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Microbial analysis of anaerobic digester reveals prevalence of manure microbiota

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ABSTRACT

Anaerobic digestion of swine manure reduces farm greenhouse gases emissions and provides a renewable gas in the agricultural sector. The particular composition of manure, with high concentrations of ammoniacal nitrogen and volatile fatty acids, often threatens the stability of the process through inhibition of methanogens. In this work, continuous production of biogas was tested under relative short hydraulic retention time (15 days). Although a strong inhibition period was detected, adaptation of the microbiota and displacement of bacteria and archaea present in the inoculum by microorganisms present in the animal manure resulted in biogas production close to the values found in standardized batch tests in absence of inhibitions. These findings suggest that in anaerobic digestion of manure, it is not necessary to inoculate, as manure itself contains a large number of active fermentative microorganisms that can even resist long-term digestion inhibition.

1. Introduction

The production of gases of renewable origin plays a crucial role in the transition to a sustainable, carbon-free energy future, becoming instrumental in shaping the energy mix [1]. These gases, such as biogas and biomethane, are generated through the anaerobic digestion of organic matter and waste, providing a clean energy source. Agricultural residues have significant potential to lead this transformation due to their abundance and constant availability [2]. The agricultural industry produces a large amount of organic waste, such as crop residues, animal manure and spoiled products. These substrates are susceptible to be converted into a valuable source of energy by processing them in anaerobic digestion plants [3]. In case of swine production, there is an increasing trend in the global production which is expected to increase by >10 % between 2020 and 2030 [4]. The high environmental impact associated with this activity and the legal requirements force the sector to implement sustainable practices in the proper management of the by-products (mainly manure), efficiency in the use of resources and the adoption of cleaner technologies [5,6]. The biological process of anaerobic digestion offers an efficient and sustainable technical solution for livestock manure management, generating renewable energy in the form of biogas and a liquid organic fertilizer. In addition, greenhouse gas (GHG) diffuse emissions in the form of CH_4 and N_2O are reduced when agricultural wastes are digested and methane is recovered. According to some estimations, GHG savings in case of wet animal manure can reach 240 % when applying anaerobic digesters and using the biogas generated for replacement of fossil-based fuels [7]. Therefore, an increasing interest in the implementation of agricultural digesters has been experimented in the recent years and foster the fossil natural gas replacement. While some European countries have achieved biogas/biomethane production that represents >15 % of the gas consumption, the percentage of replacement in this continent is expected to reach between 35 and 62 % by the year 2050, depending on the different projections based on the organic substrate availability [8]. At this point, it must be stressed that all the projections highlight the significance of animal manure in this transformation [9]. The production of biogas directly depends on the optimal operation of digesters in order to guarantee the stability and maximizes bioconversion.

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The detection of operational problems due to different inhibitions (ammonia, sulfide and heavy metals ion among others) has been studied through the different anaerobic digestion stages [10–12]. However, there has not been much research focusing specifically on examining these effects while simultaneously studying the evolution of microbiological populations including determination of organisms present in the substrates and inoculum [13,14]. The stability and efficiency of anaerobic digestion of complex substrates such as swine manure, relies on the tight equilibrium between the microbial populations responsible of the hydrolytic, acidogenic, acetogenic, and methanogenic stages [15]. This equilibrium is based on the microbial symbiotic relationships that may be importantly affected by changes in the environmental conditions (temperature, pH, nutrient content), intermediate compounds and toxic substances. In this sense, the very slow growth rates of methanogenic archaea and the lower tolerance towards some chemicals has been identified as the critical step [16,17]. The selection and maintenance of adequate anaerobic inoculum for starting the digestion processes of complex substrates such as swine manure is normally viewed as a decisive strategy for a successful process [18]. Little importance has been addressed to the manure microbiota which is already adapted to chemical conditions and can play decisive role in the bioprocess.

In the present work, two parallel reactors fed with swine manure working under semi-continuous conditions were operated with the objective of monitoring the relevant physicochemical parameters for a successful anaerobic digestion process working under a short hydraulic retention time (HRT) in order to identify possible inhibitions. Changes in the microbial populations based on 16s rRNA-based relative abundances were studied and correlated with the performance in terms of biometanization. The yields of bioconversion and rates were compared with normalized batch anaerobic test with and without anaerobic inoculum.

2. Material and methods

2.1. Substrate and inoculum

Swine manure (SM) from a feeder farm placed in Sauquillo de Boñices (Soria, Spain) was used as substrate in both semi-continuous and batch experiments. The concentration of volatile solids (VS) was between 13.41 ± 0.35 g• kg^{-1} and 62.43 ± 0.12 g• kg^{-1}, and the average Chemical Oxygen Demand (COD) was 94.01 ± 36.58 kg L^{-1}. The large variations of the organic content were due to the fact that the manure was stored in an open lagoon and exposed to ambient conditions. In case of semi-continuous manure degradation experiment, the results considered in the study correspond to the reasonable stable inlet concentrations of organic matter. In this sense, initial phase of operation when the inlet concentration of organic matter was very low was not considered (corresponding to the first 20 days of operation in a total of 140 days). The manure was collected weekly in order to provide fresh substrate and reproduce the conditions found in pig farms and it was sieved to prevent clogging and provide efficient mixing and pumping and stored at 4 °C before usage. For the Continuously Stirred Tank Reactors (CSTR) experiments the anaerobic inoculum used was sampled from a local urban wastewater treatment plant treating mixed sludge (namely WWT) located in Soria (Spain). In case of batch anaerobic tests, the WWT inoculum and another one withdrawn from a digester treating piggery wastewater (namely farm digester, FD) were used. The WWT inoculum presented a content of volatile solids (VS) value of 12.8 ± 0.1 g• kg^{-1} and the FD of 20.7 ± 0.1 g• kg^{-1}.

