Characterization of microbial community dynamics during the anaerobic co-digestion of thermally pre-treated slaughterhouse wastes with glycerin addition.

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ABSTRACT

Microbial community dynamics during the anaerobic co-digestion of pig manure, pasteurized slaughterhouse waste and glycerin, were studied in a lab-scale CSTR. The feed composition was optimized through progressive co-substrate additions for enhanced methane production and organic matter removal without accumulation of intermediate compounds. Microbial community structure of biomass samples was studied by means of qPCR and DGGE profiling of 16S rRNA genes (*Bacteria* and *Archaea*), and genus-specific qPCR of the methyl coenzyme M reductase gene (mcrA), which encodes for an enzyme universally involved in methanogenesis. The composition of the dominant bacterial populations remained relatively stable, when compared to those in the influent, but the highest changes were observed upon the introduction of glycerin. Biodiversity of archaea was restricted to a few representatives of the genera *Methanosaeta* and *Methanosarcina*, but *Methanospirillum* sp. was detected only when glycerin was introduced in the feeding. Glycerin supplementation coincided with the strongest increase in methane yield (from 0.22 to 0.64 $m^3_{CH4} \cdot m^{-3} \cdot d^{-1}$).

Keywords

Co-digestion, microbial community, slaughterhouse waste, 16S rDNA DGGE profiling, qPCR of mcrA functional genes.

Abbreviations

ABP: Animal by-products

CODt: Total chemical oxygen demand

CSTR: Continuous stirred tank reactor

DGGE: Denaturing gradient gel electrophoresis

HRT: Hydraulic retention time

mcrA: Methyl-coenzyme M reductase

Msar: Methanosarcinaceae

Msae: Methanosaetaceae

LCFA: Long chain fatty acids

OLR: Organic loading rate

PCR: Polymerase chain reaction

PM: Pig manure

PP-ABP: Pasteurized animal by-product

qPCR: Quantitative real time PCR

RG: Residual glycerin

rRNA: Ribosomal ribonucleic acid

TS: Total solids

VFA: Volatile fatty acids

VS: Volatile solids

1. Introduction

Anaerobic digesters are bioreactors designed for the conversion of residual organic matter into useful biogas by diverse and complex syntrophic microbial communities. The anaerobic digestion process involves a series of biochemical reactions that are mediated by microorganisms belonging to three trophic levels: hydrolytic-fermenting and acetogenic hydrogen-producing bacteria, both encompassed within the *Bacteria* domain, and acetoclastic and hydrogenotrophic methanogens that belong to the *Archaea* domain [1]. Knowledge on the metabolic function of the microorganisms involved in each step of the anaerobic digestion pathway, and on how they interact with the physic-chemical

parameters governing the process, is a prerequisite for an optimal and stable running of the anaerobic digester. Such microbial interactions are rather specific on the chemical composition of the feeding and still remained uncertain primarily due to the difficulty of establishing cause-effect relationships between biological and physicochemical datasets [2].

Because methanogens have a relatively low growth rate and live in a very specific set of environmental conditions, the methanogenic activity in an anaerobic digester ultimately relies on offering relatively stable operational conditions for the methanogens. However, changes in community structure may occur without detectable changes in bioreactor performance [3], which can result in severe process disruption in the longer term. Hence, the better understanding of the microbial interactions in anaerobic digesters can provide new diagnostic and monitoring tools for enhanced process monitoring. Disturbances in populations from one trophic level affect the entire community and cause an effect on bioreactor performance by a reduced efficiency or accumulation of intermediates [3]. The anaerobic digestion process is generally monitored by an exhaustive control of the ammonia and/or fatty acids. This is very important during the anaerobic digestion of complex wastes such as animal by-products (ABP), a highly biodegradable organic residue that is mainly composed by proteins and lipids with variable water content [4]. The combined release of ammonia due to protein decomposition and long chain fatty acids (LCFA) because of fat degradation might severely compromise the stability of the whole anaerobic process [5].

Co-digestion is a good strategy to prevent inhibition and optimize methane production and also allows the progressive acclimatization of the methanogenic biomass to specific inhibitors such as ammonia and/or LCFA [6,7], thus facilitating the viability of the anaerobic co-digestion process. The implementation of an adequate co-digestion regime

in industrial plants relies in the accurate selection and administration of the available co-substrates.

