Enhancing Residue Valorization Through Microbial Adaptation: A Path to Sustainable Biogas Production from Pig Manure and Corn Residues

Maria Jose Galvan (mariajosegalvan@yahoo.com.ar)
CONICET

Research Article

Keywords: Pig manure, Biogas, Stabilization ponds, Microbial adaptation, Corn residues, Waste valorization, Sustainability

Posted Date: November 23rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3620484/v1

Abstract

This research is dedicated to redefining residue valorization by maximizing biogas production potential from stabilized pig manure while promoting sustainability. This underscores the significance of microbial adaptation in expediting biogas production and waste valorization. Utilizing pig manure from stabilization ponds as a renewable substrate, this study reveals how microbial adaptation processes not only augment biogas yields, but also contribute to minimizing waste and generating clean energy. By coupling this approach with corn residue as an additional substrate, we created a synergistic model for waste valorization while reducing the environmental footprint of pig farming.

Highlights

Microbial adaptation accelerates biogas production and revolutionizes residue valorization.

Stabilized pig manure is an environmentally friendly and efficient biogas feedstock.

The synergy between pig manure and corn residues offers a promising model for sustainable waste valorisation.

Byproduct residues are transformed into valuable energy sources, which significantly reduces waste.

1. Introduction

Global meat production reached 337 million tons in 2019, 44% more than in 2000. Around 110 million tons of that was pork, i.e., 33% of the total [1]. In Argentina, pork production has increased considerably in recent years, encouraged by rising prices for imported pork and a greater domestic demand. According to the Ministry of Agriculture, Livestock and Fisheries, the sector grew over 230% between 2008 and 2019. Pig farms are mostly located in the country's main agricultural area, the Pampean region, where wide grain availability coincides with slaughterhouses and consumer markets [2]. Management of the effluents associated with this growing production has become an environmental priority, within the context of new regulations that aim to promote renewable energy, reduce nutrient losses and greenhouse gas (GHG) emissions, and protect surface water. In most establishments, these effluents are treated in large stabilization ponds, where they undergo processes like solid separation-extraction and biodigestion before being discharged into water bodies [3, 4].

Stabilized pig manure (PM) can be used for several purposes, including soil amendment. It can partially or totally replace mineral fertilizers, since it supplies the soil with nutrients such as nitrogen and phosphorus, and improves its physical properties [5]. The biggest problem created by its use is the loss of nitrogen, such as ammonia (NH_3), which increases GHG emissions [6]. These losses, and thus the actual impact of PM on the soil, vary depending on specific application practices, the dynamics of nitrogen soil, and mineralization-immobilization processes.

However, there is another use for PM: the production of biogas, for which Argentina has great potential (204,883,456 m^3/year, equivalent to 112,686 tons per year [3, 7]. This potential could be further exploited by taking advantage of corn screening waste (CSW), characterized by Galván *et al.* [8], to acclimate or pre-adapt PM. Several studies have confirmed that pre-adaptation through progressive alteration results in a specialized microbial community with improved performance and tolerance to high organic loading rates [9]. Upon its feeding pattern being changed to pulse feeding, a stirred tank reactor (STR) was found to be more tolerant to higher levels of organic loading and total ammonia nitrogen (TAN). Since this suggests that the regular application of an organic-limited pulse could promote functional stability during anaerobic digestion (AD), pulse feeding may be a good strategy to adapt and bioaugment sludge with high organic loading substrates [10–12]. The idea merits exploration, especially considering the need for more research into AD-based biorefinery, a concept which many authors point out is not yet fully mature [13–16].

Taking all of this into account, the present study characterized PM obtained from the stabilization pond of a local model farm, and subsequently acclimated it with CSW pulses. A microbiological analysis (variability and abundance) was conducted at each stage.

2. Materials and methods

2.1 Sampling and characterization of pig manure (PM)

Before sampling the pig manure (PM), the location of pig farms with treatment ponds was spatially analyzed (QGIS Geographic Information System software). The model farm, located in Bell Ville, Córdoba, Argentina (-32°40'12" S, 62°51'11" W), was selected based on its number of animals and the fact that its management of effluents is representative of typical farmer practices in the region. Sample collection and preparation followed Verein Deutscher Ingenieure, Section 5 (VDI 4630. 2016) [17]. PM was obtained from medium-depth, open-air stabilization ponds, after it had been stabilized there for 120 days in spring (an average temperature 24°C).

Total solids (TS), volatile solids (VS), and total alkalinity (TA) were respectively determined according to standard methods 2540 B, 2540 E, and 2320 B by the American Public Health Association (APHA), and Nordmann titration was performed to find out the levels of volatile fatty acids (VFA). The following parameters were measured with the instruments mentioned after each: pH with a HANNA HI 8424 electronic pH meter; chemical oxygen demand (COD), total ammonia nitrogen (TAN), and free ammonia nitrogen (FAN) with a HANNA HI 83099 spectrophotometer (adapted US EPA method 410.4 for COD and Nessler method for TAN and FAN); and biological oxygen demand (BOD_5) with a VELP BOD EVO 6 sensor system [18, 19]. Protein content was determined by multiplying the total Kjeldahl nitrogen (TKN) (APHA 4500 B) by a conversion factor of 6.25 [20], and organic carbon was determined by considering the ratio between organic matter content and organic carbon as 1.7241 [21]. The microbiological parameters were ascertained through these methods: BAM.ch.4:2002 by the FDA for total coliforms, ISO 7251:2005 for *Escherichia coli*, and ISO 6579-1:2017 for *Salmonella* spp. Finally, acid extraction and metal profiling were carried out according to methods 200.7 and 3125:2017 by the US EPA [18, 19].

2.2 Degassing and SMA tests of PM

PM was preserved, degassed, and characterized following Angelidaki *et al.* [20] and Holliger *et al.* [22]. During degassing, PM was subjected to mesophilic anaerobic conditions to assess the potential to produce biogas and methane from the remaining organic matter in the slurry. To analyze the biogas composition, the process was carried out in triplicate in 5000 mL bioreactors with volumetric displacement gasometers, which admit larger biogas volumes and enable more reproducibility than laboratory-scale tests. Each bioreactor was equipped with a temperature control device, a rotary speed control mixer, and temperature and gas volume sensors. The experiments were conducted at 37 ± 1°C and 100 rpm, and the mixer was operated continuously.

The biogas and methane volumes were measured daily. A gas chromatograph (Fuli Instrument) equipped with a thermal conductivity detector (TCD) and a GDX-502 column (4m x 3 mm) was used to determine the levels of CH_4, CO_2, H_2S, and H_2/N_2. The latter two were measured together through a characteristic biogas pattern in which these compounds were found in lower proportions. A certified mixture from LINDE (55% CH_4, 10% H_2/N_2, 32% CO_2, and 3% H_2S) was the standard. Biogas yield and degradability, VFA, TA, TAN, and FAN were determined at the end of degassing, which was stopped when daily biogas production was less than 1% of the biodigester's volume.

The methane production kinetics of degassing were compared with the performance of the stationary process under practical conditions through a first-order kinetic model.

Subsequently, the specific methanogenic activity (SMA) of the degassed pig manure (DPM), with an inoculum:substrate ratio of 5, was determined using microcrystalline cellulose following Astals *et al.* [23]. These tests were conducted at the laboratory scale in triplicate, in 500 mL bottles. The biogas generated was measured by volumetric displacement and adjusted to normal pressure and temperature conditions.

