Biogas quality was increased by pulsed feeding of acclimated pig slurry with corn screenings and microbiological analysis at each stage

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Abstract

Pig production in Argentina increased by > 230 % between 2008 and 2019. Pig farms are concentrated in the country's agricultural areas, where grain availability, slaughter, and consumption centers are also located. Based on this, a proposal involving the use of agricultural by-products to produce biogas would be economically viable. To this end, we report an increase in biogas quality by pulsed feeding of acclimated pig slurry with corn screenings. The degassing process and the pulsed feeding acclimatization of pig manure obtained from stabilization ponds using corn screenings residue at a lab scale were studied. Also, the microorganism's variability and abundance at each stage were evaluated. The results indicate that degassed pig manure results from a good alternative to use as inoculum. The methane production increased by 30 %, mainly in the lag phase, by acclimatization of pig slurry with corn screenings and the composition of the biogas is more stable in successive feeding. Although it shows that degassed pig manure can be used as inoculum, an ACoD with a high carbon source to increase daily methane yield is

needed. Microbiological analyses showed an increase in hydrolytic and fermentative populations at each acclimatization stage, indicating a positive influence on the bacterial microbiome. In addition, it has been demonstrated that pulsed feeding of acclimated pig slurry with corn screenings can increase biogas quality, and the procedure has a positive influence on the bacterial microbiome.

1. Introduction

Reports from the Food and Agriculture Organization of the United Nations (FAO), world meat production reached 337 million tons in 2019, 44% higher than in 2000. In particular, pig meat production reached 110 million tons, representing 33% of total production (Index, F. F. P. 2021). In recent years, pig production in Argentina increased considerably, due to the increase in the imported pork price and the demand for domestic consumption. Reports from the Ministry of Agriculture and Livestock indicate that such growth exceeded 230 % between 2008 and 2019 (Informe MAGyP, 2019a). Pig farms are concentrated in the councountry'sicultural area, where the availability of grains, slaughter, and consumption centers coincide (FAO, 2019). The pig production increase leds a to growth in effluents, and their management is a priority from an environmental and energy standpoint. This problem is being taken into account through policies and regulations concerning the implementation of renewable energies, reduction of nutrient losses into the environment, reduction of greenhouse gas (GHG) emissions, and protection of surface water. The treatment of these effluents, in most of establishments, is carried out by means of stabilization ponds (large extensions). This methodology presents several variants, such as theid separation, the extraction, or the use of biodigester prior to discharge into lagoons ((Informe MAGyP, 2019b; Tlustos *et al*., 2018).

Stabilized pig manure (PM) can be used for different purposes, including as a soil amendment. They can partially or totally replace mineral fertilizers, increasing soil

fertility by supplying nutrients such as nitrogen and phosphorus, and improving soil's physical properties (Damian *et al*., 2018). The major problem associated with its use is nitrogen loss as ammonia (NH_3), which increases GHG emissions (Park *et al.,* 2018). Nitrogen losses can be affected by some management practices and are related to the nitrogen soil dynamics and mineralization-immobilization processes; thus, the impact of PM on soil quality will depend on the application methods. In Argentina, owing to the predominance of no-tillage systemsthe the slurry is spread by surface diffusion, and the losses due to NH_3 volatilization can exceed 50 %. Another application method is its incorporation with prior acidification with sulfuric acid to prevent N (Pegoraro *et al.,* 2020; Fangueiro *et al.,* 2015).

Another alternative to add value to PM is to employ it as a precursor inoculum for Anaerobic Digestion (AD) and anaerobic co-digestion (ACoD) to generate biogas and digestate. Thus, the recovered water can be reused in the process (liquid phase), and the solid fraction can be subsequently pelletized. The slurry used as inoculum and properly combined in mono-and ACoD favors biogas, energy, production, and nutrient recycling, and reduces CH_4 and N_2O emissions more than conventional manure management (Holm-Nielsen et al., 2005; Kavitha et al., 2017). However, because of the high concentration of water in PM, it is rarely economically viable to operate biogas plants with animal manure alone (Treichel *and* Fongaro, 2019).

Considering the potential of biogas pig farms in Argentina (204.883.456 m^3/year, equivalent to 112.686 t/year) ((Informe MAGyP, 2019a; FAO, 2019) and corn sieving waste (CSW) residues in the region (Galván *et al.*, 2020), a huge opportunity for agroindustrial waste revalorization is emerging. This situation can be used to produce active inocula to improve the production conditions and efficiency of AD plants. Coincidentally, several authors suggest that the AD biorefinery concept is not fully

mature, and more extensive studies should be conducted on new product applications and system integration [Pivato *et al.,* 2016; Pérez-Camacho M. *and* Curry, 2018; Antoniou *et al.*, 2019; Varma *et al.,* 2021).

Another important point to revalorize this waste involves the acclimatization or adaptation process study of inocula. Several studies have confirmed that pre-adaptation through progressive disturbance results in a specialized digester microbial community with improved performance and tolerance to high organic loading rates (De Vrieze *et al.,* 2013). It was demonstrated that by changing the feeding pattern, a stirred tank reactor (STR) with pulse feeding was more tolerant to higher organic loading and total ammonia nitrogen (TAN) levels. This suggests that regular application of an organic material-limited pulse could promote greater functional stability in AD; thus, pulse feeding would be a good strategy for sludge adaptation and bioaugmentation with high organic load substrates (Tian *et al.,* 2017; Lua *et al.,* 2019; Wang *et al*. 2020).

Based on the last explanation, the objective of this work was to evaluate the PM obtained from stabilization ponds located in model swine farms and the degassing process at the lab scale. In addition, PM acclimatization by pulsed feeding using corn screening residue was analyzed. Moreover, the variability and abundance of microorganisms at each stage were analyzed.

This study contributes to the modification of pre-existing methodologies to obtain and acclimatize inoculums for AD, with the incorporation of agroindustrial wastes in the inoculums.

2. Materials and methods

2.1 Sampling and Characterization of Manure

The establishments with slurry treatment lagoon locations and their availability for evaluation were spatially analyzed (QGIS Geographic Information System software) to

carry out PM sampling and collection. Sampling and preparation were performed according to Verein Deutscher Ingenieure (VDI) 4630, section 5 (VDI 4630. 2016). The model was selected considering the animal quantity and its representativeness in terms of effluent management by producers in the region (stabilization ponds). The slurry was obtained from medium-depth stabilization ponds at a centralized swine facility located in Bell Ville, Córdoba, Argentina (Lat: S -32°40'12″ " Long: W 62°51'11""). The slurry was collected after 120 days of stabilization in open-air lagoons during spring, with an average temperature of 24°C.