Notwithstanding its low biogas production potential due to the poor organic matter content, pig manure is considered a good co-substrate because it has an important buffer capacity and contributes with a wide variety of nutrients that are necessary for the development of anaerobic microorganisms [8]. Despite of the generation of potential inhibitions, good results have been obtained in relation to the co-digestion of ABP and manure, and stable operation has been reach with high biogas and methane yields (0.7-1.0 $m^3 \cdot kg_{VS}^{-1}$ and 0.52-0.55 $m^3_{CH4} \cdot kg_{VS}^{-1}$, respectively) [9,10]. Other organic wastes such as residual glycerin from the biodiesel production process from energy crops have been mixed with nitrogen rich-substrates like manure, in order to balance the C/N ratio [6,11]. Culture independent molecular techniques have increasingly been applied to the analysis of microbial communities in anaerobic digesters and have become a useful tool for the understanding of reactor performance [12]. Some works have already been published on the microbial aspects related to the co-digestion of a significant variety of organic substrates mixed with manure [13,14]. There are two main acetotrophic archaea in anaerobic digesters: those belonging to the genus *Methanosaeta*, which are more efficient acetate metabolism at low concentrations, and those belonging to *Methanosarcina,* which are more efficient at high acetate concentrations, more tolerant to stress conditions, and which can also synthetize methane via the hydrogenotrophic pathway. The ratio between these two groups has previously been proposed as an indicator of process stability [15]. However, the reported results on microbial dynamics are often partial or too broad (e.g. only the bacterial domain is usually covered), and quantitative studies on the dynamics of specific functional microbial groups are rare. The deeper understanding of the microbial interactions inside an anaerobic digester could be of help in avoiding failure,

to predict eventual instability problems, and also to evaluate the reactor efficiency and biogas yield. Besides these practical aspects, biomonitoring of digesters using molecular methods could also lead to the identification of new and functionally relevant species.

The present work was aimed to study the microbial dynamics from methanogenic biomass in an anaerobic digester during a transition feeding from pig manure to full co-digestion regime with pasteurized ABP and glycerin. Culture-independent molecular DGGE profiling of 16S rRNA genes from both bacterial and archaeal microbial domains and quantitative PCR on specific functional target species were set. Special attention has been given to the balance between the genera *Methanosaeta* and *Methanosarcina*.

2. Material and methods

2.1. Organic substrates

The selected ABP comprised solid slaughterhouse residues classified as category 3 and pre-treated following European Community Regulations [16,17], and were described previously in Rodriguez-Abalde et al., [6]. Pig manure was obtained from a centralized pig manure facility located in Lleida (Spain) and glycerin was taken from the glycerol-containing waste discharge of biodiesel factory located in Barcelona (Spain).

2.2. Analytical methods

The pasteurized animal by-product (PP-ABP) was lyophilized before characterization in order to improve their homogeneity, while pig manure (PM) and residual glycerin (RG) were analyzed immediately after collection. Total and volatile solids (TS, VS) of all the samples were measured following standard methods [18]. Total chemical oxygen demand (CODt) was determined following the adapted method for solid samples [19]. Further information about the chemical composition of the samples (NH_4^+, volatile fatty acids (VFA), COD, etc.) can be found in Rodríguez-Abalde et al. [6].

2.3. Continuous experiment set up

A 6 liters continuous stirred tank reactor (CSTR) without recirculation was operated at 36±1ºC for 490 days (70 weeks). This bioreactor was inoculated with two mesophilic anaerobic sludges: 4 liters from the digester of a centralized plant, where the PM was also collected, and 1 liter from the digester of an urban wastewater treatment plant. An acclimation period (called P0) of the inoculum was implemented with fresh pig manure, diluted with tap water, with a hydraulic retention time (HRT) of 20 days. Details on the feeding method, biogas analysis and measurements done during the semi-continuous reactor experiment can be consulted in Rodriguez-Abalde et al., [6].

The selected operational parameters were two HRT (20 and 33 days), with an organic loading rate (OLR) of 0.8 $kg_{COD}\cdot m^{-3}\cdot d^{-1}$ during the acclimation period, and of 2.2-3.2 $kg_{COD}\cdot m^{-3}\cdot d^{-1}$ for the others periods. The performance was divided in 3 stages upon feed composition, and in 5 periods (P1 to P5) based on HRT and OLR values. For each experimental condition, the specific methane yield ($m^3_{CH4}\cdot kg_{VS}^{-1}$ and $m^3_{CH4}\cdot t^{-1}$), specific methane production rate ($m^3_{CH4}\cdot m^{-3}\cdot d^{-1}$) and COD removal efficiency were used as control parameters, as well as the biogas composition, the alkalinity ratio (ratio between intermediate and partial alkalinities), and ammonia and VFA concentrations in the effluent.

2.4. Denaturing gradient gel electrophoresis (DGGE) molecular profiling

Influent (i) and effluent (e) samples for microbial analysis were collected at the end of each period (P1 to P5), including the initial inoculum (P0). Total DNA was extracted from approx. 0.25 g of each sample with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, USA), a protocol based on a bead-beating according to the instructions of the manufacturer. Three primer sets were used to selectively amplify bacterial (F341GC/R907) and archaeal (ArchF0025/ArchR1517; nested

ArchF344/ArchR915GC) 16S rRNA gene fragments. The PCR amplification of hypervariable V3-V5 region from the 16S rRNA gene of both domains and the DGGE profiles and sequencing were performed as previously reported by Palatsi et al. [20].