2.3 Acclimation trials of degassed pig manure (DPM)

An acclimation trial was conducted as described by Tian *et al.* [10]. The pulse feeding rate was determined by considering the characteristics of the substrate (CSW) reported by Galván *et al.* [8], and 5000 mL of DPM were used. The C/N ratio was adjusted to 15 with urea (24.15:1; CSW:urea). TS values were maintained at 10% and the inoculum/substrate ratio at 2. Daily biogas production and its specific composition were analyzed at four stages in each 5000 mL biodigester, to evaluate the system's response in terms of biogas quality and degradability, as well as VFA, TA, TAN, and FAN.

2.4 Microbiological analysis

Samples for microbiological analysis were taken from each biodigester as follows: at the beginning of the assay (initial pig manure or PM); after degassing (DPM); and at the end, i.e., when the four acclimation pulses had been completed (APM). They were stored at -20°C. DNA was extracted from each with the FastSpin for Soil Kit (MP Biomedicals EE. UU), according to the manufacturer's instructions, and sequenced at the Genomics Unit of the Biotechnology Institute of INTA (National Institute of Agricultural Technology). The sequencing of 16S rRNA in the V3-V4 hypervariable region was performed with universal primers 341F and 805R, using 2 × 300 PE reads on the Illumina MiSeq platform. The data were analyzed with the DADA2 package on R, to obtain ASVs (amplicon sequence variants) [24, 25].

3. Results and discussion

3.1 Characterization of initial pig manure (PM) and degassed pig manure (DPM)

Table 1 shows the values for physicochemical parameters and bacterial pathogen counts in pig manure, before and after degassing (PM and DPM).

Table 1
Physicochemical parameters and bacterial pathogen counts in initial pig manure (PM) and after degassing (DPM). Values represent mean ± SD of three different experiments.

Physicochemical parameters	**PM**	**DPM**
Total Solids TS (% m/m)	5.15 ± 0.05	2.25 ± 0.07
Volatile Solids, VS (% m/m)	33.35 ± 0.69	28.06 ± 0.38
Chemical Oxygen Demand, COD (g/l)	58,81 ± 0.95	16.39 ± 0.50
Biochemical Oxygen Demand, BOD (g/l)	23.90 ± 0.76	8.65 ± 0.66
pH	7.44 ± 0.03	7.69 ± 0.09
Total Ammoniacal Nitrogen TAN(g/l)	2.02 ± 0.06	1.17 ± 0.03
Free Ammoniacal Nitrogen FAN(g/l)	2.08 ± 0.04	1.01 ± 0.05
Total Kjeldahl Nitrogen, TKN (% m/m)	2.24 ± 0.05	1.54 ± 0.07
Total Carbon, C (% m/m)	19.39 ± 0.40	16.31 ± 0.38
C/N	8.69 ± 0.18	10.59 ± 0.20
Volatile Fatty Acids VFA (g/l)	0.97 ± 0.01	0.51 ± 0.02
Total Alkalinity TA (g/l)	2.96 ± 0.03	2.87 ± 0.03
Sodium Absorption Ratio SAR	35.11	31.54
Percentage exchangeable sodium PES	35.82	33.21
Potassium (mg/kg DM)	72 ± 3	80 ± 2.2
Phosphorus (mg/kg DM)	134 ± 0.20	175 ± 0.16
Sodium (mg/kg DM)	2322 ± 3	1978 ± 2.1
Arsenic (mg/kg DM)	0.82 ± 0.01	0.90 ± 0.01
Zinc (mg/kg DM)	5.50 ± 0.10	5.20 ± 0.04
Chromium (mg/kg DM)	1.70 ± 0.02	1.54 ± 0.04
Magnesium (mg/kg DM)	55 ± 3	47.30 ± 1.2
Mercury (mg/kg DM)	0.002 ± 0.001	ND
Nickel (mg/kg DM)	0.88 ± 0.001	0.72 ± 0.009
Lead (mg/kg DM)	0.04 ± 0.001	0.02 ± 0.001
Bacterial pathogens		
Escherichia coli (UFC/100ml)	0.80	ND
Fecal coliforms (NMP/100ml)	0.84	ND
Salmonella spp. (UFC/100ml)	ND	ND
Yield parameters		
Organic matter removal OMR (% VS)	63.23 ± 1.07	-

Physicochemical parameters	PM	DPM
Biogas yield (Nml/g $_{VS}$)	298.77 ± 1.04	-
Methane yield (Nml/g $_{VS}$)	138.02 ± 1.03	-

Analysis of samples in triplicate. N: STP. ND: not detected

The parameters that decreased most significantly after degassing included chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5). In the first case, the difference between PM (58.81 g/l) and DPM (16.39 g/l) was 42.42 g/l. In the second case, the difference between PM (23.90 g/l) and DPM (8.65 g/l) was 15.25 g/l. Moreover, total solids (TS) decreased by 2.9%, (from 5.15% m/m in PM to 2.25% m/m in DPM). The changes in these three parameters (COD, BOD5, and TS) were likely due to a reduction in organic matter after degassing.

On the other hand, the increase in the C/N ratio in DPM can be attributed to a reduction in total nitrogen, among other factors. As described by Shen *et al.* [26], a low C/N ratio hinders continuous biogas production. Consequently, high biogas yields would not be possible with PM as the sole substrate, and supplementary substrates would be necessary to improve the C/N balance [26–28].

The values for AGV, TA, pH, and TAN in DPM were adequate for the production of biomethane, according to reference values by Holliger *et al.* [22]

and the VS value exceeded TS by 50% (VDI 4630. 2016) [17]. Other factors also point to DPM being an appropriate inoculum for AD. First, its VFA/TA ratio (which indicates the organic load) was 0.17. Values between 0.3 and 0.4 signal optimal biogas production; those above 0.4 indicate substrate overload, while those below 0.3 correspond to low substrate input [29]. In other words, DPM appears to have reached a high degree of starvation. Additionally, due to supplements added to animal feed, DPM contains and preserves macro- and micronutrients and metals that optimize the metabolic reactions in AD. Cai *et al.* [30] reported improvements in biomethane yield after incorporating micronutrients into animal feed.

Moreover, starvation under anaerobic and mesophilic conditions reduces or eliminates pathogens, which explains why pathogenic bacteria were detected in the initial slurry but not in DPM.

During degassing, PM yielded 298.77 NmL/g VS of biogas and 138.02 NmL/g VS of methane. The latter was approximated to the volume calculated on the basis of chemical composition (157.84 NmL/g VS) [31]. Even higher potential values than these (181 NmL/g VS) were reported by Flotats *et al.* [32]. The organic matter removal rate (OMR) in our assay was also significant (63.23%).

Figures 1a and 1b respectively show the daily and cumulative production of biogas and methane in each batch (or biodigester), as well as their positive controls. The test lasted 30 ± 2 days, until less than 1% of the total volume was produced. In terms of composition, 46.21% of the biogas before degassing was made up of CH_4, which is 9% lower than what was reported by Regueiro *et al.* [33]. During degassing (Fig. 1c), the percentage of CH_4 peaked on day eight (64.08%). That same day, the rest of its composition consisted of 31.87% CO_2, 3.86% H_2/N_2, and 0.19% H_2S. In general, CH_4 percentages were at their highest when production was also at its highest (days 8–15), while CO_2 and N_2/H_2 remained stable in the ranges of 20–30% and 10–15%, respectively.

The calorific value of biogas is known to climb when its CH_4 content increases, and to drop as CO_2 rises. A possible cause for CO_2 increasing in our trial might be the accumulation of VFA during AD. This shifts the bicarbonate balance

towards CO_2 to maintain the pH, as long as the alkalinity of the system supports it. Moreover, high percentages of CO_2 may create an acidic environment in engines during the transformation of biogas into electricity [34].