2.2. Semi-continuous manure degradation

The experimental set up consisted of two continuously stirred tank reactors (CSTR) with a total volume of 1.5 L and 0.75 L of liquid. Mesophilic conditions were used for the digestion (35 ± 1 °C) using a thermal bath (Selecta, Termotronic-100). The reactor was operated for a period of 5 months with an HRT of 15 days. A 1-L gas trap containing water was used to measure the biogas produced. Reactors were initially filled with the WWT inoculum described in Section 2.1 and the swine manure feeding was done manually every 24 h since the day "0". The reactor was mixed continuously with a magnetic stirrer at 100 rpm (Barnstead Thermolyne, SP131320-33). Final effluent from the digesters were collected to measure total volatile fatty acids (VFAs) concentration, chemistry oxygen demand (COD), total and partial alkalinity, pH and ammoniacal nitrogen. Biogas composition was also measured with a gas analyser once per week. Fig. 1 shows a scheme of the experimental setup.

2.3. Biomethane potential test

In view of the results of the continuous operation and the impact of microbial communities present in the animal manure (see Section 2.5), standardized BMP (biomethane potential tests) were performed to evaluate the effect of the inoculation over biogas productivity. Three different essays were performed: with WWT inoculum, with the farm inoculum adapted to piggery wastewater (FD) and uninoculated. Glass serum bottles with a total working volume of 120 mL were used as batch reactors for BMP experiments with an inoculum/substrate ratio of 1:1 for 60 days [19]. Temperature conditions were maintained at 35 ± 0.5 °C in an incubator (Selecta, Hotcold-GL) provided with an orbital stirring plate (Selecta, Rotabit). Control tests with the inoculum were included to measure the endogenous production of biogas in inoculated essays. 0.5 g•L^{-1} of $CaCO_3$ were introduced as a buffer to prevent alterations in pH and to ensure anaerobic conditions. Bottles were flushed with N_2 gas (99.9 % purity) and immediately sealed with butyl rubber stoppers and aluminium crimps [20,21]. The amount biogas production and its composition were measured by water displacement and biogas composition was periodically determined.

2.4. Analytical methods

The characterization of the substrate and inoculum was carried out following the standardized methodology by The American Public Health Association (APHA) in order to measure total solids (TS), volatile solids (VS), pH, total and partial alkalinity, Total Kjeldahl Nitrogen (TKN), ammoniacal nitrogen and PO_4^{3-} [22]. Samples for volatile fatty acids (VFA) determination were prepared following the procedure described in [23] and determined through a gas chromatograph (Agilent 7820). Biogas composition was analysed with a (GeoTech, Biogas 5000) gas analyser. The characteristics of inoculum and diluted substrates used in the experiments are presented in Table 1.

2.5. Microbial communities

Microbial characterization was performed in samples taken from the CSTR at the different stages identified during the operation: after 41 days of operation, corresponding to a period of low biogas productivity and after 136 days of operation, when biogas production reached maximum levels. Additionally, the microbial composition was also analysed in samples of the swine manure and the WWT inoculum. Samples were preserved at −20 °C until DNA extraction procedures. After DNA extraction, a total of 50 ng of high-quality DNA was amplified following the 16S metagenomic sequencing library Illumina 15044223 B protocol (ILLUMINA). Two sets of primers were used to amplify 16S regions. The V3-V4 region of the 16S rRNA gene was amplified using the universal 341F-805R set of primers [24], additionally, the V4 region of 16S was amplified to study archaeal communities, using the primer pair combination 344F-1041R/519F-806R [25].

The retrieved 16s rRNA sequences were analysed using the software package DADA2 v1.6 in the R environment [26]. Forward and reverse reads were filtered and truncated to 290 and 220 nucleotides, respectively, and then paired reads were assembled. Subsequently, paired-end reads underwent denoising, and singleton and chimera sequences were

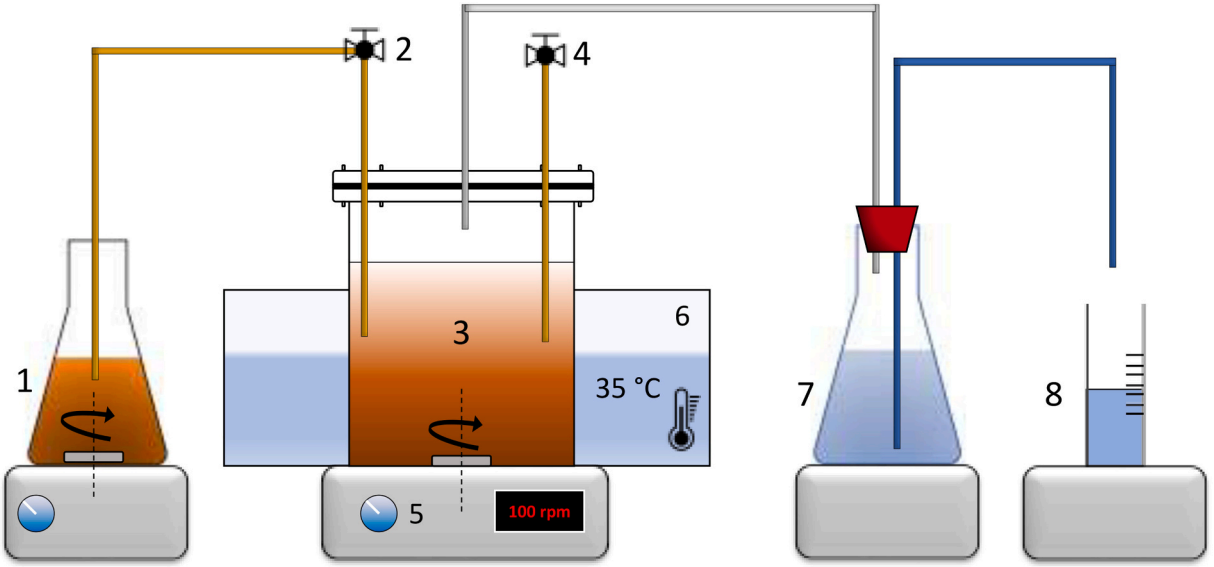

Fig. 1. Schematic representation of the experimental set-up and components: (1) swine manure (substrate); (2) feeding inlet valve; (3) anaerobic reactor; (4) digestate outlet valve; (5) stirring plate; (6) thermal bath; (7) water trap; (8) measuring cylinder.