2.5. Quantitative PCR assay

The different ratios between bacterial and methanogenic communities were realized by quantifying the 16S ribosomal DNA and the alpha subunit of methyl-coenzyme M reductase (*mcrA*) for total bacterial population and methanogenic archaea, respectively. Gene copy numbers both fragments were quantified with the quantitative real time PCR (qPCR). System MX3000P (Stratagene, La Jolla, CA) operated with the protocol described in Sotres et al. [21]. Each sample was analyzed in triplicate by means of three independent DNA extracts.

The ration between representatives of the *Methanosarcina* and *Methanosaeta* genera was analyzed by developing qPCR probe-based assays by using Brilliant II qPCR Master Mix (Stratagene). The genus-specific *mcrA* genes were obtained from the type strains *Methanosarcina barkeri* DSM 800 and *Methanosaeta concilii* DSM 2139. The target genes were cloned onto the PGEM plasmid vector using PGEM-T Easy Vector System II (Promega, Madison, WI, USA). In this assay the analysis were performed by PrimeTime® qPCR Probes (IDT DNA Technologies, Coralville, IA) and the protocol was configured by: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s; annealing and image captured for 1 min at 55ºC and 60ºC (for *Methanosarcina* and *Methanosaeta mcrA* genes, respectively). The specificity of PCR amplification was determined by observations on gel electrophoresis profile.

For the genus *Methanosarcina*, the *PrimeTim*e set was: forward primer Meth-r-F (5'-CAC TTY GGW GGW TCH CAR-3'), reverse primer Me2b-R (5'- TCC TGS AGG TCG WAR CCG AAG AA-3'), and the double-quenched probe msar_Probe (5'-/6-

FAM/TC TCT CWG G/ZEN/C TGG TAY CTC TCC ATG TAC /IBFQ/-3'). For the genus *Methanosaeta*, the *PrimeTime* set was: forward primer msa-F (5'-WCG GMG GAT TYG CCA AGG- 3'), reverse primer Me2b-R (5'- TCC TGS AGG TCG WAR CCG AAG AA-3'), and the double-quenched probe Sae716Taq_Probe (5'-/6-FAM/AG GCC TTC C/ZEN/C CAC TCT GCT TGA GGA T/IBFQ/-3'). Both reactions were performed in 10 µl volume containing 1 µl of DNA template, 500 nM for one couple of primers and 300 nM for the others ones, 300nM and 200nM of the each probe (both for *Methanosarcina* and *Methanosaeta* assay, respectively), 5 µl of the ready reaction mix and 30 nM of ROX reference dye. The qPCR efficiencies of amplification were greater than 90%, while the Pearson Correlation Coefficients (R^2) of the standard curves were between 0.999 and 0.997, and the slopes were between -3,570 and -3,583 for *Methanosarcina* and *Methanosaeta mcrA* genes, respectively.

3. Results and discussion

3.1. Characterization of organic substrates

RG and PP-ABP were characterized by high concentrations of COD, in relation to PM (1517.0 and 1318.0 versus 45 $g_{COD} \cdot kg^{-1}$). On the other hand, PM and PP-ABP presented a relatively high amount of nitrogen, 3.4 and 19.2 $g_{NT} \cdot kg^{-1}$, respectively, being organic nitrogen the predominant form in PP-ABP, while being almost inexistent in RG. This fact is also reflected in the carbon to nitrogen ratio (C/N of 5.7 and 14.1 for PM and PP-ABP), while the C/N ratio was particularly high in RG (C/N=587.5). The content of volatile solids (VS) was very different in the three substrates but was particularly low in PM (Table 1), reason why this substrate was very suited as dilution media in co-digestion mixtures.

Table 1. *Waste characterization of pig manure (PM), pasteurized pig waste (PP-ABP) and residual glycerin (RG), including their anaerobic biodegradability (AB) and methane yields. Nomenclature: nd - not detected, nm – not measured. Note: *Calculated value from elemental analysis.*

Parameter	**PM**	**PP-ABP**	**RG**
TS ($g \cdot kg^{-1}$)	36.7 ±10.2	551.7 ±3.1	926.1 ±0.1
VS ($g \cdot kg^{-1}$)	26.0 ±8.3	542.5 ±2.1	924.4 ±1.2
C/N ($g \cdot g^{-1}$)	5.7 ±2.3	14.1 ±2.3	587.5 ±2.3
CODt ($g \cdot kg^{-1}$)	45.4 ±7.1	1318.0*	1517.0 ±12.9
VFA ($g_{COD} \cdot kg^{-1}$)	8.3 ±4.9	2.5 ±0.1	Nd
NH_4^+ ($g \cdot kg^{-1}$)	2.5 ±0.3	1.5 ±0.3	Nm
TKN ($g \cdot kg^{-1}$)	3.4 ±0.3	19.2 ±2.3	Nm
Protein ($g \cdot kg^{-1}$)	3.1 ±1.2	110.6 ±2.6	Nm
Fat ($g \cdot kg^{-1}$)	Nm	363.4 ±0.6	Nm
SO_4^{2-} ($g \cdot kg^{-1}$)	Nd	Nd	1.7 ±0.1
AB (%COD_t)	41.0 ±0.7	94.3 ±3.0	65.3 ±4.8
CH_4 (%v/v biogas)	65%	70%	60%
$m^3_{CH4} \cdot kg_{VS}^{-1}$	0.2 ±0.0	0.9 ±0.0	0.3 ±0.0
$m^3_{CH4} \cdot t^{-1}$	6.0 ±0.1	476.3 ±7.2	201.9 ±29.3