The percentages registered for H_2/N_2 during degassing were also high. Such values make partial pressure higher than what is suitable for acetogenesis, and thus render it thermodynamically impossible. High H_2 concentrations may indicate that the digester is overloaded [35]. Finally, the specific methanogenic activity (SMA) of DPM was 0.12 CH_4. g_{COD}/g_{VSinoc}, despite its degree of dilution and its relatively low content of VS. This value is higher than that reported by Angelidaki *et al*. [20] and Holliger *et al*. [22] (0.1), and that the one obtained by Astals *et al.* [23] for inocula from effluent ponds.

3.2 Acclimation of the inoculum

Figures 2a and 2b respectively show the daily and the cumulative production of biogas/CH_4 during each acclimation pulse (P1, P2, P3, and P4). Between P1 and P4, the volume of biogas doubled and that of methane tripled. The lag phase for CH_4 production shortened as the trial went on (Fig. 2b), which means that the system was able to withstand the stress caused by the organic load of each pulse. This increase in production and the ability to tolerate high organic loading rates may be attributed to the specialized microbial community that emerges in the digester during acclimation [9].

Besides, due to the low solid content applied with each pulse and the characteristics of CSW (high percentage of starch), organic matter was transformed into biogas more quickly. In other words, the pulse feeding pattern appears to have made the biodigesters more tolerant to organic loads and higher levels of total ammonia nitrogen (TAN), and thus more efficient at producing biogas.

The composition of biogas in each batch during each acclimation pulse, in terms of component percentages, is shown in Fig. 3c. After an initial increase, the percentage of CH_4 remained at around 60–64% from P3 onwards. This was correlated with a decrease in CO_2, whose values then stabilized between 20 and 25%. Although H_2/N_2 rose dramatically upon the application of P1, they rapidly dropped to 10% and remained stable during the other three pulses. This may have prevented additional stress on the system, and better allowed the microbial biomass to adapt. Syntrophic microorganisms can proliferate during acetate oxidation (SAO) by transforming acetic acid into H_2 and CO_2 [36] while CH_4 production remains stable. The biogas/methane yield was 1.6 and 2.8 times higher than the initial values after P1 and P2, respectively (Fig. 2d). The values for OMR based on VS remained between 55 and 64% throughout the four pulses (Fig. 2e).

Even though VFA increased at the end of P1, they then decreased considerably and from thereon stayed more or less the same during P2, P3, and P4 (Fig. 3a). Put otherwise, there was an initial spike in VFA associated with the system's rapid adaptation to substrate consumption, but once this had been achieved, the levels fell and each successive pulse brought about slightly decreasing values. This reduction was correlated with an increase in TA, whose values then stayed at around 6 g $CaCO_3$/L throughout the trial (Fig. 3b). Thus, the VFA/TA ratio (which is widely taken to indicate biodigester performance) was balanced enough to allow the system to withstand pH variations. Similarly, Kim *et al*. [37], who attempted to reduce the AD dormancy phase and attain higher yields by using substrates with a high organic load, concluded that for this to happen the VFA/TA ratio should be below 0.4 and the initial VFA/TS ratio should be less than 10%. Finally, the values registered for FAN, TAN, and NH_3 in our trial were within the stable range reported for other inocula (0.5–1.2 g/L) [22].

Although, as seen in Figs. 3a and 3b, the percentages of CH_4 had tripled by the end of the trial, they increased mainly during the first two pulses and did not significantly rise after P2. This means the system reached its maximum CH_4 production at that point, and it is the reason why the test was stopped after four pulses. In addition to the lag phase being shorter with each successive pulse, the composition of the biogas also became progressively less variable, which is advantageous in terms of final biogas quality.

In general, these findings demonstrate that biodigesters exposed to perturbation in the form of organic feeding achieve better biogas and methane yields (without considering endogenous biogas production), a similar conclusion to that reported by Wang *et al.* [12]. Other authors propose using disturbance as a strategy to influence methanogenic microbiomes and improve the co-digestion of critical waste, and suggest that acclimated inocula are essential to enhance biogas production by anaerobic co-digestion [38].

All these parameters described so far are important and complement each other. However, it is biogas composition in particular which is essential to implement this kind of feeding regime at a larger scale, since it determines biogas quality. Higher solid concentrations than those used here (< 10%) might produce higher yields, but the system's stability might not be guaranteed. Nevertheless, each AD system has its own working limits and interactions that arise between variables.

3.3 Microbial characterization

The microbiological characterization was based on 227,590 sequences found in the three samples analyzed: PM (initial pig manure), DPM (degassed manure), and APM (acclimated manure). The average number of sequenced fragments (251 bp) was consistent with the sequencing method used. PM contained 17219 sequences and DPM, 14993. In contrast, APM had a smaller number (9151). Amplicon sequence variants (ASVs) were obtained on DADA2, and taxonomically classified (with 100% identity) using the SILVA database (v 138.1).

Table 2 shows the number of ASVs and the values of Shannon's diversity index and inverse Simpson's index for the three samples. APM had fewer taxa and lower diversity than PM and DPM, which is consistent with its smaller number of sequences. The change in conditions in the biodigesters (acclimation with pulse feeding) was likely responsible for this reduction in microbial diversity.

Table 2
Number of amplicon sequence variants (ASVs), Shannon's diversity index (richness), and inverse Simpson's index (equity) for PM, DPM, and APM.

Sample	Number of ASVs	Shannon	1/Simpson
PM	418	5.68	232
DPM	417	5.68	228
APM	256	5.12	132

Figure 4a shows the relative abundance of phylotypes (% RA), separated into taxonomic levels (domain (K), phylum (P), order (O), and genus (G)) in PM, DPM, and APM. Bacteria were the most prevalent domain in all three. This does not mean there were no Archaea, but rather that the sequencing primers likely originated from bacteria and thus have less affinity with Archaea. The predominant phyla were Bacteroidetes, Proteobacteria, Firmicutes, and Desulfobacterota, particularly in PM and DPM. Bacteroidales and Burkholderiales were the most relevant orders, the latter mainly in DPM. At the genus level, *Sulfuritalea* was only present in PM (9% RA).

The relative abundance of the 70 most significant ASVs in the samples can be seen in Fig. 5b. Abundance was low for all the taxa and none of them was dominant. Moreover, the most abundant sequences represented only 2% of the total. The samples did not share many sequences, but instead each had its own specific composition.

These 70 ASVs were put through BLAST + to find matches (99% identity) with full-length ASVs available on the NCBI (National Center for Biotechnology Information) database. Sixty of them matched 1269 sequences on the database, while the other ten had no matches and might correspond to as yet unreported microorganisms. Most of the sequences belonged to uncultured microorganisms.

Figure 5 shows the sources from which were isolated the sequences on the NCBI database that matched the ASVs in our study. Most of them came from anaerobic bioreactors, followed by a large percentage that is typical of wastewater treatment processes ("WWTP"). Others were found in anaerobic environments unrelated to reactors, or were associated with livestock farming (manure lagoons, slurry, etc.) [39]. Importantly, all these environments are related to the process studied here, and they cover most of the sequences found (~ 80%). There were, nevertheless, other matching sequences that came from the petrochemical industry ("Oil & Gas"); lakes, rivers, and rhizospheric environments associated with agriculture; mining activity, urban landfills, and other laboratory processes ("Other").