Table 1
Characteristics of substrates and inoculum.

Analitic parameter	Pig manure	Inoculum	Digestate
Total solids (g/kg)	45.3 ± 28.9	17.9 ± 0.1	35.8 ± 12.2
Volatile solids (g/kg)	33.3 ± 23.1	12.8 ± 0.1	25.01 ± 8.6
Total Kjeldahl nitrogen (g/L)	3.3 ± 1.3	4941.1 ± 854.8	3.06 ± 0.77
Total phosphorus PO_4^{3-} (mg/L)	1024.5 ± 819.7		751.25 ± 317.2
Electrical conductivity (mS)	13.9 ± 2.8		16.3 ± 2.73
Chemistry oxygen demand (mg/L)	95.3 ± 37.6		72.08 ± 31
Total alkalinity (g $CaCO_3$/L)	13.775 ± 6.186		13.528 ± 2.842
Alkalinity ratio	1.1 ± 0.2		0.41 ± 0.35
pH	7.5 ± 0.4		7.87 ± 1.43

removed. Taxonomy was assigned to representative sequences taken from an amplicon sequence variant (ASV) table using the Naïve Bayesian classifier trained against the SILVA database release v132. Data analysis was performed using R through RStudio software (R Core Team, 2021). The sequences obtained in this work were deposited in the Genbank Sequence Read Archive under BioProject number PRJNA1025111.

3. Results and discussion

3.1. Biogas production in CSTR

Three different phases were observed through the experiment: an initial short-duration adaptation stage characterized by a continuous increased in the organic loading rate (OLR), unstable biogas production and a OLR of 2.45 ± 1.01 kg COD $m^{-3}d^{-1}$ (stage 1). This stage was followed by a second phase (2) marked by a minimal biogas production and a constant value of OLR of 7.81 ± 2.11 kg COD $m^{-3}d^{-1}$. Finally, the third stage when biogas production gradually increased reaching the yields achieved in the BMP tests (stage 3) presented an average value of OLR of 5.98 ± 1.66 kg COD $m^{-3}d^{-1}$ (Fig. 2b). The unstable conditions of stage 1 could be attributed to microbial adaptation and substrate availability still present in the WWT inoculum. A significant decline in biogas production commenced after day 15 (stage 2), when a period corresponding to one HRT was completed. This inhibition period was marked by a very low biogas production, decreasing from initial values of about 196.57 mL• g VS^{-1} to only 25.20 mL• g VS^{-1} and corresponded to 55 days of continuous operation (until day 70) (Fig. 2b). In stage 2, the decrease in methane yield is attributed to the methanogenesis inhibition as consequence of the relative short HRT, high ammonia concentration and high OLR, which leaded to the accumulation of volatile fatty acids (VFA) and subsequently resulted in a decline in methane production activity.

From day 70 onwards (stage 3), biogas production experienced a significant increase which lasted until the end of the experiment with an average value of biogas production of 375 mL• g VS^{-1} during the second half of the stage (Fig. 2b). In stage 3 the accumulation of VFA gradually diminishes, and the biogas production was resumed. Although a slight decrease in OLR was detected between stage 2 and 3 (from 7.81 to 5.98 kg COD $m^{-3}d^{-1}$) reducing the load of inhibitors, the improvement in biogas productions was more probably due to the adaptation of the microbial communities to the presence of inhibitors (see Section 3.2). The biomethanization levels at the end of stage 3 were close to the values achieved in the BMP tests (410, vs. 435 mL• g VS^{-1}) (see Section 3.3).

The inhibition of the methanogenic process was evidenced by the VFA accumulation and the considerable high values of the alkalinity ratio. In case of VFA, average concentration of the stage 2 was above the inhibition thresholds with average values of 14.6 ± 2.2 g L^{-1} of total VFA per litre. In this sense, concentration above 5 g•L^{-1} can potentially inhibit microbial activity in the anaerobic digestion process, and subsequently reducing the capacity to produce biogas [27]. VFAs, such as acetic acid, propionic acid, and butyric acid are intermediate products of anaerobic fermentation of organic matter. At low concentrations, these VFAs are utilized by methanogenic bacteria to produce methane, the primary component of biogas. However, at elevated concentrations, VFAs can have a toxic effect on methanogenic bacteria, reducing their activity and resulting in decreased biogas production [28]. Yeole T. (1996) and Yuan H. (1999) stablished the VFA concentrations of 5–6 g•L^{-1} as inhibitory level using cattle dung and sewage sludge as substrate [29,30]. Alkalinity ratio is often used to monitor the digester stability [31]. Stage 2 was characterized by values above 0.4, and reaching values beyond 1, which are normally associated with inhibition processes and the insufficient capacity to neutralize or remove organic acids (Fig. 2c). A high alkalinity ratio, above 0.3, may indicate an excess of alkalinity, signifying an imbalance of the anaerobic digestion. In the same manner that VFA concentration, the alkalinity ratio showed a