3.2. Reactor performance

The reactor feeding strategy was implemented for the biomass adaptation from PM alone to a complex mixture of PM, PP-ABP, and RG. This continuous experiment lasted 70 weeks and was divided in 3 stages based on feed composition and reactor control parameters (Table 2). It consisted on progressively increasing the PP-ABP concentration in a first stage, adding then carbohydrates (RG) in the last stage.

Table 2. *Operation and control parameters during the continuous co-digestion with different feeding mixtures. Nomenclature: PM - pig manure, PP-ABP - pasteurized pig waste, RG - residual glycerin.*

Step	**1**	**2**		**3**	
Period	**P1**	**P2**	**P3**	**P4**	**P5**
Influent					
PM:PP-ABP:RG (%inlet-VS)	100:0:0	93:7:0	64:36:0	34:50:1	35:47:1
C/N ($g \cdot g^{-1}$)	6.3	6.1	5.9	8.0	10.3
TAN ($g_N\ kg^{-1}$)	2.69	3.08	2.62	1.61	2.14
OLR					
$kg_{COD} \cdot m^{-3} \cdot d^{-1}$	0.8	3.0	2.6	2.5	3.2
	0.5	1.3	0.9	1.3	1.6
Control parameters					
COD removal (%)	30%	48%	44%	51%	55%
CH_4 (%v/v)	65%	73%	73%	71%	71%
Yields:					
$Nm^3_{CH4} \cdot kg_{VSin}^{-1}$	0.15	0.35	0.43	0.38	0.38
$Nm^3_{CH4} \cdot t^{-1}$	3.6	9.7	13.6	16.0	18.7
TAN ($g_N \cdot l^{-1}$)	1.81	2.95	3.28	2.30	2.42
FAN ($g_N \cdot l^{-1}$)	0.13	0.33	0.31	0.14	0.11
Total VFA (% effluent-COD)*	1.5%	3.6%	2.8%	4.2%	1.9%
TA ($g_{CaCO3} \cdot l^{-1}$)	8.95	13.80	17.47	10.18	8.66
Alkalinity ratio**	0.19	0.19	0.22	0.22	0.24
pH	7.8	7.7	7.9	7.8	8.0
Propionic to acetic ratio	0.17	0.32	0.21	0.82	0.88
Micro. samples (week)	8	23	55	73	86

*Conversion factors: 1.07 $g_{COD} \cdot g_{acetic}^{-1}$; 1.51 $g_{COD} \cdot g_{propionic}^{-1}$; 2.03 $g_{COD} \cdot g_{butyric}^{-1}$; 2.21 $g_{COD} \cdot g_{valeric}^{-1}$; 2.34 $g_{COD} \cdot g_{heptanoic}^{-1}$ from Soto et al. (1993).
**Alkalinity ratio is the ratio between intermediate and partial alkalinity (IA/PA)

The first period (P1) was the starting up of the reactor, feeding only with PM. The second stage consisted on supplementations with PP-ABP (expressed as percentage in relation to VS), which were added increasingly in three subsequent periods: 7% (P2), 36% (P3) and 60% (P4). The HRT in P2 was maintained at 21 days and the OLR was increased

until 3.0 $kg_{COD}\cdot m^{-3}\cdot d^{-1}$. The methane yield and production rate raised up to 9.7 $m^3_{CH4}\cdot t^{-1}$ and 0.47 $m^3_{CH4}\cdot m^{-3}\cdot d^{-1}$, representing an improvement of 168% and 114%, respectively, in relation to the previous phase in which only PM was applied (P1). The CH_4 biogas fraction also increased from 65 till 73% $v\cdot v^{-1}$. Despite the higher concentration of ammonia and VFA measured in the effluent (0.33 $g_{NH3}\cdot l^{-1}$ and 3.6% COD), the system was considered stable because of the relatively high methane production rate.

In period P3, the HRT was increased from 21 till 33 days (Figure 1) in order to prevent potential problems due to the increase of the PP-ABP content from 7 to 36% VS in the feed. The COD removal efficiency experimented a slight decrease when compared to P2, despite the fact that there was neither VFA accumulation (2.8% COD) nor high ammonia concentration ($0.31 g\cdot l^{-1}$) in P3. As expected, with respect to P1, the gas production was higher, achieving values of 0.43 $m^3_{CH4}\cdot kg_{VS}^{-1}$, which are slightly greater than the ranges of 0.27-0.35 $m^3_{CH4}\cdot kg_{VS}^{-1}$ previously reported for the co-digestion of slaughterhouse and fruit wastes with pig manures [4].