Total solids (TS), volatile solids (VS), and total alkalinity (TA) were determined according to American Public Health and Association (APHA) standard methods 2540 B, 2540 E, and 2320 B, respectively [23]. Volatile fatty acids (VFA) were measured using the Nordmann titration method. In addition, the pH was measured using a HANNA HI 8424 electronic pH meter. Chemical oxygen demand (COD), total ammonia nitrogen (TAN), and free ammonia nitrogen (FAN) was measured using a HANNA HI 83099 spectrophotometer (adaptation of USEPA method 410.4 for COD and Nessler method for TAN and FAN). The biological oxygen demand (BOD_5) was analyzed using a VELP BOD EVO 6 sensor system. Proteins were determined by multiplying the total Kjeldahl nitrogen (TKN) (APHA 4500 B) with a conversion factor of 6.25 (Angelidaki *et al.*, 2009). Organic carbon was determined by considering a ratio of organic matter content to organic carbon of 1.7241 (Cuetos *et al.,* 2008). The following methods were used for microbiological testing: fecal coliforms (FDA: BAM.ch.4:2002), *Escherichia coli* (ISO 7251:2005), and *Salmonella spp.* (ISO 6579-1:2017). The metal profile was determined using the SM 3125:2017 methodology.

2.2 Degassing and SMA tests

The PM was preserved, degassed, and characterized according to the methodology proposed by Angelidaki *et al.* (2009) and Holliger *et al.* (2016). To analyze the bioreactor compositions, the batch test experimental configuration during degassing was carried out in triplicate in 5 L bioreactors with volumetric displacement gasometers ensuring biogas larger volumes and higher reproducibility than the laboratory-scale tests. Each bioreactor was equipped with a temperature control device, speed control rotary mixer, and temperature and gas volume sensors. For the experiments, the bioreactors were set at 37 ±1 °C and 100 rpm, and the mixer worked continuously. The biogas and methane volumes were measured daily. The CH_4, CO_2, H_2, and N_2O contents in the biogas were analyzed using a gas chromatograph (Fuli Instrument) equipped with a thermal conductivity detector (TCD) and a GDX-502 column (4m x 3 mm). The biogas yield, degradability, VFA, TA, TAN, and FAN parameters were determined at the end of manure degassing.

To evaluate the kinetics of methane production, a first-order kinetic model was used to compare the performance of the stationary process under practical conditions. Cumulative methane production can be described by Equation 1, representing the modified Gompertz model, which has been used in numerous studies.

$$B(t) = B_m * e^{-e^{-\frac{R_m * e}{B_m} * (\lambda - t) + 1}} \qquad \text{Equation 1}$$

Where B(t) is the cumulative biogas production at a given time t, B_m is the maximum biogas production potential of the substrate, t is the time, R_m is the maximum specific biogas production rate, e is exp (1) = 2.7183, and λ is the lag phase. The cumulative methane production experimental data were fitted to the Gompertz equation using Origin PRO® 2018.

Additionally, degassed pig manure (DPM)-specific methanogenic activity (SMA) was determined after starvation, with an inoculum-to-substrate ratio of 5, using microcrystalline cellulose, according to Astals *et al.* (2015).

2.3 Acclimatization essays

The acclimatization assay was performed as described by Tian *et al.* (2017). The pulsed feed rate was determined by considering the corn sieving (CSW) substrate characteristics as reported by Galván *et al.* (2020). The acclimatization consisted of CSW pulses adjusting the C/N ratio to 15 using urea (24.15:1; CSW: urea) and keeping the TS system values lower than 10 % of the final TS and an inoculum-to-substrate ratio of 2. Daily biogas production and its specific composition were measured, repeated in four stages in each 5 L biodigester, to evaluate the adaptation of the system based on its response to biogas production and quality, in addition to the degradability and the parameters VFA, TA, TAN, and FAN.

2.3 Microbiological analysis

Microbiological analysis samples were collected from the 5 L biodigesters at the end of each stage and stored at -20 °C prior to DNA extraction. Analyses were performed on the starting PM, DPM, and after PM acclimatization (APM). DNA was extracted from each sample using the FastSpin for Soil Kit (MP Biomedicals, USA) according to the manufacturer's protocol. Sequencing was performed at the Genomics Unit of the Biotechnology Institute of INTA (Instituto Nacional de Tecnología Agropecuaria). The 16S rRNA was sequenced in the hypervariable region V3-V4 with the universal primers 341F and 806R. The sequencing method used was 2 × 250 PE reads on Illumina MiSeq.

Data were analyzed using the DADA2 package in R to form ASVs (*Amplicon Sequence Variants*) (Engelbrektson *et al*., 2010; Bolger *et al.,* 2014).

3. Results and discussions

3.1 Inoculum evaluation. Characterization and pilot scale tests

The physicochemical data and bacterial pathogens identified in the slurry from stabilization ponds before (PM) and after the degassing process (DPM) in 5 L Bach starvation are shown in Table 1.

Table 1: Physicochemical data and bacterial pathogens of slurry from stabilization ponds before (PM) and after degassing process from 5 L Bach. The values shown are the average ± 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
Ammoniac (g/l)	2.07±0.06	1.19±0.020
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
Cadmium (mg/kg DM)	ND	ND
Cadmium (mg/kg DM)	245±3	224±2
Cinc (mg/kg DM)	5.50±0.10	5.20±0.04
Copper (mg/kg DM)	1.20±0.10	0.89±0.12
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
Plumb (mg/kg DM)	0.04±0.001	0.02±0.001
Bacterial pathogens		
Escherichia coli (UFC/100ml)	ND	ND
Coliformes fecales (NMP/100ml)	0.84	ND
Salmonella spp. (UFC/100ml)	ND	ND
Yield parameters		
Organic matter removal OMR (% VS)	63.23±1.07	-
Biogas yield (Nml/g $_{VS}$)	298.77±1.04	-
Methane yield (Nml/g $_{VS}$)	138.02±1.03	-

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

Analyzing the data obtained from the DPM experiments, it is possible to observe that the OMR (%VS) value presents a reduction of 63.23 % versus PM, and the C/N ratio remained in the range of 9-10, similar to that reported by other authors (7.4-12.96) (Ren et al., 2014; Zhou *et al.*, 2016; Shen., 2014), and Zeng *et al.* (2015) showed a C/N ratio of 10.47.