The most important ASVs detected in PM were ASV 7, ASV 12, ASV 4, and ASV 17. The first two are *Sulfuritalea* spp., and neither of them matched the NCBI data. This makes sense considering that only one species of this genus has been isolated so far (*Sulfuritalea hydrogenivorans*) [40]. This is an autolithotrophic, sulfur-oxidizing, and nitrate-reducing neutrophil which has been described not only in several aquatic environments with low carbon loads, but also in activated sludge and hydrocarbon-contaminated sites [41].

ASV 4 is a member of *Novosphingobium*, a genus of facultative aerobic organotrophs that can reduce nitrate and are involved in the degradation of aromatic compounds [42]. Sequences identical to ASV 4 were isolated from landfills and WWTP. ASV 17, a *Pelospora sp.*, showed identity with sequences from an activated sludge, an anaerobic digester of livestock waste, and municipal landfills. Only one species of this genus, a strictly anaerobic glutarate fermenter, is known so far (*Pelospora glutarica*).

The most predominant ASV in DPM was ASV 1, from the family *Anaerolineaceae*, which represents only 2% of the community. Furthermore, the same sample featured ASV 9 (genus *Mesotoga*), ASV 11 (also belonging to *Anaerolineaceae*), ASV 32 (genus *Pseudomonas*), and ASV 6 (a taxon of the class Clostridia). Sequences similar to those of ASV 1 and ASV 11 have been widely reported in wastewater treatment systems and anaerobic digesters, including biogas plants [43]. Members of the *Anaerolineaceae* family include strict anaerobes, mesophiles, thermophiles, and chemoheterotrophs. In addition, some studies have shown that they can interact syntrophically with methanogenic microorganisms and generate hydrogen [44].

ASV 9 was classified as *Mesotoga infera*, a species within the order *Thermotogales* which has been found in anaerobic digesters containing high loads of carbonaceous compounds (including hydrocarbons such as toluene, benzene, and xylene) at high temperatures (65–85°C). *Mesotoga* spp. are mesophilic, and they use sulfur compounds as electron acceptors to produce sulfur, acetate, and CO_2, but do not seem able to produce hydrogen [45].

The genus *Pseudomonas*, to which ASV 32 belongs, comprises a wide variety of species that are capable of obtaining energy from complex carbon compounds. Sequences similar to ASV 32 have been found in bioreactors, rhizospheric environments, sludge, and wastewater. As degraders of complex carbon sources, *Pseudomonas* spp. may be some of the most important microorganisms in anaerobic digesters [46]. ASV 6, from the class Clostridia, is related to anaerobic reactors as well.

The most relevant ASV in APM was ASV 13, another *Pseudomonas* sp. (2.1% RA). Other ASVs which were identified in this sample are ASV 2 and ASV 3 (both from the order Bacteroidales), ASV 19 (from the order Synergistales), and ASV 39 (from the class Clostridia). ASV 2 and 3 were similar to each other and had matches on the NCBI database, which corresponded to uncultured microorganisms from anaerobic bioreactors that produce biogas from agricultural waste (lignocellulolytic waste, bovine albumin, and pig farming waste). ASV-19 like sequences (synergists) have been reported in anaerobic bioreactors used for municipal wastewater treatment. Synergists are associated with animal microbiota and frequently found in anaerobic digesters that produce amino acids and degrade proteins, where they establish a syntrophic relationship with methanogens [47–48]. Finally, only five sequences on the NCBI database matched ASV 39 (*Clostridia*),

four of which came from an anaerobic reactor fed with swine waste (the same one where the sequences that matched ASV 2 and 3 were detected).

4. Conclusions

The physicochemical characterization of pig manure after degassing (DPM) showed that it had adequate OMR, VFA, TA, pH, and TAN values to be used as an inoculum for the production of biogas through AD. Its appropriateness for this purpose was further corroborated by the levels measured for SMA, the VFA/TA ratio, and micronutrients, and by its lack of pathogens. However, its low daily yield of biogas/methane and low C/N ratio indicate that one or more substrates should be incorporated as sources of carbon to enhance production. When DPM was acclimated by pulse feeding with CSW (APM), the methane concentration improved by 30% and the dormancy phase for its generation was shorter. Moreover, the composition of biogas became less variable, i.e., more stable.

Both DPM and APM, as well as the stabilized manure in its original state (PM), were microbiologically analyzed at the genetic level. Out of the top 70 amplicon sequence variants detected, 60 were matched to 1269 sequences available at the NCBI database. The other ten had no matches and could correspond to unreported microorganisms. Most of the sequences correspond to uncultured microorganisms found in anaerobic bioreactors. The increase in hydrolytic and fermentative populations as a result of acclimation appears to have positively influenced the bacterial microbiome in the digesters.

This study offers a general picture of microbial evolution at each stage of acclimation, and also explores the possibility of incorporating agroindustrial waste into the biorefinery system. Further research could attempt to obtain inocula that improve the efficiency and conditions of biogas production.

Declarations

Competing interests: The authors declare no competing interests.

References

1. Index, F. F. P. (2021). World Food Situation. FAO: Rome, Italy. https://doi.org/10.4060/cb4477es
2. Informe 2019a. Ministerio de Agricultura, Ganadería y Pesca Argentina. Subsecretaría de Ganadería y Producción Animal. Área Porcinos-Dirección de porcinos, aves de granja y no tradicionales. https://www.magyp.gob.ar/sitio/areas/porcinos/estadistica/_archivos/000005 Anuario/190000-Anuario%202019.pdf
3. Informe 2019b. Ministerio de Agricultura, Ganadería y Pesca Argentina. Subsecretaría de Ganadería y Producción Animal. Área Porcinos-Dirección de porcinos, aves de granja y no tradicionales. https://www.magyp.gob.ar/sitio/areas/porcinos/estadistica/_archivos/000005-Anuario/190000-Anuario%202019.pdf
4. Tlustoš P., Hejcman M., Kunzová E., Hlisnikovský L., Zámečníková H., Száková J. 2018. Nutrient status of soil and winter wheat (Triti-cum aestivum L.) in response to long-term farmyard manure application under different climatic and soil physicochemical conditions in the Chec Republic. *Arch Agron Soil Sci* 64:70-83. https://doi.org/10.1080/03650340.2017.1331297
5. Damian J., Muraro D., Basso C., Da Silva N., Pinto M., Santi A. 2018 Strategies for fertilization with pig and cattle slurry in wheat crop. *Rev Bras Eng Agrícola e Ambient* 261-266. http://dx.doi.org/10.4067/S0718-58392014000300009