RG was introduced during the third and final stage, up to 16% and 18% of the fed VS for the periods P4 and P5, respectively. The difference between P4 and P5 was the OLR, which was increased from 2.5 to 3.2 $kg_{COD}\cdot m^{-3}d^{-1}$. In this period, glycerin was added as a mean for increasing C/N ratio of the influent from 6 till 10. An optimum C/N range of 20 to 30 has commonly been reported for an efficient use of nutrients and maximum methane yield. Yet, lower C/N ratios have also been suggested as optimal, particularly in the anaerobic digestion of swine manure. In an early study, Sievers and Brune [22] revealed that the optimal C/N range for swine manure digestion was 15-19 in terms of maximum methane production. They also reported that, along with an increasing loading rate, the biogas production was stable in the digesters when the C/N was maintained between 6-16, when compared with digesters that were operated with a C/N of 20.

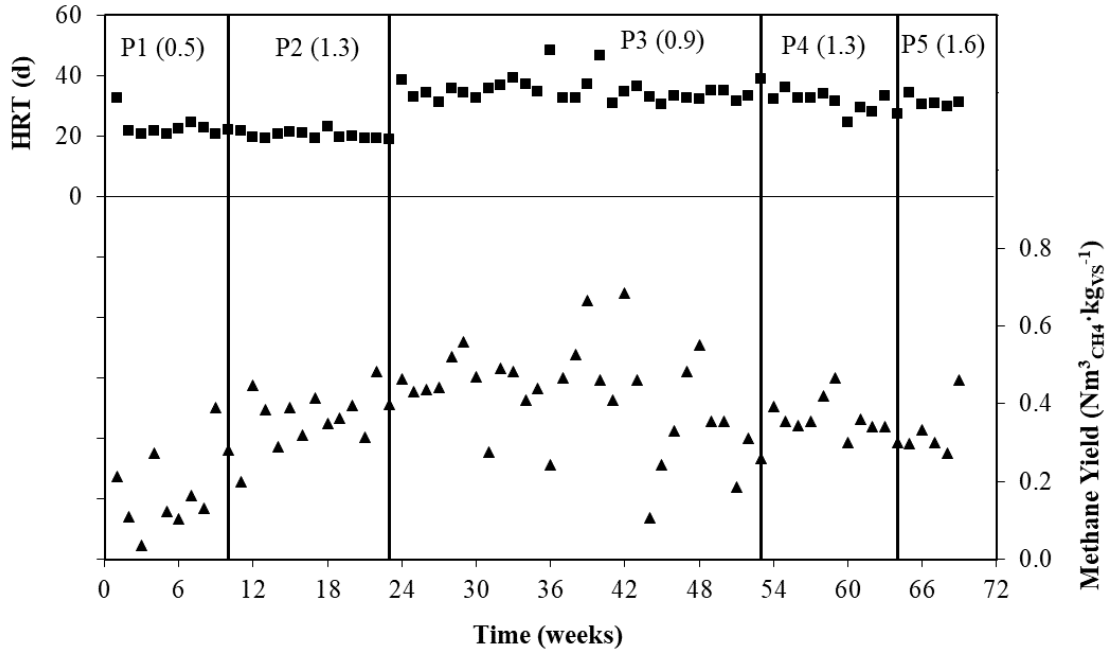

Figure 1. *Continuous operation: HRT and methane yield of every period (OLR is shown between brackets in* $kg_{VS} \cdot m^{-3} \cdot d^{-1}$*).*

Both methane yield and production rate in the digester improved: 18% and 11% in P4 with respect to P3. Amon et al. [11] also used glycerin as carbon supplementation in the digestion of pig manure and maize silage, with an increment of 19% in methane yield (from 0.57 to 0.68 $m^3_{CH4} \cdot kg_{VS}^{-1}$) after adding a 6%VS of glycerin in the total feeding, while Robra et al. [23] found that maximum methane yields (0.52 $m^3_{CH4} \cdot kg_{VS}^{-1}$) were attained when 5-10%VS-fed of glycerin was added to cattle slurry. There was an increase in VFA concentration (equivalent to 4.2% COD) in P4, but it decreased down to 1.9% COD in P5 due to a better COD removal efficiency, values that were in the range of the VFA concentration observed previously during P1. When compared to the feeding with pig manure (period P1), the achieved methane yield was increased by 344% and 419%

for P4 and P5, respectively. The COD removal efficiency also increased accordingly (51% and 55% COD removal in P4 and P5), regarding P1.