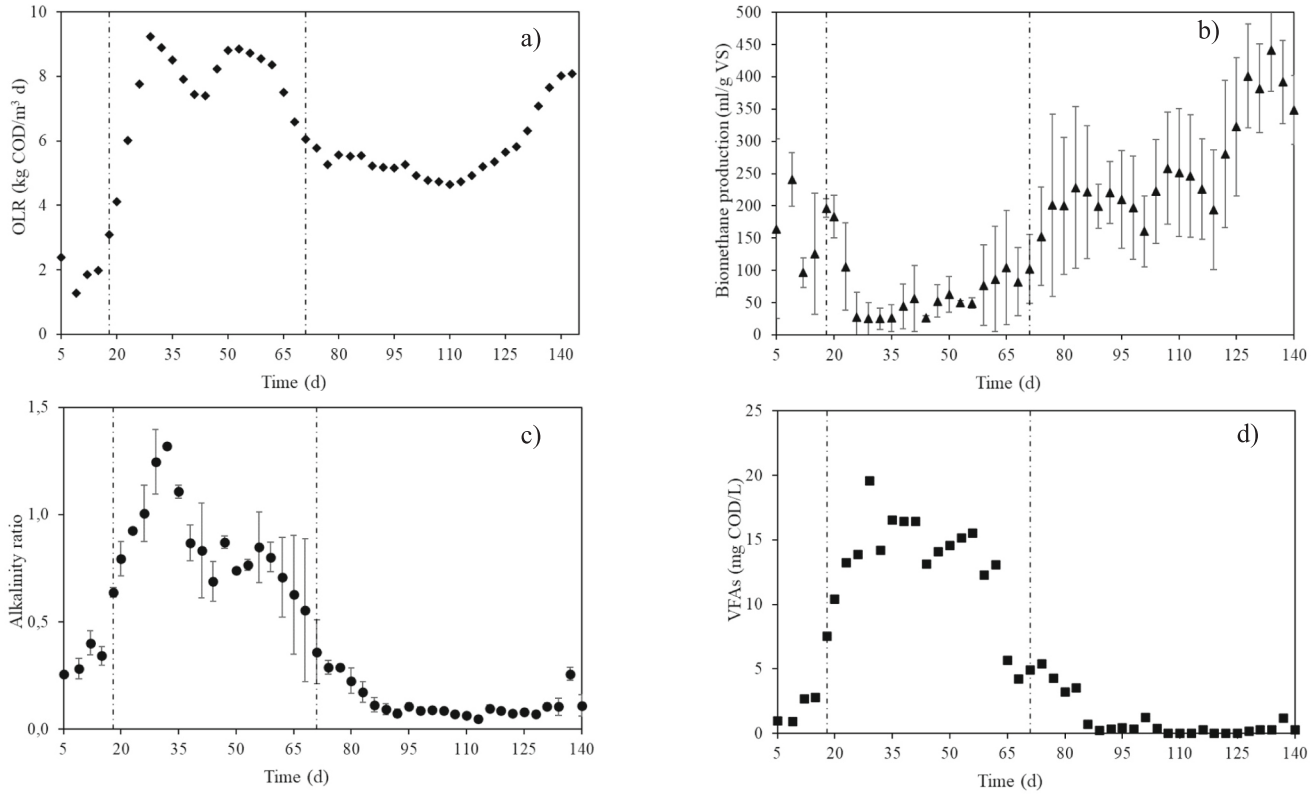

Fig. 2. Time course of the operational parameters during the experiment inside the anaerobic reactors: a) organic loading rate (OLR); b) biomethane production; c) alkalinity ratio; d) volatile fatty acids (VFAs).

remarkable decrease along stage 3, evidencing the increase of the methanogenic activity.

Animal waste products, such as swine manure, frequently contain exceptionally high concentrations of total ammoniacal nitrogen due to the presence of ammonia, as well as proteins and urea that readily release ammonia after anaerobic treatment [32,33]. While sudden increases in ammonia concentration in the feedstock are uncommon [34], stored feed slurries, like the substrate under study, often contain elevated levels of ammonia released during the organic nitrogen decomposition process. Ammoniacal nitrogen together with the VFA are main inhibitors found during swine manure treatment in digesters.

Fig. 3 illustrates the evolution in total Kjeldahl nitrogen (TKN) and pH in the CSTRs over the course of the experiment. In stage 2, the TKN concentration averaged in 2.72 ± 0.29 g•L^{-1}, while slightly lower concentrations were detected in stage 3, 2.03 ± 0.19 g•L^{-1}. These conditions were detected together with mild basic conditions, which increases the free ammonia form. Stable pH values of 8.21 ± 0.3 and 8.14 ± 0.25 were measured in stages 2 and 3, respectively. Ammonia accumulation often triggers process instability through inhibition of the methanogenesis, leading to the accumulation of VFAs, which subsequently inhibits the process. The interplay between free ammonia, VFAs, and pH can result in what is termed an "inhibited steady state" - a situation where the process operates steadily but with a reduced methane yield [35,36] and corresponding to the conditions described in stage 2. Given that basic pH was registered during the hole experimentation, NH_3 was probably the main inhibitor, since VFA suppress the methanogenic activity only in their undissociated form, which are not present in basic pH (pka values of VFA averages between 4.7 and 4.9). Nevertheless, microbial acclimatization to these conditions can lead to a resume of the biomethane production. Such adaptation may arise from internal changes within the dominant fermentative or methanogenic species or shifts in the population [37]. In this experiment, the adaptation period seemed to occur within a duration spanning between 4 and 5 HRT (stage 3). Among the four types of anaerobic microorganisms involved in the anaerobic digestion process, methanogens are the least resilient and are prone to halting their growth in response to ammonia inhibition [38]. Numerous studies have examined this issue, suggesting that a concentration of 4 g N-NH_3 L^{-1} is already sufficient to affect certain methanogenic microorganisms. Once acclimated, microorganisms can maintain their viability even at concentrations well above the initially inhibitory levels. Koster & Koomen, (1988) reported that non-acclimated methanogens failed to produce methane at ammonia concentrations of 1.9–2 g N-NH_3 L^{-1}, but they did resume methane production at a concentration of 11 g N-NH_3 L^{-1} after the adaptation process [39]. Experiments have unequivocally demonstrated the feasibility of achieving stable manure digestion at ammonia concentrations exceeding 5 g NH_3-N•L^{-1} following an initial adaptation period.