The increase in the C/N ratio can mainly be attributed to total nitrogen reduction and other factors. *Shen et al.* (2014), proposed that a relatively low global C/N ratio makes continuous biogas production difficult, consequently, the use of PM in monodigestion will not be efficient.

After degassing, the PM presented adequate VFA, TA, pH, and TAN values to produce biomethane in accordance with reference values published by *Holliger et al.* (2016), and the VS value exceeded 50 % of TS (VDI 4630. 2016). Taking into account that the VFA/TA ratio indicates the organic load, and the DPM VFA/TA ratio reached a value of 0.17, is possible to conclude that the DPM inoculum reached a starvation-high degree.

Radis Steinmetz determined that VFA/TA values between 0.3 and 0.4 indicate optimal biogas production, values lower than 0.3 suggest low substrate input and a VFA/TA ratio higher than 0.4 indicates overloading conditions (Radis Steinmetz et al., 2016). Based on this, the DPM inoculum presents optimal conditions for digestion, with adequate amounts of macro-and micronutrients and the necessary metals (from supplements added to the animal feed) to optimize AD metabolic reactions.

Cai *et al.* (2017) reported biomethane yield improvements by incorporating micronutrients in the animal feed, among them metals such as (Co, Cu, Mg, I, Se, Zn, etc.). In addition, the inoculum contained N, P, K, Ca, Mg, Na, and trace metals that can be used as bacterial micronutrients.

214 Moreover, the swine effluent presents high content of organic matter; the DBO value is
215 30 more polluting than the sewage effluent. In addition, the TKN content of the slurry
216 was reduced by 55 % after (stabilization or degassing), and the TKN highest proportion
217 was found as TAN in PM and as FAN in DPM. The high TKN value is mainly due to
218 protein degradation; a percentage is used for animal tissue formation, and the rest is
219 removed as by-products containing macronutrients N, P, and K, generating high total
220 ammonia nitrogen concentrations.

221 The stabilized inoculum (DPM) has saline-sodium characteristics; therefore, direct
222 application as a soil amendment should be controlled. In addition, no pathogenic bacteria
223 were detected in DPM (Table 1). Bacteriological counts show that the starvation process
224 under anaerobic and mesophilic conditions reduces or eliminates pathogens in the initial
225 slurry. This result indicates that a correct DA process would reduce or eliminate
226 pathogens from the initial pig manure.

227 Figure 1 shows the daily (1a) and cumulative (1b) biogas production averages of triplicate
228 batches and their respective positive controls. The assay duration in 5 L Bach was 30 ± 2
229 days until less than 1 % of the total volume was produced.

230 The PM methane yield obtained (138.02 NmL/g VS) based on the chemical composition
231 was similar to the theoretical methane yield (157.84 NmL/g VS). The biogas composition
232 corresponds to 46.21 % CH_4, which is 9 % lower than that reported by Regueiro *et al*.
233 (2012). The maximum production peak was observed between days 10-13, also methane
234 yield values were higher than those reported by Bonmatí (96 NmL/g VS) (Bonmatí *et*
235 *al.,*2003). Moreover, Flotats *et al.* (2009) reported potential production values similar to
236 those obtained in their assay (181 NmL/g VS).

The daily biogas production reached is relatively low 298.77 NmL/g VS, making the biogas continuous and homogeneous production difficult, and its use as a mono substrate economically unviable.

Nguyen et al. proposed that an acclimatized inoculum is essential for biogas improvement using (CoDA) anaerobic co-digestion (Nguyen *et al.,* 2021; Wang *et al*., 2020). Moreover, the organic matter removal percentage was 63.23 % higher than that of the VS. Lendormi *et al.* (2022) indicated that pig manure stored briefly (2 months) proved to be optimal as inoculums, and in Table 1 is possible to observe that after the starvation process the possible DA inhibitory compounds values decrease significantly, improving the starting inoculum quality.

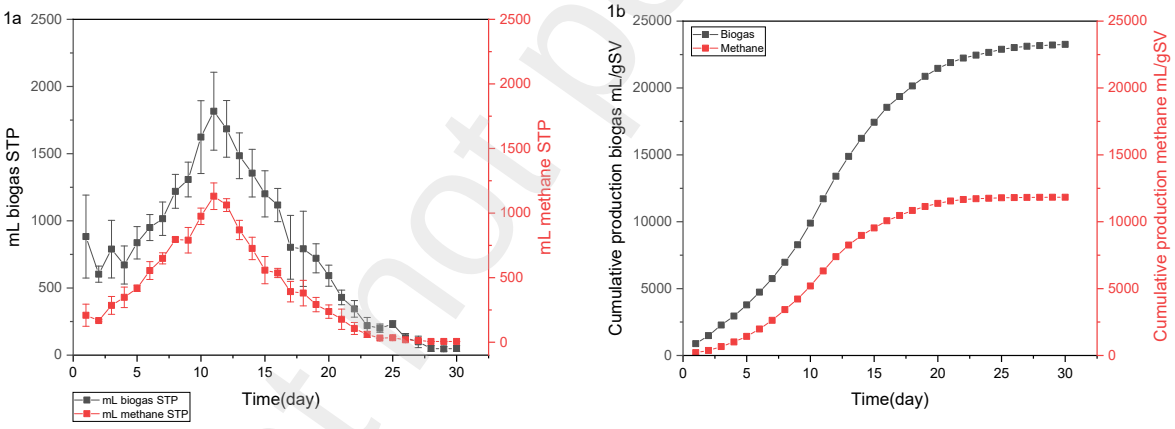


Figure 1: (a) Daily mean biogas and methane production of 5L batches in triplicate; (b) Mean cumulative biogas and methane production of 5L batches in triplicate.

During the starvation process, the obtained biogas composition presents a maximum peak on day 8, corresponding to 64.08 % CH_4, 31.87 % CO_2, 3.86 % H_2/N_2, and 0.19 % H_2S (Figure 2). Also, is possible to observe that the highest production days (8-15) present the

highest CH_4 percentage, and the other analyzed products are stable in a range of 20-30 % for CO_2 and 10-15 % for N_2/H_2.