Despite an increment in methane yield of 17% in P5 in relation to P4, the addition of RG above 18% fed VS was discarded because of the imbalance in propionic/acetic acid inside the reactor. The inhibitory effect of methanogenesis of propionic acid at concentrations of 1-6 $g \cdot l^{-1}$ was previously manifested with the sporadically RG addition [24]. Fountoulakis et al., [25] concluded that crude glycerol addition at 1% v/v to sewage sludge co-digestion increased CH_4 production above the expected theoretical value. Yet, when glycerol in the feed exceeded 1%, the digestion process was not stable. They also observed that the metabolism of glycerol occurs at a rate that is faster than that of propionate, so that a glycerol overloading might result in propionate accumulation in the reactor. In fact, Angelidaki et al. [26] assumed that glycerol conversion to propionate took place instantly, as an integral part of lipid hydrolysis.

The question on whether a bioreactor remains stable over time is not easy to answer, as more than 140 different definitions of "stability" (properties and measure of stability) exist in ecology [3]. So, the definition of ecosystem stability is referenced in many cases either to measurable parameters describing the function of the whole system or to the community composition [3]. For anaerobic digesters, stable performance implies steady-state production and consumption of metabolites along the trophic chain. Selected metabolites that are generally monitored over time, as VFA/NH_3/H_2/CH_4, besides the COD removal efficiency, were chosen as good functional stability indicators. From this point of view, the described co-digestion experiment was run for 470 days and functional stability in all the periods was confirmed by a constant performance with respect to COD reduction and methane production.

3.3. Microbial community dynamics

The structure of the microbial populations along the co-digestion experiment was characterized by DGGE molecular profiles of bacterial and archaeal 16S rRNA genes. Different ribotypes were depicted during each operational period and predominant bands were successfully excised and sequenced (Figure 2 and Tables 3, 4).

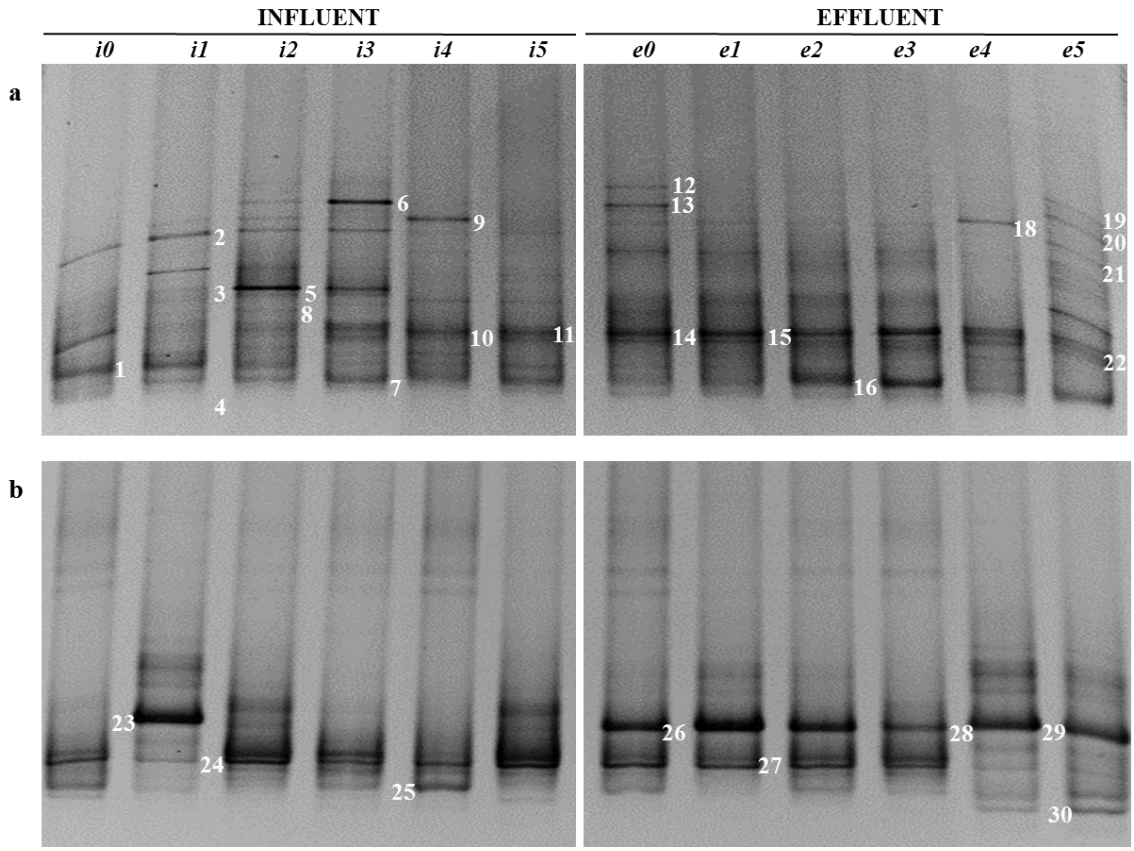

Figure 2. *DGGE profiles on 16S rRNA gene sequences from bacteria (a) and archaea (b), amplified from total DNA extracts on influent and effluent samples taken during the co-digestion experiment. These samples corresponded to the different feed composition, as detailed in Table 2. Numbered bands were successfully excised and sequenced.*