3.2. Microbial communities in CSTR

Microbial community analysis based on universal primers (V3-V4) revealed a significant difference in the composition of microbial lineages between swine manure and the WWT used as inoculum (Supplementary information, Figs. S1A and 4). While the swine manure was mainly composed of three phyla: Firmicutes, Bacteroidota, and Actinobacteriota, accounting for approximately 82, 8 and 6 % of the microbial community, respectively, the WWT inoculum displayed a broader microbial diversity. Firmicutes and Actinobacteriota were the dominant groups each comprising approximately 17 % of the microbial community, followed by Bacteroidota, Patescibacteria and Chloroflexi, each representing 12 to 13 % of the community found in the WWT sample. Regarding archaeal members of the communities, only members of the Haloarchaeota phylum were detected by the V3-V4 primers in the inoculum, making up 3.7 % of the microbial community. However, no archaea were detected in the swine manure by these primers (Fig. S1A). Conversely, when archaea-targeted V4 primers were employed,

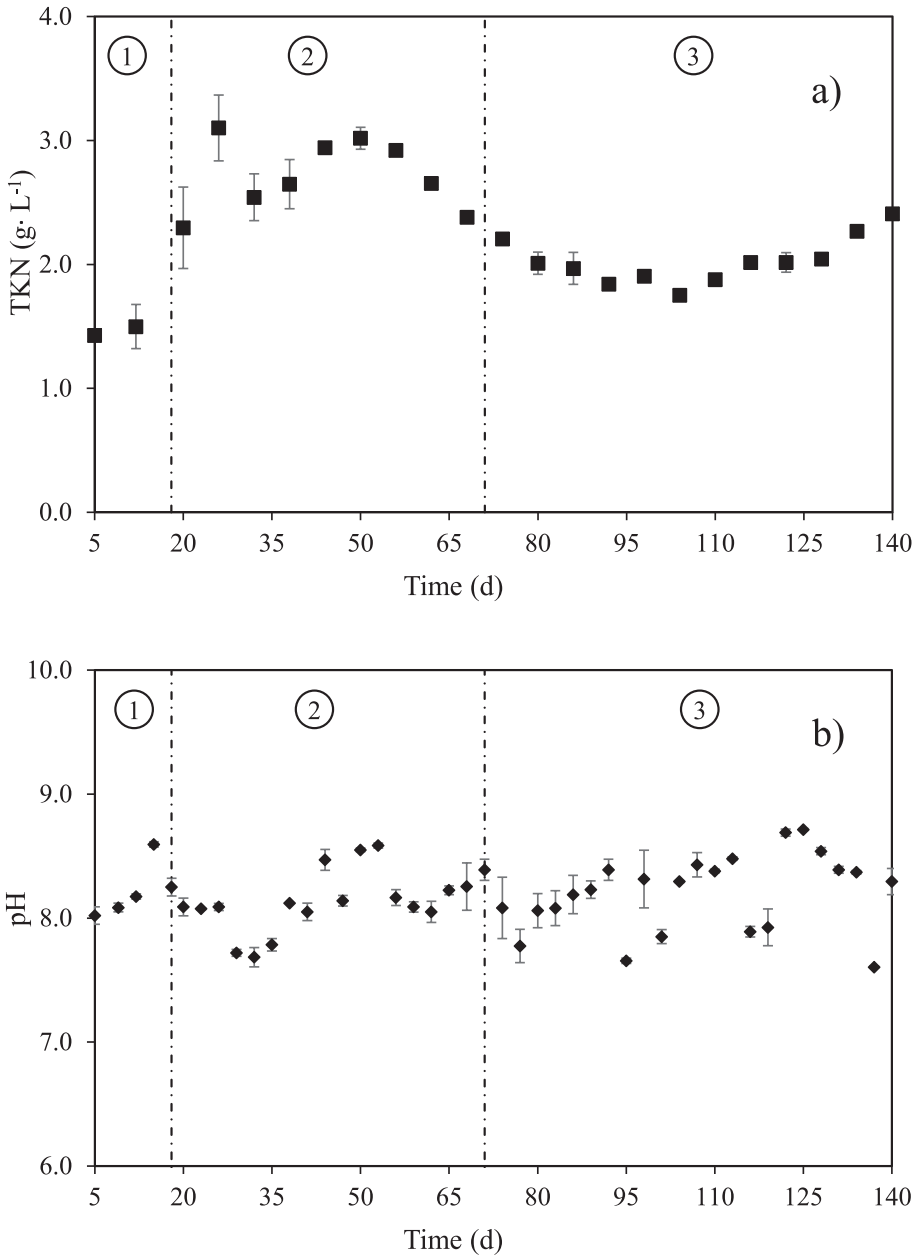

Fig. 3. Time course of the operational parameters during the experiment inside the anaerobic reactors: a) total Kjeldahl nitrogen; b) pH.

Euryarchaeota were predominant in the swine manure (80 %), while the WWT sample was also dominated by Euryarchaeota (50 %) followed by Halobacterota, comprising 42 % of the archaeal community (Fig. S1B).

Interestingly, bacterial groups originally found in the swine manure showed stability and resilience throughout the anaerobic digestion process, whereas those originally found in the anaerobic sludge tended to diminish over time (Fig. 4A).

For instance, members of the Peptostreptococcaceae and Clostridiaceae families (specifically *Terrisporobacter* and *Clostridium* sensu stricto 1 genera) from the Firmicutes demonstrated minimal fluctuations in their relative abundance maintaining levels of approximately 10 % and 40 %, respectively throughout the entirety of the anaerobic digestion process (Fig. 4). Furthermore, an unidentified member of the Rikenellaceae family (Bacteroidota), increased its relative abundance from 0.5 in the swine manure, to 2.5, and 8 % in days 41 and 136 of the anaerobic digestion operation. These facts prove that the microorganisms originally found in the swine manure possess metabolic capabilities contributing to anaerobic digestion. Supporting this fact, several recent studies have detected *Terrisporobacter* and *Clostridium* sensu stricto 1 as key bacteria responsible of fermentation reactions during the digestion of swine manure [40–42]. Historically, members of the Firmicutes phyla have been recognized due to their fermentative metabolism which allows them to provide acetoclastic and methanogenic microbes with substrates for biogas production [43,44]. Furthermore, members of the Rikeneallaceae family have been reported as H_2 producing microorganisms and thus they could have contributed to hydrogenotrophic methanogenesis in the studied system [45,46].

In contrast, members of *Longilinea*, *Tetrasphaera*, unclassified *Intrasporangiaceae*, and *Thermovirga* genera, that were present in the WWT inoculum, significantly diminished their relative abundance by 9, 6, and 5 % respectively by the end of the anaerobic digestion process (Fig. 5). This suggests a less prominent role in the anaerobic digestion of the swine manure, even though members of these genera have been previously reported to play significant roles in digesters [47–49].