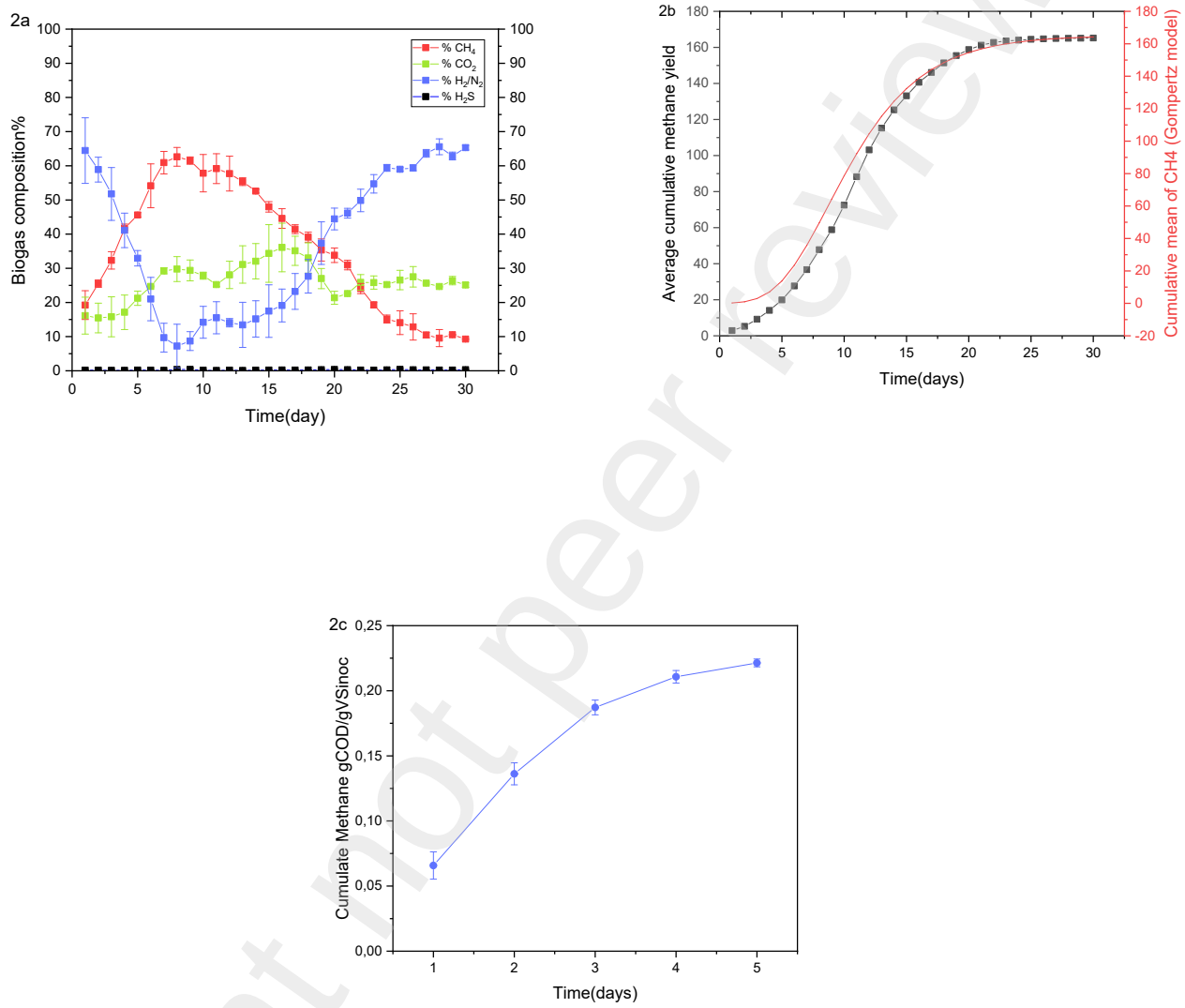


Figure 2: (a) Mean composition of biogas from 5L batches (triplicate) versus time, (b) Cumulative mean methane yield and fit to the Gompertz model, (c) DPM methanogenic activity after the starvation process.

It is known that the biogas calorific value increases not only with the CH_4 content but also with the increment in the CO_2 and CH_4 percentages, thereby increasing the Wobbe index.

In addition, CO_2 is an important component of biogas, and its increase is due to VFA accumulation during AD, shifting the bicarbonate balance towards CO_2 to maintain the pH, as long as the alkalinity of the system supports it. In addition, the CO_2 in biogas reduces the heat output; however, a high CO_2 content results in an acidic environment in engines during the transformation of biogas into electricity (Solera del Rio, 2014). Furthermore, during degassing, not only a high concentration of CO_2 but also high percentages of H_2/N_2 were found. This situation increases the partial pressure above the appropriate values for acetogenesis, making it thermodynamically impossible. Ward *et al.* (2008) state that higher H_2 concentration may indicate digester overloading. A high NH_3 concentration also causes problems in combustion engines. The inoculums at the end of the starvation process presented adequate characteristics in terms of nutrients, pH, VFA, and TA necessary to withstand pH variations during the hydrolysis stages. (Cuetos *et al.,* 2008; Holliguer *et al.,* 2016).

Figure 2b shows the CH_4 cumulative mean, fitting using the Gompertz model with values of 14.68 mL/g VS d for R_m (maximum specific CH_4 production rate) and 4.62 days for λ (phase lag time), the adjusted R^2 value was 0.9974. Thus, at this scale and composition analysis, a low CH_4 production rate is achieved; therefore, to obtain biogas rich in CH_4, mono digestion is not recommended. The DPM samples tested presented an SMA of 0.22 CH_4 gCOD/gVS_{inoc} despite their degree of dilution and relatively low volatile solids (VS) content (Figure 2 c). The SMA value obtained is higher than those obtained by Astals *et al.* [27] for swine effluent lagoon inoculums and higher than the SMA value (0.1) reported by Angelidaki *et al* (2009) and Holliger *et al* (2016). Based on the control of inoculum activity yield reported by the VDI 4630/16 standards, the triplicate test yielded 92 % yield. This value was higher than those obtained by Astals *et al*. (2015) for inoculums from swine effluent lagoons and higher than the 0,1 SMA reported by Angelidaki *et al*.

(2009) and Holliger *et al.* (2016). Considering the yield reported by the VDI 4630/16 standards for the control of inoculum activity, the triplicate tests yielded 92 % yield.