Table 3*. DGGE bands of bacteria (Figure 2a): designations ,accession numbers and levels of similarity to related organisms. Note: T Type strain.*

Band	Sample	Phylum/ Family	Reference species, strain or uncultivated microorganism (environmental source)	Accession	H
1	i0	*Firmicutes/ Clostridiaceae*	Uncultured (anaerobic reactor treating pig slurry) *Syntrophomonas sapovorans DSM3441*^{T}	HQ155840 NR_028684	98 97
2	i0-i5	*Firmicutes/ Erysipelotrichaeae*	Uncultured (anaerobic reactor treating pig slurry) *Erysipelothrix tonsillarum ATCC43339*^{T}	HQ156132 NR_040871	95 91
3	i1-i3; e5	*Bacteroidetes/ Sphingobacteriaceae*	Uncultured (anaerobic reactor treating pig slurry) *Parapedobacter soli DCY14*^{T}	GQ139189 NR_044119	98 86
4	i0-i3, i4, i5; e4	*Firmicutes/ Clostridiaceae*	Uncultured (anaerobic reactor treating pig slurry) *Syntrophomonas zehnderi OL-4*^{T}	GQ133946 NR_044008	99 94
5	i1-i3	*Bacteroidetes/ Bacteroidaceae*	*Bacteroidescoprosuis* JCM13475^T	AB510699	100
6, 12, 13, 17, 19, 20	i2, i3; e0, e5	*Bacteroidetes/ Porphyromonadaceae*	Uncultured (anaerobic reactor treating pig slurry) *Petrimonas sulfuriphila BN3*^{T}	GQ137794 NR_042987	99 92
7, 16	i1-i3, i5; e0-e3, e4, e5	*Firmicutes/ Carnobacteriaceae*	*Trichococcus flocculiformis DSM2094*^{T} *Trichococcus palustris DSM9172*^{T} *Trichococcus pasteurii DSM 2381*^{T}	NR_042060 NR_025435 NR_036793	99 99 99
8	i2-i3; e0	*Proteobacteria/ Pseudomonadaceae*	Uncultured (aerobic reactor treating pig slurry) *Pseudomonas pertucinogena IFO 1416*^{T}	HM069956 NR_040799	99 95
9, 18	i1,i2,i4; e0-e4	*Bacteroidetes/ Rikenellaceae*	*Ruminofilibacter xylanolyticum S1*^{T}	DQ141183	100
10, 11, 14, 15	i2-i5; e0-e5	*Firmicutes/ Clostridiaceae*	Unidentified (swine feces) *Clostridium disporicum DS1*^{T}	FJ753830 NR_026491	98 98
21	E5	*Bacteroidetes/ Bacteroidaceae*	Uncultured (aerobic reactor treating pig slurry) *Bacteroides propionicifaciens JCM14649*^{T}	GQ137107 AB510706	95 91
22	I4, i5; e1-e3, e5	*Bacteroidetes/ Sphingobacteriaceae*	Uncultured (aerobic reactor treating pig slurry) *Solitalea canadensis DSM3403*^{T}	GQ134100 NR_040906	98 85

Table 4. *DGGE bands of archaea (Figure 2b): designations and accession numbers for the band sequences and levels of similarity to related organisms. Note: T Type strain.*

Band	Sample	Phylum/Order	Reference species, strain or uncultivated microorganism (environmental source)	Accession number	H
23, 26, 28, 29	i1,e0-e5	Euryarchaeota / Methanosarcinales	Uncultured (activated sludge) Methanosaeta concilii DSM2139^T	AB489236 NR_028242	100 99
24, 27	i0, i2-i5, e0-e5	Euryarchaeota / Methanosarcinales	Uncultured (anaerobic reactor treating pig slurry) Methanosarcina barkeri DSM800^T	JN173201 AJ012094	100 98
25	i3, i4	Euryarchaeota / Methanosarcinales	Methanosarcina mazei DSM2053^T	NR_041956	99
30	e5	Euryarchaeota / Methanomicrobiales	Uncultured (anaerobic reactor treating MWS) Methanospirillum hungatei NBRC100397^T	CU917418 AB517987	99 96

The biomass ratio in the different periods (Figure 3) was estimated in terms of gene copy numbers per gram of fresh sample of bacterial 16S rRNA genes and methanogenic archaea by *mcrA* genes (*mcrA* of total methanogenic archaea, mcrA of *Methanosarcinaceae* (*Msar*) and *mcrA* of *Methanosaetaceae* (*Msae*)). The functional *mcrA* gene encodes for the alpha subunit of the methyl-coenzyme M reductase, which catalyzes the last step in methanogenesis and is present in all methanogens [27]. In all studied periods, the bacterial community structure was more diversified and abundant than that of the archaeal population. Total methanogenic population remained relatively stable at about 10^7 *mcrA* gene copy numbers · mL^{-1} for raw sample in all stages, just one magnitude order below bacterial 16S rDNA gene counts. Most of the dominant bacterial ribotypes were associated to uncultured heterotrophic bacteria that are characteristic of anaerobic reactors fed with pig slurry. The obtained phylogenetic archaeal assignments were similar to other works in that *Methanosarcinales* and *Methanomicrobiales* were dominant in swine manure biogas reactors [28].