From the archaeal counterpart of the microbial communities, taxa originally identified in both, the swine manure and WWT sludge,

A.

Phylum	Family	Genus	SM	WWT	Day 41	Day 136
Universal V3-V4 primers						
Actinobacteriota	Corynebacteriaceae	*Corynebacterium*	2.5	0.0	1.9	1.6
	Intrasporangiaceae	*Tetrasphaera*	0.0	2.5	0.0	0.0
		NA	0.0	2.3	0.0	0.0
Bacteroidota	Dysgonomonadaceae	*Proteiniphilum*	3.2	0.3	1.9	1.4
	Prolixibacteraceae	NA	0.0	2.2	0.1	0.1
	Rikenellaceae	NA	0.5	0.0	2.5	7.8
Chloroflexi	Anaerolineaceae	*Longilinea*	0.0	5.5	0.0	0.2
		NA	0.0	2.2	0.7	0.3
Firmicutes	Erysipelotrichaceae	*Turicibacter*	4.5	0.7	4.2	4.4
	Lactobacillaceae	HT002	2.0	0.0	0.5	0.7
	Christensenellaceae	Christensenellaceae R-7 group	0.1	2.1	0.8	0.7
	Clostridiaceae	*Clostridium sensu stricto* 1	39.4	4.4	38.7	36.1
	Hungateiclostridiaceae	*Fastidiosipila*	2.6	0.1	2.7	3.3
	Peptostreptococcaceae	*Romboutsia*	2.5	2.6	2.0	2.3
		Terrisporobacter	9.8	0.8	9.1	8.9
Halobacterota	Methanosaetaceae	*Methanosaeta*	0.0	3.0	0.1	0.1
Patescibacteria	NA	NA	0.0	5.5	4.5	1.2
	NA	NA	0.0	3.5	0.0	0.1
Synergistota	Synergistaceae	*Thermovirga*	0.0	4.1	0.0	0.3
		Others (<2%)	32.8	58.1	30.1	30.5

Color key (%): 0, 5, 10, 20, 30, 40

B.

Archaeal V4 primers						
Euryarchaeota	Methanobacteriaceae	*Methanobacterium*	0.1	41.3	13.9	26.5
		Methanobrevibacter	22.3	5.2	7.7	8.6
		Methanosphaera	57.4	3.2	37.7	22.3
Halobacterota	Methanomicrobiaceae	*Methanoculleus*	0.0	0.0	0.5	0.0
		Methanogenium	1.7	0.0	0.4	0.1
	NA	NA	0.0	23.5	0.8	20.0
	Methanosaetaceae	*Methanosaeta*	0.0	18.9	2.3	2.1
	Methanosarcinaceae	*Methanosarcina*	0.2	0.1	28.8	16.3
Thermoplasmatota	Methanomassiliicoccaceae	*Methanomassiliicoccus*	0.1	5.2	0.0	0.1
	Methanomethylophilaceae	*Candidatus Methanoplasma*	6.5	0.0	0.3	1.2
		Others*	11.5	2.6	7.7	2.9

Color key (%): 0, 15, 30, 45, 60

Fig. 4. Heatmap displaying the microbial community analysis employing 16s-based universal (Panel A) and archaeal (Panel B) primers. For the V3-V4 universal primers, all taxa with relative abundances of less than 2 % were summarized in the "others" group. *For the V4 archaeal primers, all taxa not belonging to the archaea kingdom were summarized in the "others" group. SM stands for swine manure and WWT stands for wastewater treatment plant inoculum. Day 41 and 136 columns correspond to the samples taken from CSTRs.

showed prevalence during anaerobic digestion (Fig. 4). From the swine manure, members from the *Methanobrevibacter* and *Methanosphaera* genera reached relative abundances of 9 and 22 %, respectively, at stage 3 when biogas production reached its maximum value, albeit their dominance was considerable higher in the manure, 22 and 57 %, respectively. From the WWT sludge, members of *Methanobacterium*, and unclassified Halobacterota, comprised 27 and 20 % of the archaeal community in stage 3, respectively. However, the practical absence of the unclassified member of the Halobacterota phylum in the sample of the stage 2 (inhibition period) suggest that the inoculated organisms belonging to this group were probably inhibited by high concentrations of ammonia and/or VFA. The subsequent adaptation of these organisms probably conducted to the most favourable conditions detected during stage 3. The genus *Methanosaeta*, strongly present in the WWT inoculum (up to 19 %) were practically absent in the steady state conditions of stage 3 (2 %). Interestingly, the most drastic increase in relative abundance was observed for *Methanosarcina* (final abundance of 16 %) which was present at very low levels (<0.2 %) in both the swine manure and in the inoculum (Fig. 6).

Overall, the microbial community analysis suggests that bacterial taxa with fermentative metabolism originally present in the swine manure do prevail during anaerobic digestion and provide to the hydrogenotrophic and acetoclastic methanogens from both the swine manure and the inoculum with substrates for biogas production. In case of archaeal, responsible of the methane production, the intermediate microbial population found in the steady state of the reactors, comprising organisms present in both manure and the WWT inoculum, suggested that inoculation could played a relative minor role. This observation is in agreement with the results found in the BMP tests, where uninoculated tests showed similar levels or even higher levels of biomethane production.

