can control the horizontal transfer of ARGs during the enzymatic hydrolysis and AD process in situ.

Regarding prokaryotic microorganisms, *Archaea* were more affected by ABs than *Bacteria* and, in particular, by a mixture (Mix_L and Mix_H) of ABs with different mechanisms of action, in accordance with other works [58]. In a previous study, the removal of ABs occurred mainly in the hydrolytic and fermentation phases of AD, especially in the case of AB mixtures, while no removal of ABs was detected in the phase with prevalent CH_4 production [23]. These results confirm that microorganisms of the domain *Bacteria* are able to counteract ABs' toxic effects by almost entirely (SMX) or partially (enrofloxacin and CIP) removing them. After SMX degradation, *Archaea*'s relative abundances increased (day 51), showing values even above those of the Control, as was particularly evident in Mix_L, confirming that low concentrations of ABs may not hinder methanogenesis activity [57]. The lack of acetic acid accumulation and H_2 detection during the AD process did not

allow identification of the metabolic pathways of CH_4 production (acetoclastic and/or hydrogenotrophic). This aspect requires further investigation, considering that acetoclastic methanogens are more sensitive to ABs than hydrogenotrophs [59].

3.4. Antibiotic Resistance Genes

ARGs were detected at the start of the experiment in all conditions (Figure 5). However, Mix_H was found to have significantly higher relative abundances of ARGs than the others. In particular, the highest value was for the for *aac-(6′)-Ib-cr* fluoroquinolone resistance gene (Tukey HSD test, $p < 0.01$).

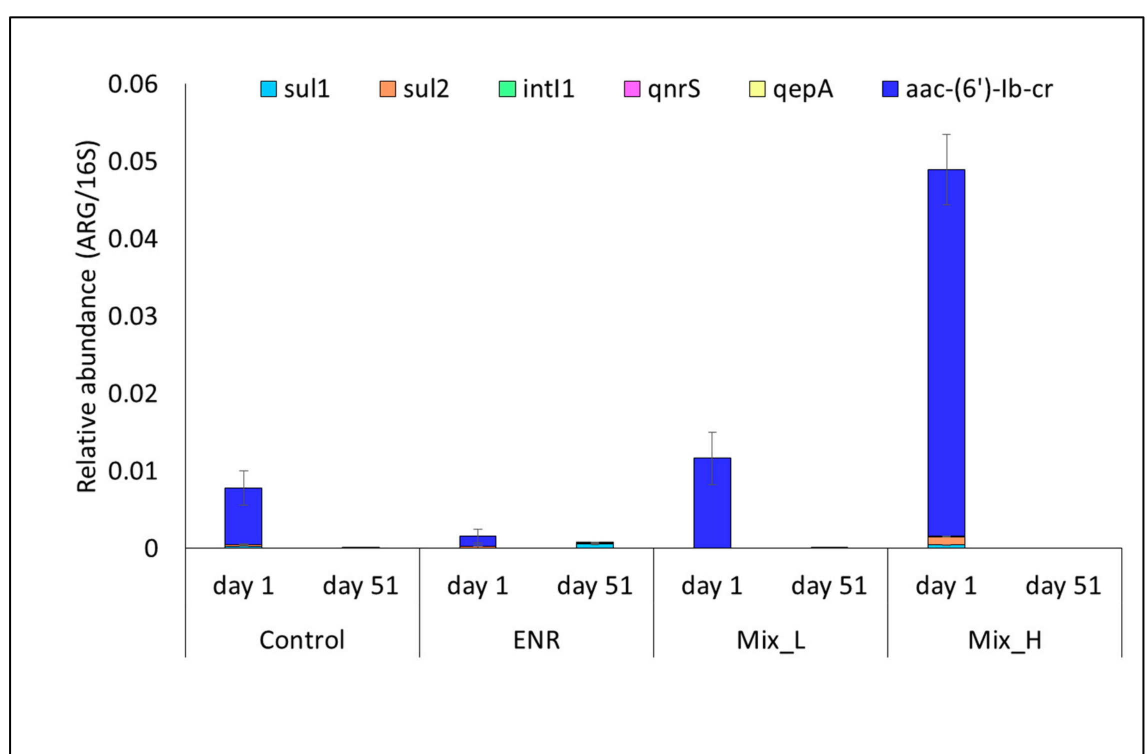

Figure 5. Relative gene abundances (ARGs/16S) in the Control, ENR, Mix_L and Mix_H conditions at 1 and 51 days. The vertical bars represent the standard errors.

Interestingly, at day 51 an overall decrease in ARGs was observed in all conditions. Thus, this work confirms that ARGs and ABs tend to decrease under AD in a timeframe close to its HRT, as found in a previous work [23]. This provide interesting information for the management of manure contaminated with ABs. However, this experiment lasted longer than the HRT used in the AD plant of origin (ca. 30–50 days), because the process was still producing CH_4 (ca. 6 months). On the other hand, this long experimental time in a batch configuration, i.e., without any nutrient renewal, was presumably stressful and acted as "selective force" that promoted, in a second window of time (from day 51), new AB-resistant microorganisms. In fact, at the end of the experiment, ARGs were detected in all conditions, with the highest values for the *aac-(6′)-Ib-cr* fluoroquinolone gene in Mix_H (*aac-(6′)-Ib-cr/16S* copies: 0.0526), corresponding to the highest residual fluoroquinolone concentrations (Mix_H: 15.8 ± 0.7 mg kg^{-1}). SMX genes were also detected in this condition, but at very low abundances (*sul1/16S* copies: 0.0094 and *sul2/16S* copies: 0.0043).

These results suggest that the HRTs (i.e., 30–50 days) commonly used in AD plants favour substantial AB and ARG decreases. On the other hand, longer HRTs and closed systems could promote the growth of bacteria with resistance to persistent antibiotics such as fluoroquinolones.

4. Conclusions

This multi-disciplinary study provides new insights into the impact of an antibiotic mixture on AD efficiency, as well as the ability of the AD process to degrade ABs and reduce ARGs in manure digestate. It also offers valuable information on whether disposal of zootechnical residues in AD plants is the most effective strategy for managing this kind of waste.

The antibiotic mixture tested did not significantly affect the AD process, except at the highest concentration (7.5 mg kg^{-1} of each antibiotic). The AD process remained metabolically balanced for an extended period, although CH_4 production was mainly concentrated in the first 51 days. The AB mixture initially decreased both *Archaea* and *Fungi*; however, the negative effect persisted over time only for *Fungi*. The AD process was effective in reducing both ABs and ARGs, suggesting that the prokaryotic microbial community exhibited plasticity and resilience to ABs, maintaining efficient CH_4 production at the tested concentrations.

In conclusion, this multidisciplinary study indicates that the disposal of cattle manure in anaerobic digesters is preferable to storage in open ponds. AD not only enhances the value of zootechnical waste from an energy perspective but also improves the quality of the digestate for its use as an organic fertiliser, while counteracting the spread of emerging contaminants, such as ABs and ARGs. Finally, this nature-based solution reduces atmospheric emissions of CH_4, recognised as a potent greenhouse gas and dangerous for its contribution to climate change.

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