6. Park S., Lee B., Jung K., Kim T. 2018 Acidification of pig slurry effects on ammonia and nitrous oxide emissions, nitrate leaching, and perennial ryegrass regrowth as estimated by N-urea flux. Asian-Australasian J Anim Sci 31:457-466. https://doi.org/10.5713/ajas.17.0556
7. FAO. 2019. Estudio de cuencas de biogás. Colección Informes Técnicos N° 4. Buenos Aires.http://www.probiomasa.gob.ar/_pdf/Informe%20Tecnico%20Nro4Estudio%20de%20cuencas%20de%20biogas-19-08-22.pdf
8. Galván, M. J., Degano, S., Cagnolo, M., Becker, A., Hilbert, J., Fuentes, M., & Acevedo, D. 2021. Batch optimization of biogas yield from pasteurized slaughterhouse by-products incorporating residues from corn sieving. *Biomass and Bioenergy*, 151, 106136.
9. De Vrieze J., Verstraete W., Boon N. 2013. Repeated pulse feeding induces functional stability in anaerobic digestion. *Microbial Biotechnology* 6(4), 414-424. https://doi.org/10.1111/1751-7915.12025
10. Tian H., Fotidis I., Mancini E., Angelidaki I. 2017. Different cultivation methods to acclimatize ammonia-tolerant methanogenic consortia, *Bioresource Technology*. http://dx.doi.org/ 10.1016/j.biortech.2017.02.034
11. Lua F., Jianga Q., Qiana F., Zhoue Q., Jiang C, Shen P. 2019. Semi-continuous feeding combined with traditional domestication improved anaerobic performance during the treatment of cassava stillage. *Bioresource. Technology* 291: 121807.
12. Wang L., Hossen E., Aziz T., Ducoste J., De los Reyes III F. 2020. Increased loading stress leads to the convergence of microbial communities and high methane yields in adapted anaerobic co-digesters. *Water Research.* 169, 115-155.
13. Pivato A., Vanin S., Raga R., Lavagnolo M., Barausse A., Rieple A., Laurent A, Cossu R. 2016. Use of digestate from a decentralized on-farm biogas plant as fertilizer in soils: An ecotoxicological study for future indicators in risk and life cycle assessment, *Waste Management,* Vol: 49, 378-389. https://doi.org/10.1016/j.wasman.2015.12.009
14. Pérez-Camacho M. & Curry R. 2018. Regional assessment of bioeconomy options using the anaerobic biorefinery concept. Proc. Inst. Civ. *Eng.-Wast Resour. Manag.* 171, 1700015, 104-113. https://doi.org/10.1680/jwarm.17.00015
15. Antoniou N., Monlau F., Sambusiti C., Ficara E., Barakat A., Zabaniotou A. 2019. Contribution to Circular Economy options of mixed agricultural wastes management: coupling anaerobic digestion with gasification for enhanced energy and material recovery. *J. Clean. Prod*. 209, 505–514. https://doi.org/10.1016/j.jclepro.2018.10.055.
16. Varma V., Parajuli R., Scott E., Canter T., Lim T., Popp J., & Thoma, G. 2021. Dairy and swine manure management–Challenges and perspectives for sustainable treatment technology. *Science of The Total Environment*, 146319. https://doi.org/10.1016/j.scitotenv.2021.146319
17. VDI 4630. 2016. Fermentation of organic materials-Characterization of the substrate, sampling, collection of material data, fermentation tests, VDI-Handbuch Energietechnik.
18. APHA. (2005). Standard methods for the examination of water & wastewater. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC, USA.
19. USEPA-ICP Users Group (Edited by T.D. Martin and J.F. Kopp) - Method 200.7, Revision 1.0, (Printed 1979, Published 1982.
20. Angelidaki I., Alves M., Bolzonella D., Bprzacconi L., Campos J. L., Guwy A. J., Kalyuzhnyi S., Jenicek P., Van Lier J. B. 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. *Water Sci. and Technol.*, 59, 927-934.
21. Cuetos M. J., Gómez X., Otero M., Morán A. 2008. Anaerobic digestion of solid slaughterhouse waste (SHW) at laboratory scale: Influence of co-digestion with the organic fraction of municipal solid waste (OFMSW). *Bioch. Eng*. 40, 99-106.
22. Holliger C., Alves M., Andrade D., Angelidaki I., Astals S., Baier U., Bougrier C., Buffiere P., Carballa M., Wilde V., Ebertseder F., Fernández B., Ficara E., Fotidis I., Frigon J., Fruteau de Laclos H., Ghasimi D. S. M., Hack G., Hartel M.,

Heerenklage J., Horvarth I. S., Jenicek P., Koch K., Krautwald J., Lizasoain J., Liu J., Mosberger L., Nistor M., Oechsner H., Oliveira J. V., Peterson M., Pauss A., Pommier S., Porqueddu I., Raposo F., Ribeiro T., Pfund F. R., Stromberg S., Torrijos M., van Eekert M., van Lier J., Wedwitschka H., Wierinck I. 2016. Towards a standardization of biomethane potential tests. *Water Sci. and Technol*. 74, 2515-2522.

23. Astals, S., Batstone, D. J., Tait, S., & Jensen, P. D. 2015. Development and validation of a rapid test for anaerobic inhibition and toxicity. *Water Research*, 81, 208-215.
24. Bolger A., Lohse M., Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data, *Bioinformatics,* 30 (2014) 2114-2120.
25. Engelbrektson, A., Kunin, V., Wrighton, K. C., Zvenigorodsky, N., Chen, F., Ochman, H., & Hugenholtz, P. 2010. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. *The ISME journal*, 4(5), 642-647.
26. Shen P., Han F., Su S., Zhang J., Chen Z., Li J., Gan J., Feng B., Wu B. 2014. Using pig manure to promote fermentation of sugarcane molasses alcohol wastewater and its effects on microbial community structure. *Bioresour. Technol*. 155, 323-329. https://doi.org/10.1016/j.biortech.2013.12.073
27. Ren J., Yuan X., Li J., Ma X., Zhao Y., Zhu W., Wang, X., Cui Z. 2014. Performance and microbial community dynamics in a two-phase anaerobic co-digestion system using cassava dregs and pig manure. *Bioresour. Technol*. 155, 342-351.
28. Zhou S., Nikolausz M., Zhang J., Riya S., Terada A., Hosomi M. 2016. Variation of the microbial community in thermophilic anaerobic digestion of pig manure mixed with different ratios of rice straw. J. *Biosci. Bioeng*. 122 (3), 334-340.
29. Radis Steinmetz R., Mezzari M., Busi da Silva M., Kunz A., Cestonaro do Amaral A., Tápparo D., Moreira Soares H. 2016. Enrichment and acclimation of an anaerobic mesophilic microorganism's inoculum for standardization of BMP assays. *Bioresource Technology* 219: 21–28. https://doi.org/10.1016/j.biortech.2016.07.031
30. Cai, Y., Hua, B., Gao, L., Hu, Y., Yuan, X., Cui, Z., ... & Wang, X. 2017. Effects of adding trace elements on rice straw anaerobic mono-digestion: Focus on changes in microbial communities using high-throughput sequencing. *Bioresource Technology,* 239, 454-463.
31. Hejnfelt, A., & Angelidaki, I. (2009). Anaerobic digestion of slaughterhouse by-products. *Biomass and bioenergy*, *33*(8), 1046-1054.
32. Flotats X., Bonmatí A., Fernández B. Magrí, A. 2009. Manure treatment technologies: on-farm versus centralized strategies. NE Spain as a case study. *Bioresour. Technol*. 100, 5519-5526. https://doi.org/10.1016/j.biortech.2008.12.050
33. Regueiro, L, Carballa, M, Álvarez, J. A., Lema, J. 2012. Enhanced methane production from pig manure anaerobic digestion using fish and biodiesel wastes as co-substrates. *Bioresource Technology*, 123, 507-513. https://doi.org/10.1016/j.biortech.2012.07.109
34. Solera del Rio R. 2014. De Residuo a Recurso. II Procesos de biotransformación de la materia orgánica. II Aspectos biológicos de la digestión anaeróbica. Madrid, España: Ediciones Mundi-prensa.
35. Ward A., Hobbs P., Holliman P., Jones D. 2008. Optimization of the anaerobic digestion of agricultural resources, Bioresour. Technol. 99: 7928-7940.
36. Rinzema, A., Boone, M., van Knippenberg, K., & Lettinga, G. 1994. Bactericidal effect of long chain fatty acids in anaerobic digestion. *Water Environment Research*, 66(1), 40-49.
37. Kim, M. J., & Kim, S. H. 2020. Conditions of lag-phase reduction during anaerobic digestion of protein for high-efficiency biogas production. *Biomass and Bioenergy*, 143, 105813.