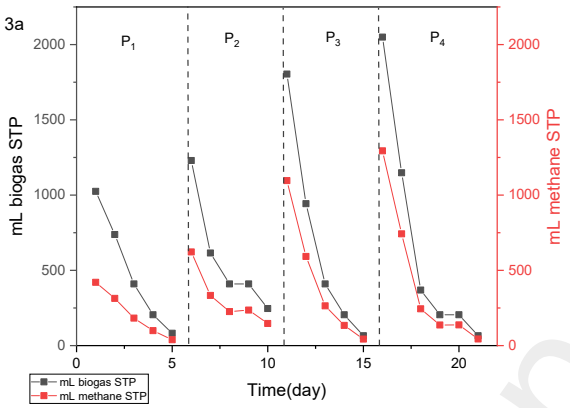
3.2 Acclimatization of inoculum

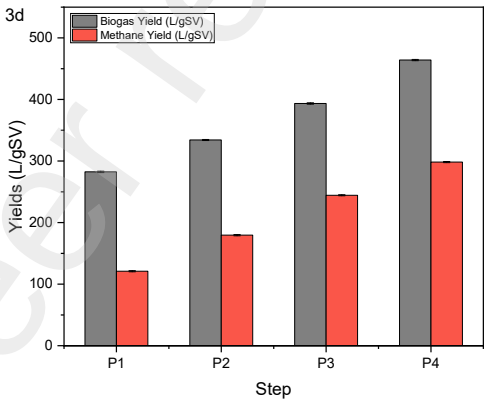
The average daily and cumulative biogas/CH_4 production of each acclimatization pulse performed in 5 L batches are shown in Fig. 3a and 3b, respectively. The biogas volume increases with the succession of pulses; for P4, the volume obtained duplicates concerning P1, and between P1 and P4, the methane volume triples. A lag phase reduction was observed in CH_4 production (Figure 3 b) indicating that the system withstood the stress caused by the organic load in each pulse.

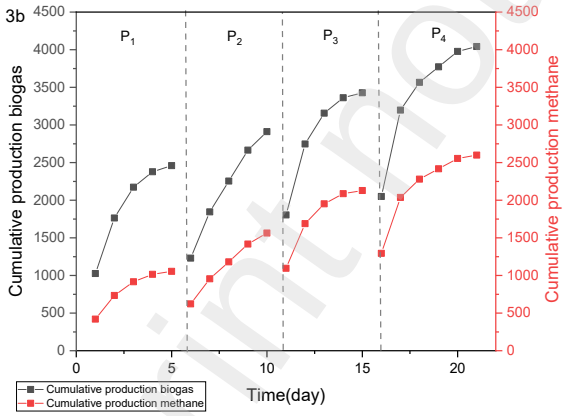
Several studies have confirmed that pre-adaptation through progressive disturbance results in a specialized digester microbial community, improving performance and tolerance to high organic loading rates (De Vrieze *et al.,* 2013). By changing the feeding pattern, the pulse-feeding batch reactor became more tolerant to higher organic loads and total ammonia nitrogen (TAN) levels. This result suggests that regular application of a limited pulse of organic material could promote greater functional stability during anaerobic digestion. Thus, pulse feeding is a good strategy for sludge adaptation and bioaugmentation with high-organic-loading substrates (Tian *et al.*, 2017; Lua *et al*., 2019; Wang *et al.,*2020).

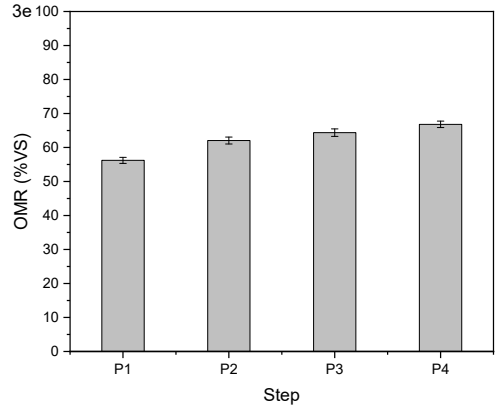
The percentage composition of biogas from each batch acclimatization pulse is shown in Fig. 3c. The percentage of CH_4 from pulse P3 onwards remains in the range of 60-64 %, and the CO_2 values first decrease and then stabilize between 20-25 %; this reduction can be correlated with the increase in the CH_4 percentages. The H_2/N_2 values in the first pulse exhibit a high jump, and with successive pulses, the values decrease to 10%. Subsequently, the H_2/N_2 values remained stable because no jumps were generated with successive pulse applications. In this manner, additional system stress is avoided, and

possible biomass adaptation to NH_3 can be achieved. Moreover, the results demonstrated that syntrophic microorganisms can proliferate in acetate (SAO) oxidation, transforming acetic acid into H_2 and CO_2 (Rinzema *et al.*, 1994), while CH_4 production remained stable. These increases can also be observed in the biogas/methane yields 1.6 and 2.8 times higher than the initial values (Figure 3d). Figure 3e shows the OMR based on VS; the values were maintained between 55-64 % during different pulses.









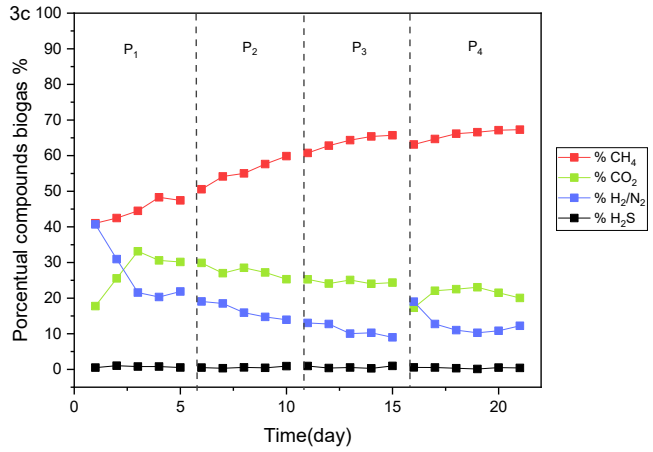


Figure 3: (a) Mean daily biogas and methane production of each acclimation pulse in 5L batches; (b) Mean cumulative biogas and methane production of each acclimation pulse in 5L batches; (c) Mean percent biogas composition of each acclimation pulse in 5L batches.

VFA values increased at the end of the first pulse and decreased with successive pulses (Fig. 4a). The TA behavior increased to 6 $gCaCO_3/l$ with pulses (Fig. 4b). This reduction in VFA concentration with each pulse was related to an increase in the TA of the system (Fig. 4a and 4b). Thus, the VFA/TA ratio remained balanced, allowing the system to withstand pH variations.

Although this ratio is widely used as an indicator of biodigester performance, it is important to note the progressive increase of VFA during the system adaptation to substrate consumption, and the decreasing trend with each feeding is maintained. Figure 4c shows that the values of FAN, TAN, and NH_3, are within the stability limits for reported inoculums in the range (0.5-1.2 g/l) (Holliger *et al.* 2016).