3.3. BMP tests

The biomethane production data was monitored over a time span extending for 60 days shown in Fig. 7 with the three inoculums (WWT, FA and without inoculation). Significant discrepancies emerged between the trials concerning the rate of production and the total biogas output. In the case of tests inoculated with WWT sludge, biogas production showed a rapid increase without lag phase increasing until it peaked at 435 mL $CH_4 \bullet g\ VS^{-1}$ after 18 days of incubation. The trial with only pig sludge (uninoculated) showed a slower production during the

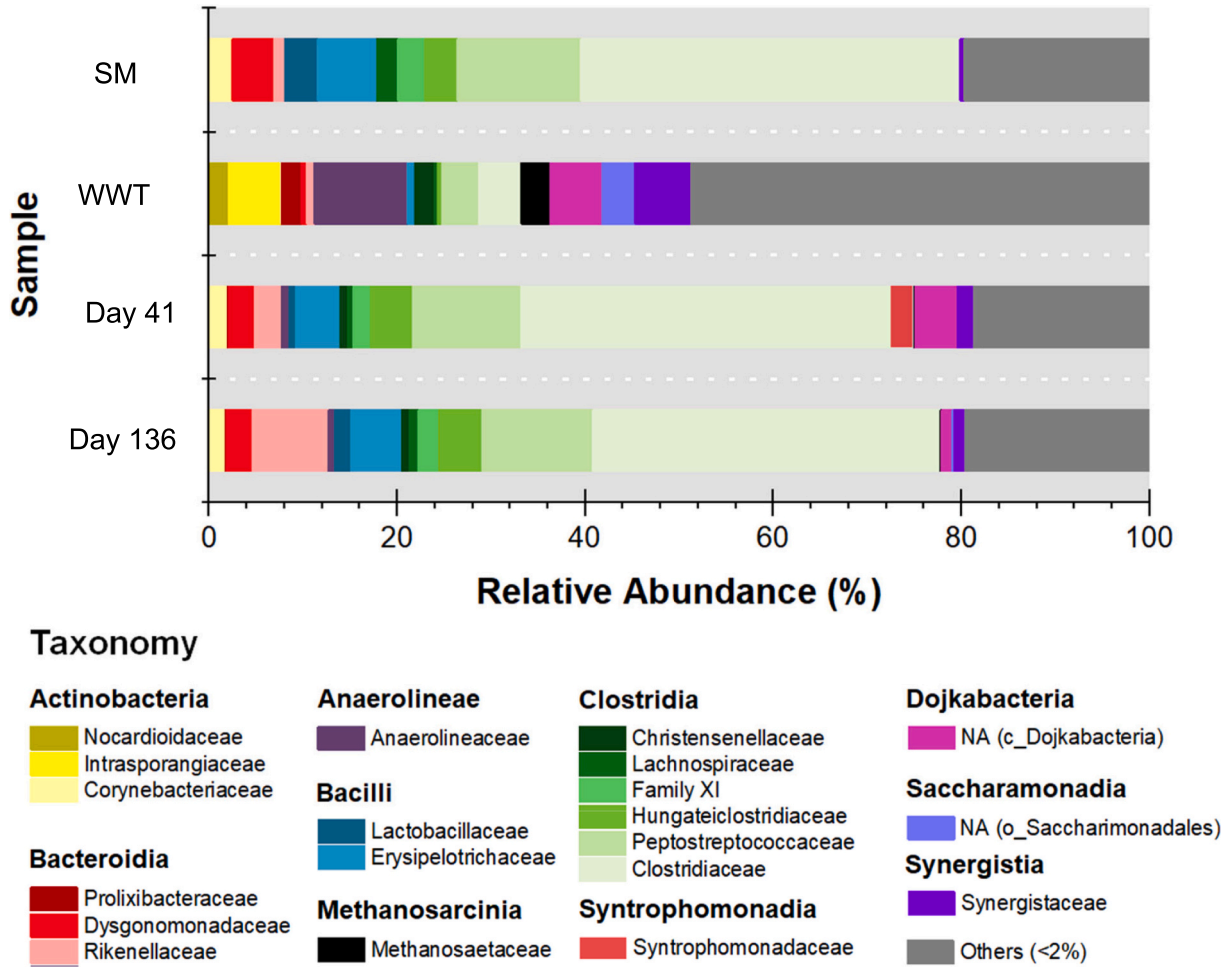

Fig. 5. 16s-based rRNA analysis showing the relative abundance of bacteria retrieved by V3-V4 universal primers from biomass and swine wastewater samples. Taxa not identified at the family level include the next identified taxonomic level in parenthesis. All taxa with relative abundances of <2 % were summarized in the "others" group. *Abbreviations: o_: order, c_:class.

first 10 days of the essay with a notable lag phase. However, a higher accumulated biomethane production was reached with 517.5 mL CH_4•g VS^{-1} on day 55. The flasks inoculated with FD (taken from the farm digester) showed an intermediate rate of biogas production but the higher bioconversion of organic matter to methane with a value of 534 mL•g VS^{-1} by day 57. Taking in consideration that the same substrate was employed in the three essays, these variations between the trials should be attributed to the inoculum use. Although the test with a WWT inoculum exhibits the fastest methane production rate (up to 18 %), its methane potential is lower than that of the essays without inoculum or with a manure adapted inoculum (FD test). Even though higher rates were achieved in WWT inoculated tests during the first 10 days, this fact is not directly related with a better digester performance since continuous operation conditions could modify the initial microbial population composition (see Section 3.3). It is important to consider that in BMP essays, inhibitions mediated by NH_3 or VFA (chronic inhibition) are unlikely to occur because of the optimal doses of inoculum and substrate, resulting in lower concentrations of inhibitors compared to continuous processes [50]. This fact suggests that inhibition phenomena observed in CSTRs during stage 2 has been overcome during stage 3.

4. Conclusions

In summary, biomethane production in continuous mode showed a strong inhibition due to high ammonia that was overcome after >70 days of operation. These trends indicate changing reactor conditions over time and underscore the importance of careful monitoring and control of system parameters (chemical and biological) to optimize biogas production in anaerobic digestion applications. Furthermore, the 16s analyses performed revealed that clades of bacteria and archaea intrinsic to the swine manure display resilience and might play key roles during the anaerobic digestion process presumably due to acclimation to the substrates found in the swine manure. This suggests that microbial population find in this substrate might be sufficient for the quick start-up of anaerobic digestion without the need of the addition of external sources of microorganisms (i.e., anaerobic sludge).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2024.105162.