38. Nguyen, L. N., Kumar, J., Vu, M. T., Mohammed, J. A., Pathak, N., Commault, A. S., Nghiem, L. D. 2021. Biomethane production from anaerobic co-digestion at wastewater treatment plants: A critical review on development and innovations in biogas upgrading techniques. *Science of the Total Environment*, 765, 142753. https://doi.org/10.1016/j.scitotenv.2020.142753
39. Pasalari, H., Gholami, M., Rezaee, A., Esrafili, A., & Farzadkia, M. 2021. Perspectives on microbial community in anaerobic digestion with emphasis on environmental parameters: A systematic review. *Chemosphere*, 270, 128618.
40. Kojima, H., & Fukui, M. 2011. *Sulfuritalea hydrogenivorans* gen. nov., sp. nov., a facultative autotroph isolated from a freshwater lake. *International journal of systematic and evolutionary microbiology*, 61(7), 1651-1655.
41. Sperfeld, M., Diekert, G., & Studenik, S. 2019. Anaerobic aromatic compound degradation in *Sulfuritalea hydrogenivorans* sk43H. *FEMS microbiology ecology,* 95(1), fiy199.
42. Liu, Y., Pei, T., Du, J., Huang, H., Deng, M. R., & Zhu, H. 2021. Comparative genomic analysis of the genus *Novosphingobium* and the description of two novel species *Novosphingobium aerophilum sp. nov. and Novosphingobium jiangmenense sp. nov*. *Systematic and Applied Microbiology*, 44(3), 126202.
43. McIlroy, S. J., Kirkegaard, R. H., Dueholm, M. S., Fernando, E., Karst, S. M., Albertsen, M., & Nielsen, P. H. 2017. Culture-independent analyses reveal novel anaerolineaceae as abundant primary fermenters in anaerobic digesters treating waste activated sludge. *Frontiers in microbiology*, 8, 1134.
44. Sun, L., Toyonaga, M., Ohashi, A., Matsuura, N., Tourlousse, D. M., Meng, X. Y., ... & Sekiguchi, Y. 2016. Isolation and characterization of *Flexilinea floccu*li gen. nov., sp. nov., a filamentous, anaerobic bacterium belonging to the class Anaerolineae in the phylum Chloroflexi. *International journal of systematic and evolutionary microbiology*, 66(2), 988-996.
45. Hania, W. B., Postec, A., Aüllo, T., Ranchou-Peyruse, A., Erauso, G., Brochier-Armanet, C., ... & Fardeau, M. L. 2013. *Mesotogainfera sp*. nov., a mesophilic member of the order Thermotogales, isolated from an underground gas storage aquifer. *International journal of systematic and evolutionary microbiology*, 63(8), 3003-3008.
46. Buettner, C., von Bergen, M., Jehmlich, N., & Noll, M. 2019. *Pseudomonas spp*. are key players in agricultural biogas substrate degradation. *Scientific reports*, 9(1), 1-13.
47. Godon, J. J., Morinière, J., Moletta, M., Gaillac, M., Bru, V., & Delgènes, J. P. 2005. Rarity associated with specific ecological niches in the bacterial world: the 'Synergistes' example. *Environmental Microbiology*, 7(2), 213-224.
48. Vartoukian, S. R., Palmer, R. M., & Wade, W. G. 2007. The division "synergistes". *Anaerobe*, 13(3-4), 99-106.

Figures

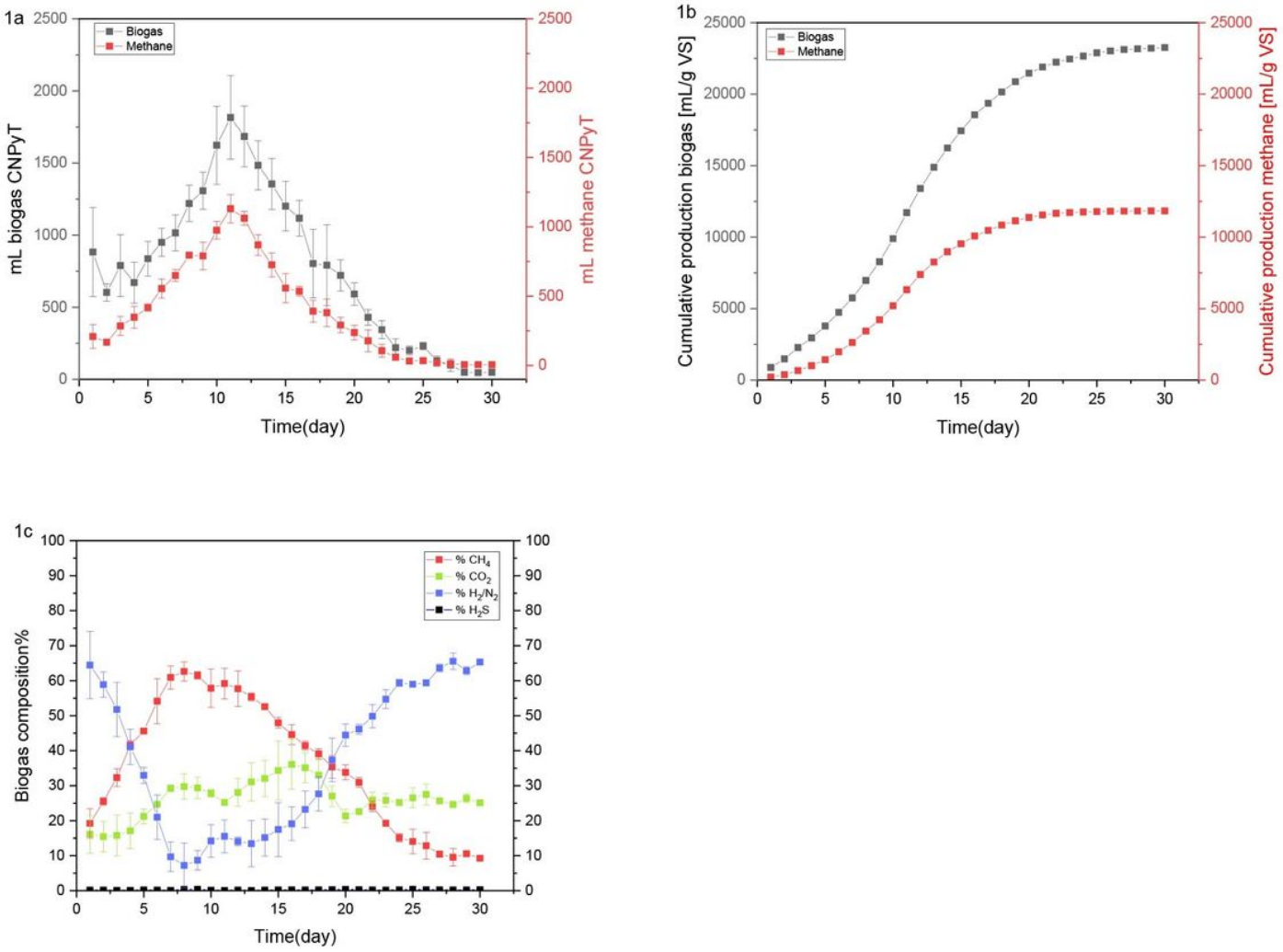

Figure 1

(a) Daily production of biogas/methane, and (b) cumulative production of biogas and methane in 5000 mL batches, measured in triplicate. (c) Biogas composition in 5000 mL batches during degassing.