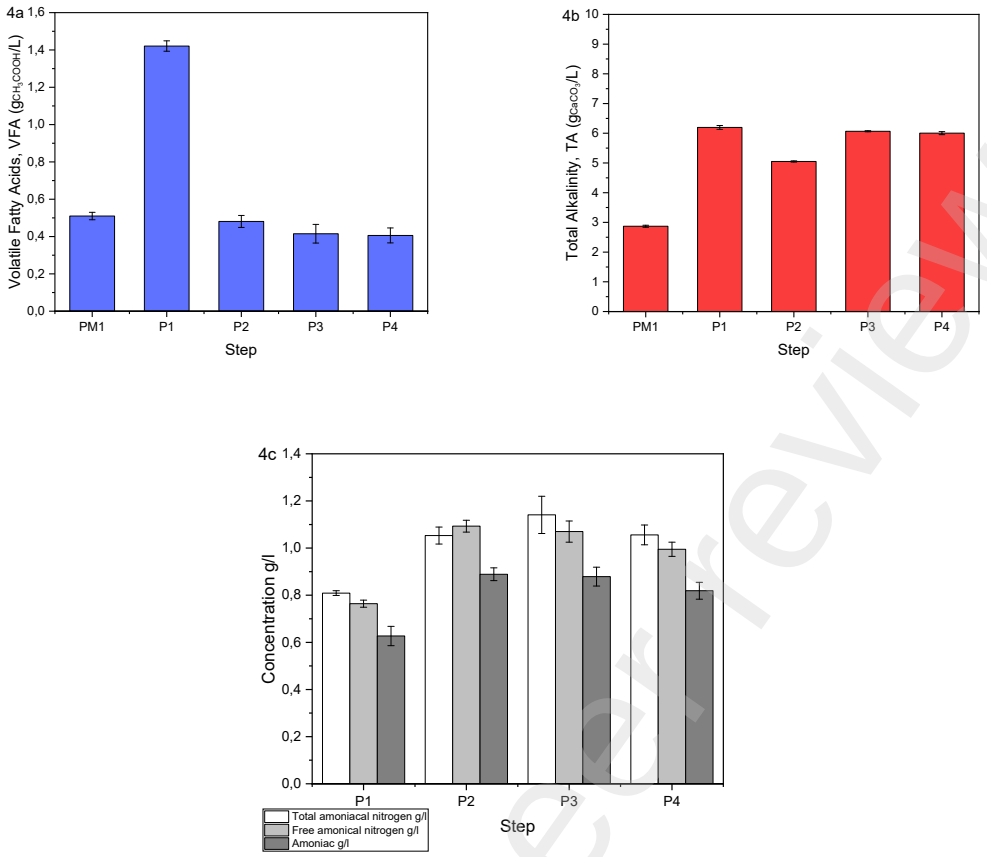


Figure 4: (a) Volatile fatty acids in each acclimation pulse in 5L batches; (b) Alkalinity in each acclimation pulse in 5L batches; (c) Total ammonia nitrogen (TAN), free ammonia nitrogen (FAN) and ammonia (NH_3) in each acclimation pulse in 5L batches.

The assay was carried out for up to four pulses because the CH_4 percentages did not present a further increase after the second pulse, indicating that the system reaches the maximum CH_4 production. In addition, it was observed that with successive feeds, the lag phase was shortened, and the range of composition was less variable, assigning a higher quality to the resulting biogas.

The results obtained were close to those published by Kim *et al.*(2020), who determined the lag phase reduction conditions of AD using high organic load substrates. They concluded that to reduce the lag phase, the VFA/TA ratio should be kept below 0.4 and an initial VFA/VS ratio below 10 %, thus improving the AD yield and reducing the digestion time.

All these parameters are important and complement each other; however, the biogas composition is critical in validating a larger-scale feeding regime to ensure biogas quality in its subsequent use.

Yield values could be increased by working at a higher solid concentration (<10 %), without guaranteeing that the stability indicator parameters would be altered. However, it is still necessary to work on the limits of each particular ACoD system and determine the different variables that interact.

The obtained results demonstrate that biodigesters were exposed to a similar perturbation; the pre-adapted digester achieved better performance, while the non-adapted digester was inhibited. An identical conclusion was reported recently by Wang *et al.*(2020). The authors indicated that this perturbation could be used strategically to influence methanogenic microbiomes and improve the co-digestion of critical waste.

3.3 Microbial characterization

Microbiological characterization was performed using a total sequence number of 227590, with an equal number of sequences in the PM and DPM samples (~87800) and a lower number (~60 %) in the APM. The median number of sequenced fragments (251 bp) was consistent with the sequencing method used. Based on the results, it was possible to determine 9151 APM sequences, 14993 DPM sequences, and 17219 PM sequences. DADA2 software was employed to obtain Amplicon Sequence Variants (ASVs), followed by taxonomical classification using the Silva database (v 138.1) at 100 % identity.

Table 2 shows the net number of species, strains, taxa, Shannon's diversity index, and inverse Simpson's index for PM, DPM, and APM. Based on the results, it can be observed that the APM sample shows a lower number of taxa and diversity. These results are in

agreement with the lower sequence number, suggesting a lower microorganism diversity. This could be attributed to a selection process consequence owing to the change in conditions.

Table 2: ASV number, Shannon's diversity index (richness), and inverse Simpson's index (equity) of PM, DPM, and APM.

Sample	Numbers ASVs	Shannon	1/Simpson
PI	418	5.68	232
II	417	5.68	228
IA	256	5.12	132

Figure 5a shows the relative abundance of phylotypes (%AR) separated by taxonomic level (domain (K), phylum (P), order (O), and genus (G)) in the initial manure sample (PM), after the starvation process (DPM), and after acclimatization (APM). As shown in Figure 5a, the bacterial domain was dominant in all samples. However, this result does not mean that there are no *archaea*, but rather that, most likely, the sequencing primers used in this study came from bacteria, presenting a low affinity with the *Archaea* domain. In addition, the predominant phyla were *Bacteroidetes, Proteobacteria, Firmicutes*, and *Desulfobacterota*, particularly in the DPM and PM samples. Related to the order level, Bacteroidales and Burkholderiales predominate, the latter mainly in the DPM. Analyzing the genus level, it is possible to confirm that *Sulfuritalea* is only present in PM (9% AR) and not in the other stages.