CRediT authorship contribution statement

Alfonso García Álvaro: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **César Ruiz Palomar:** Validation, Methodology. **Edgardo I. Valenzuela:** Writing – review & editing, Investigation, Conceptualization. **Daphne Hermosilla Redondo:** Supervision, Funding acquisition. **Raúl Muñoz Torre:** Supervision, Methodology. **Ignacio de Godos Crespo:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

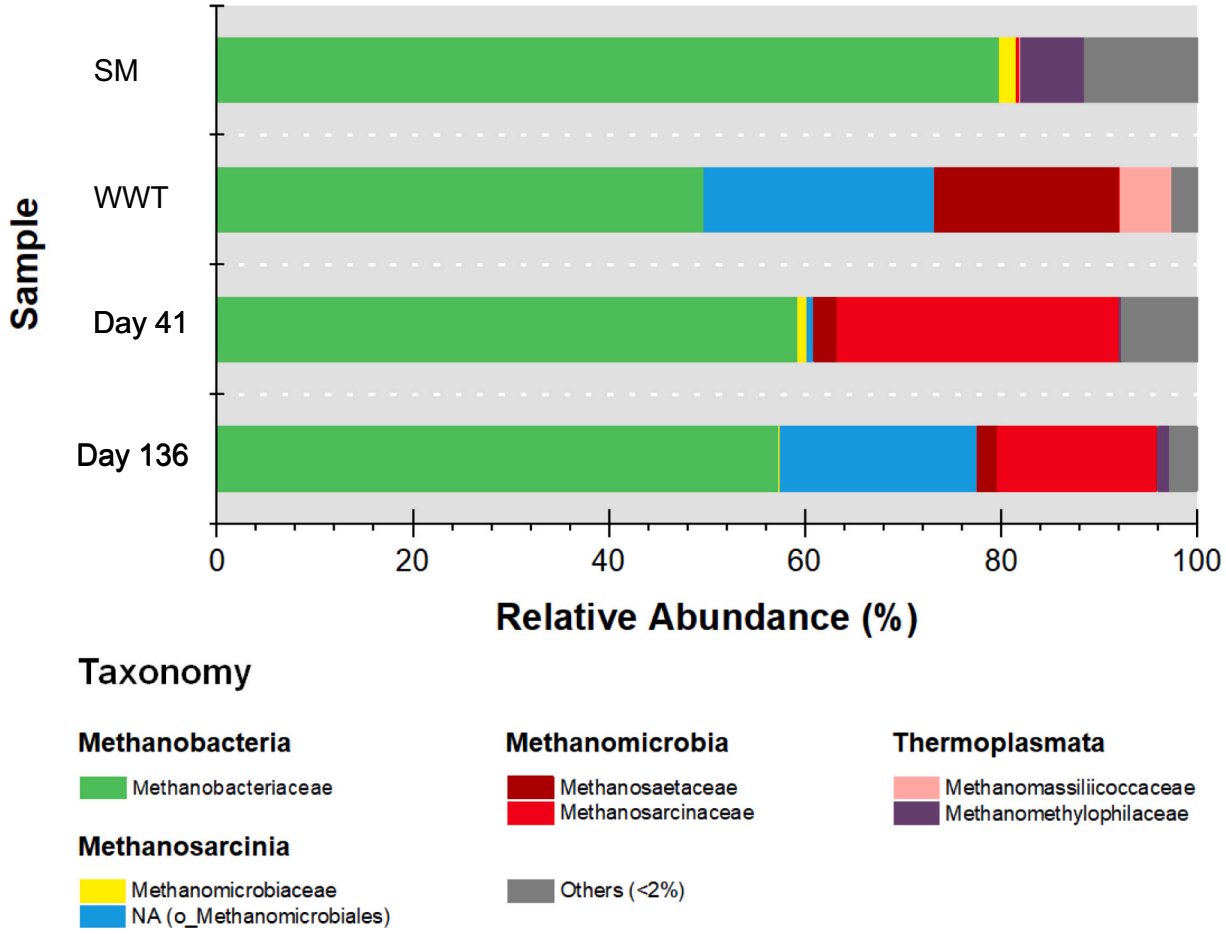

Fig. 6. 16s-based rRNA analysis showing the relative abundance of archaea retrieved by V4-V5 archaeal primers from biomass and swine wastewater samples. Taxa not identified at the family level include the next identified taxonomic level in parenthesis. All taxa with relative abundances of <2 % were summarized in the "others" group. *Abbreviations: o_: order.

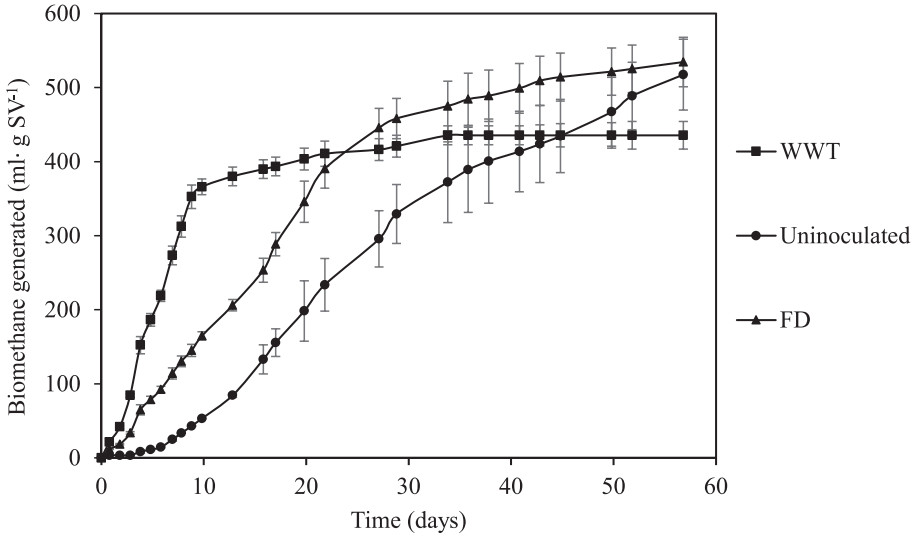

Fig. 7. Biogas production in the BMP tests: ■ (WWT- swine manure with WWT sludge); ● (uninoculated- swine manure without inoculum); ▲ FD- (swine manure with farm digester digestate).

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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