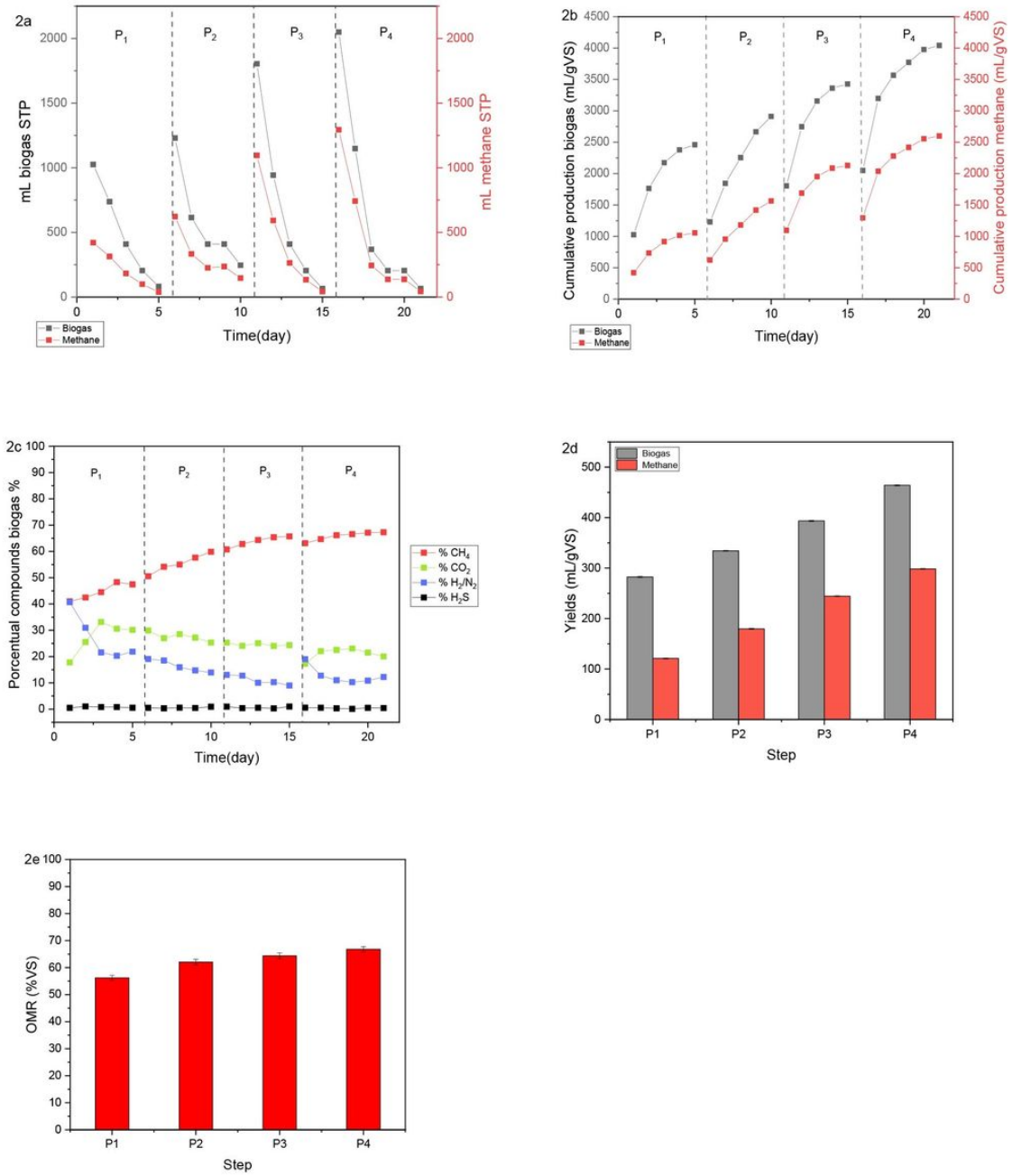

Figure 2

(a) Average daily biogas and methane production during each acclimation pulse; (b) average cumulative biogas and methane production during each acclimation pulse; (c) average biogas composition (%) during each acclimation pulse; (d) organic matter removal (OMR) VS%; (e) biogas/methane yieldsduring each acclimation pulse. All the values were measured in 5000 mL batches.

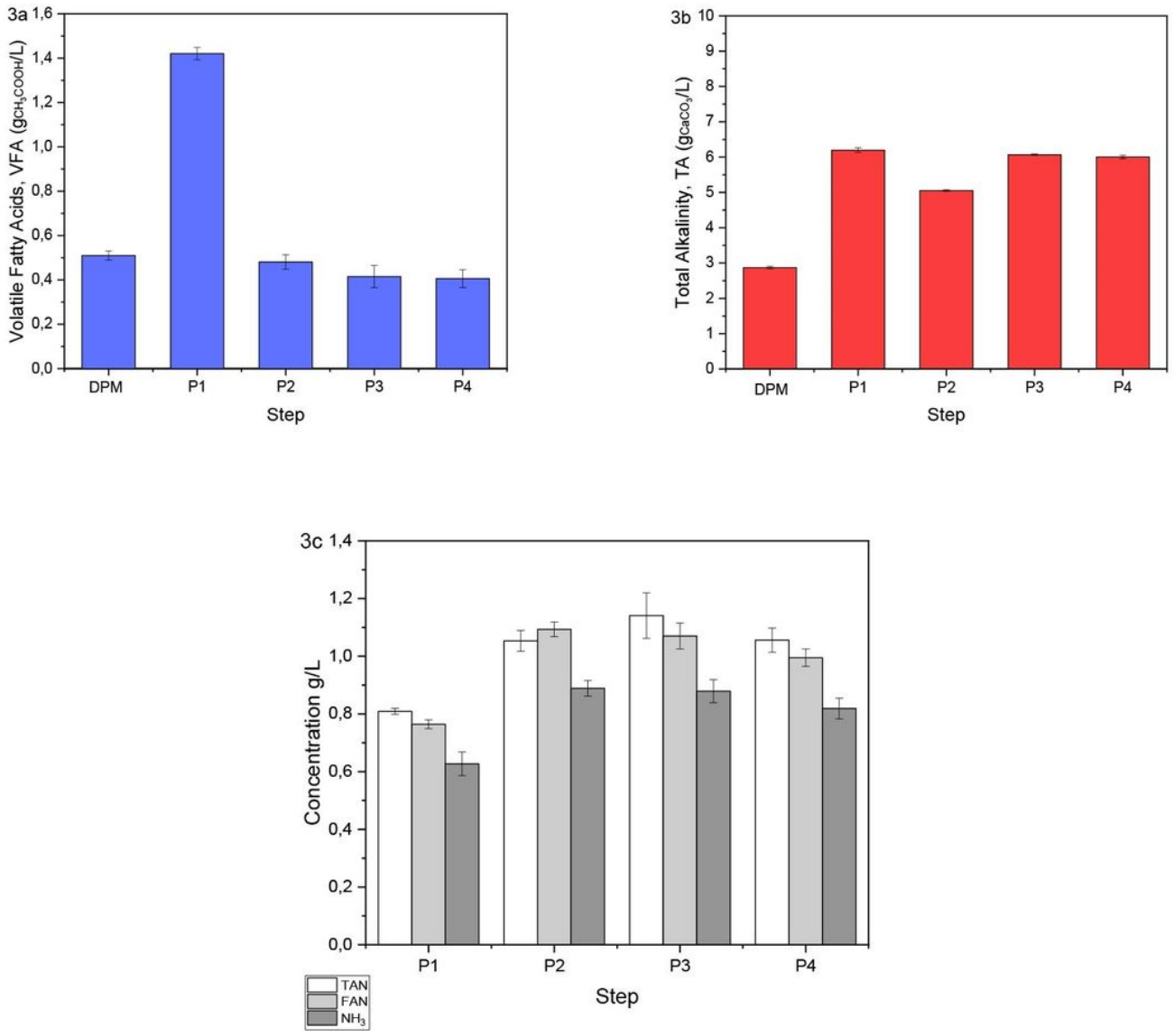

Figure 3

(a) Volatile fatty acids (VFA) during each acclimation pulse; (b) total alkalinity (TA) during each acclimation pulse; (c) total ammoniacal nitrogen (TAN), free ammoniacal nitrogen (FAN), and ammonia (NH_3) during each acclimation pulse. All the values were measured in 5000 mL batches.