Figure 5b shows the relative abundances of the 70 most significant ASVs in the PM, DPM, and APM samples. There was a low abundance of all taxa and an absence of dominant taxa; moreover, the highest abundance sequences represented only 2 %.

Furthermore, in Figure 5b, it is observed that there are not many shared sequences among the three samples, but, on the contrary, each stage of the bioreactor has its specific composition. Based on this result, the sequences of the 70 most relevant ASVs were determined by matching with more than 99 % identity to the full-length ASV in the National Center for Biotechnology Information (NCBI) database using the BLAST+ tool. In this way, 1269 matched sequences were obtained for 60 ASVs, and 10 ASVs did not present identical sequences in NCBI and could correspond to microorganisms not yet reported; more than most of the sequences corresponded to noncultured microorganisms.

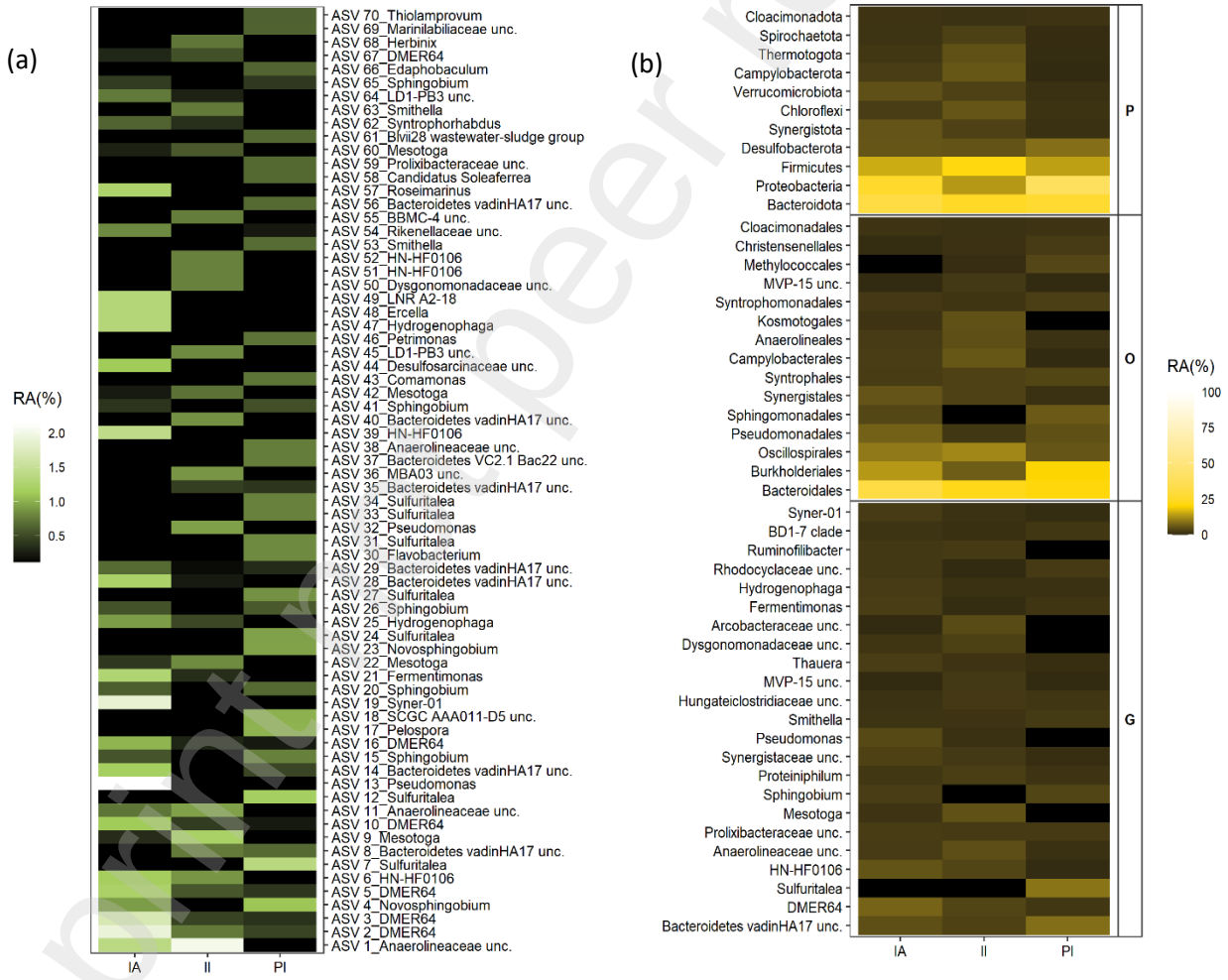


Figure 5: the (a) abundance of the most significant SVs and (b) relative abundance of phylotypes in initial manure samples PM, after the starvation process DPM and acclimatized APM.

The insolation source in which these matched sequences were found and/or reported is analyzed (Figure 6).

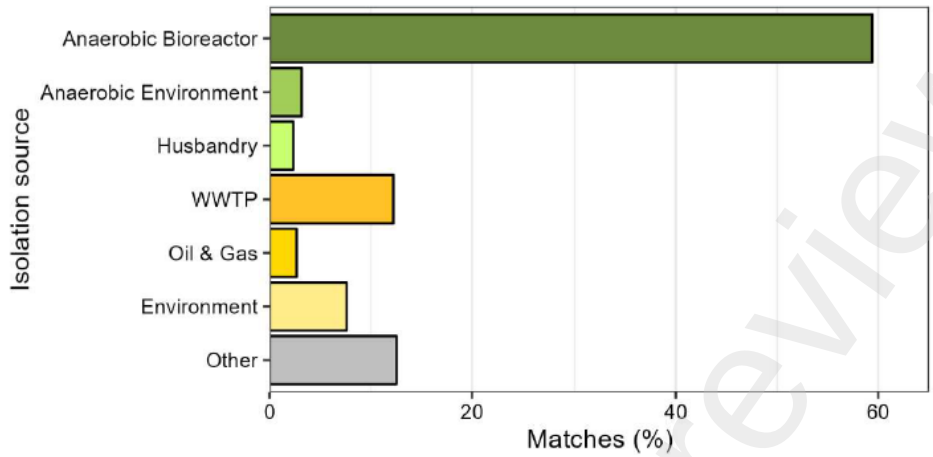


Figure 6: Provenance of NCBI sequences identical to the top 70 ASVs.