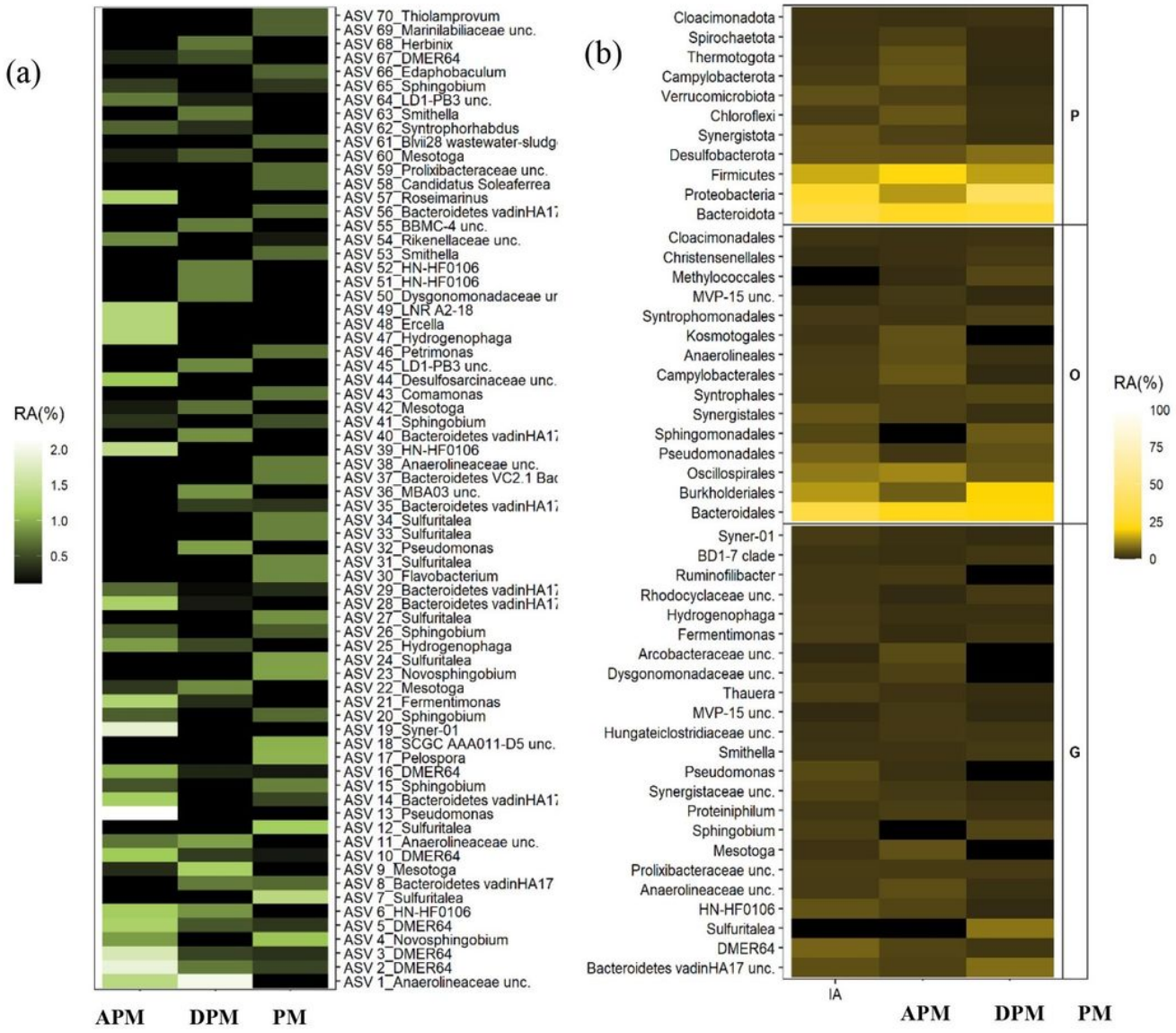

Figure 4

(a) Abundance of the most significant ASVs, and (b) relative abundance of phylotypes in PM, DPM, and APM.

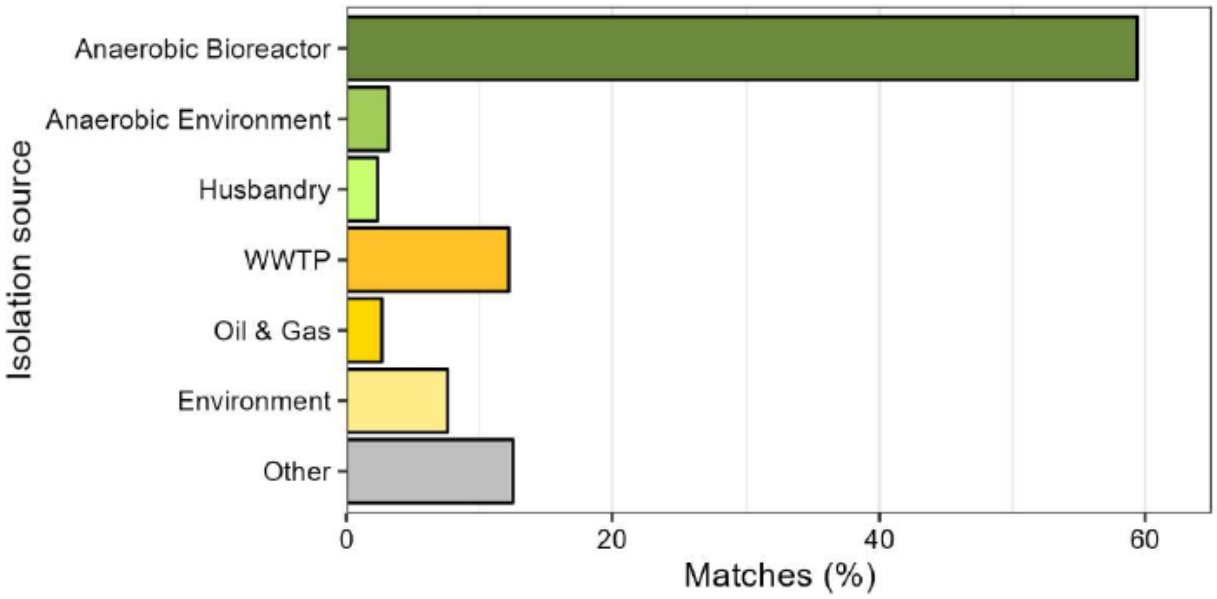

Figure 5

Provenance of NCBI sequences identical to the top 70 ASVs.