In Figure 6 is possible to observe that the NCBI sequences found in the analyzed samples have been reported mostly related to anaerobic bioreactors. In addition, a high percentage of the corresponding matched sequences are typical in wastewater treatment processes (WWTP), while the remaining sequences have been found in other anaerobic environments (not involving reactors). Sequences associated with livestock farming (manure lagoons, manure, slurry, etc.) have also been reported (Pasalari *et al.,* 2021). It is important to note that all these environments are related to the studied process in this work and include most of the sequences found (~ 80 %); however, other matched sequences came from environments related to petrochemical processes (Oil & Gas), natural environment lakes, rivers, and rhizospheric environments (agriculture), mining activity, urban landfills, and other laboratory processes (Others).

Based on this information, it was possible to describe the samples corresponding to the three biodigester stages. The most important ASVs detected in PM were ASV 7, ASV 12, ASV 4 and ASV 17. The ASV 7, and ASV 12 can be classified as *Sulfuritalea sp.*; also, none ASVs presented matches in the NCBI, which is consistent with the fact that, so far,

406 only one species of this genus has been isolated (*Sulfuritalea hydrogenivorans*) (Kojima.
407 *et al.,* 2011). This microorganism belongs to a sulfur-oxidizing autolithotrophic
408 neutrophil genus and a nitrate reducer and has been reported not only in diverse low-
409 carbon-loading aquatic environments but also in activated sludge and hydrocarbon-
410 contaminated sites (Sperfeld *et al.,* 2019).
411 ASV 4 can be classified as *Novosphingobium sp.* Another important ASV is present in
412 PM. This genus of facultative aerobic organotrofy, capable of reducing nitrate, is related
413 to the degradation of aromatic compounds (Liu *et al.,* 2021). The identical sequences of
414 ASV 4 were isolated from landfills and WWTP. Furthermore, ASV 17 (*Pelospora sp*.)
415 isolated from municipal landfills also presented identity with a sequence obtained from
416 an activated sludge process, and with another sequence found in a livestock waste
417 anaerobic digester. The genus *Pelospora* has only one species (*Pelospora glutaric*) and
418 is a strict anaerobic glutarate fermenter. The most predominant ASVs found in sample II
419 is ASV 1, which n be assigned to the *Anaerolineaceae* family, representing only 2 % of
420 the community, Moreover, in the same sample ASV 9 (*Mesinfrainfera*), ASV 11
421 (*Anaerolineaceae*), ASV 32 (*Pseudomonas*), and an ASV 6 (taxon of the class
422 *Clostridia*). Similar sequences to ASV 1 and ASV 11 (family Anaerolineaceae) have been
423 reported to be related to wastewater treatment systems and anaerobic digesters, including
424 biogas plants; specifically, this family is widely reported in this kind of process (McIlroy
425 *et al.*, 2017). Microorganisms of the *Anaerolineaceae* family are strict anaerobes,
426 mesophilic or thermophilic, and chemoheterotrophs. In addition, some have shown
427 syntrophic associations with methanogenic microorganisms through hydrogen generation
428 (Sun *et al.,* 2016). ASV 9 can be classified as *Mesotoga infera*, a species of the order
429 *Thermotogales* associated with anaerobic digesters with a carbonaceous compound high
430 load (including hydrocarbons, such as toluene, benzene, and xylene) at high temperatures

(65°C-85°C). The genus *Mesotoga* is mesophilic and employs sulfur compounds as electron acceptors, producing sulfur, acetate, and CO_2, and no hydrogen generation is detected (Hania *et al.,* 2013). ASV 32 is classified within the genus *Pseudomonas*, which includes a wide variety of species capable of obtaining energy from complex carbon compounds. Sequences similar to ASV 32 were found in diverse environments (bioreactors, rhizospheric environments, sludge, and wastewater). *Pseudomonads* have been proposed as one of the most important microorganisms in anaerobic digesters as degraders of complex energy (Buettner *et al.,* 2019). Finally, ASV 6, which belongs to the *Clostridia* class, is linked to anaerobic reactors.

The most relevant ASV identified in the APM sample was ASV 13, which was classified as Pseudomonas sp. (2.1 % AR). Moreover, a taxon of the order *Bacteroidales* (ASV 2), ASV 19 (within the order Synergistales), another ASV of the order *Bacteroidales* (ASV 3), and ASV 39 belonging to the class *Clostridia* are present. ASV 2 and 3 were strongly similar to each other and shared matches in the NCBI search, and the reported sequences corresponded to non-cultured microorganisms from anaerobic bioreactors to obtain biogas from agricultural wastes (lignocellulolytic wastes, bovine albumin, and pig farming wastes). Moreover, sequences similar to ASV 19 (synergists) have been reported in anaerobic bioreactors for municipal wastewater treatment. Synergists are microorganisms associated with animal microbiota and are frequently found in anaerobic digesters producing amino acids and protein degradation, and ASV 19 presents a syntrophic relationship with methanogens (Godon *et al.*, 2005; Vartoukian et al., 2007). Finally, ASV 39 (*Clostridia*) only presented five similar sequences in NCBI, four of which came from an anaerobic reactor fed with swine waste (the same reactor as the sequences reported for ASV 2 and 3).

4. Conclusions

The final results achieved using DPM show adequate values of OMR, VFA, TA, pH, and TAN. Also, values of SMA, VFA/TA ratio, micronutrients, and the absence of pathogens, obtained from the DPM probe that degasified PM is a good alternative to using as inoculum. However, to obtain high biogas production a high carbon source due to low daily biogas (methane) yield and C/N ratio of DPM is suggested for an ACoD. Furthermore, the methane concentration is improved by 30 % using DPM, it was found that the methane concentration increased up to the second feeding pulse, after that, no significant changes were observed. In addition, it was observed that successive feeding pulses shortened the lag phase and the biogas composition was less variable, allowing higher quality to the resulting biogas. Most of the genomic sequences collected from the biodigesters (70 %) correspond to previously non-cultivated microorganisms reported in anaerobic bioreactors, 16 % of the matching sequences corresponded to WWTP, and the rest correspond to other sequences found in anaerobic environments. Therefore, the genomic sequences collected were identified in reactors for biogas production from agricultural residues (lignocellulolytic residues, bovine albumin, and residues from pig farming). Also, the results showed that an increase in hydrolytic and fermentative populations at each stage of acclimation produces a positive influence on the bacterial microbiome. Based on the results is possible to conclude that is possible to increase the biogas quality by pulsed feeding of acclimated pig slurry with corn screenings, microbiological analysis of each stage

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