The bacterial composition of the influent experimented significant fluctuations along the continuous reactor experiments, which might primarily be attributed to the changing nature of the pig slurry. Microbial community of effluent samples appears to be more stable and less diverse than that of the influent. The most abundant ribotype (band 7 and 16) has a 99% sequence homology to the species cluster formed by *Trichococcus flocculiformis*, *T. palustris* and *T. pasteurii*. These species have been characterized as fermentative, aero-tolerant and gram-positive filamentous bacteria, which mainly degrade monomeric and dimeric carbon sources and that has been isolated from bulking sludge [29]. They have also been reported to ferment glucose by producing lactate, formate, acetate and ethanol as organic end products [30].

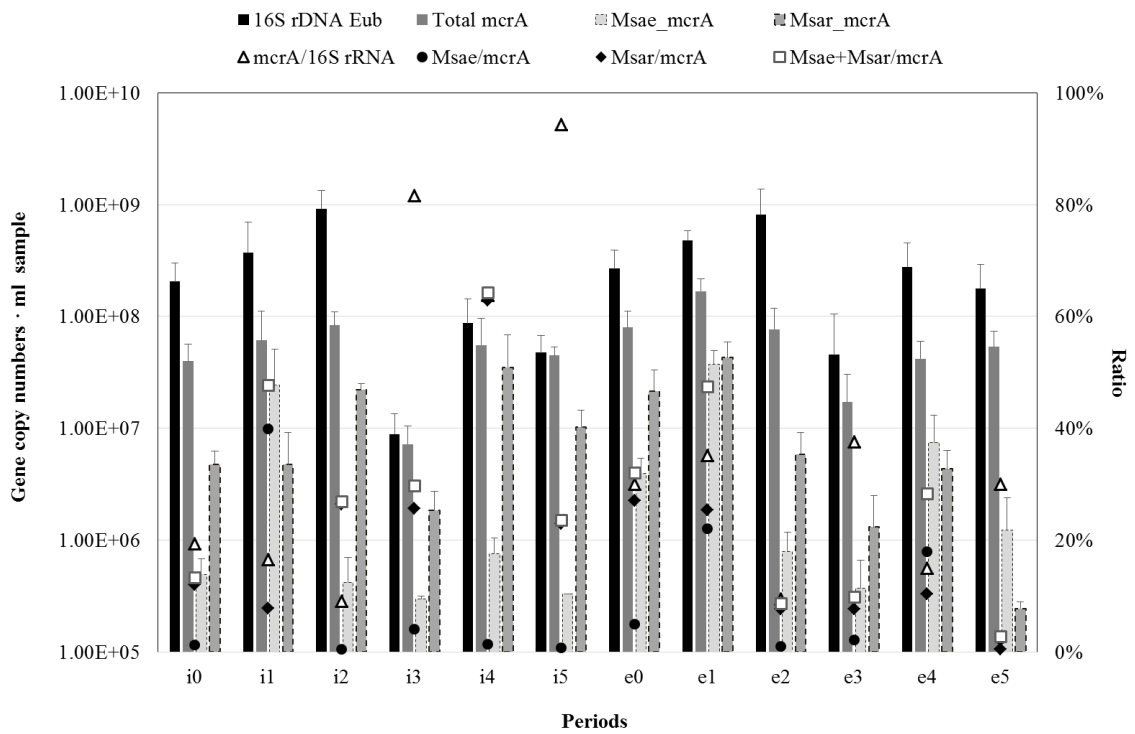

Figure 3. *Gene copy numbers of Bacteria and Archaea per mL of fresh sample and ratio between Methanosaeta and Methanoosarcina.*

Other ribotypes that were present in both influent and effluent samples were associated to *Petrimonas sulfuriphila* (band 6, 12, 13, 17, 19 and 20: sequence homology 92%), *Clostridium disporicum* (band 10, 11, 14 and 15: sequence homology 98%), *Pseudomonas pertucinogena* (band 8: sequence homology 95%) and *Ruminofilibacter xylanolyticum* (band 9 and 18: sequence homology 100%). *P. sulfuriphila* is a mesophilic, strictly anaerobic, fermentative bacterium that was isolated previously from a biodegraded oil reservoir [31]. This species is known to ferment carbohydrates and some organic acids, producing acetate, H_2 and CO_2. Elemental sulphur and nitrate can be used as electron acceptors, being reduced to sulphide and ammonium, respectively. *Clostridium disporicum* is a starch hydrolyzing bacteria that ferments sugars to acids [32]; it was described as a resistant bacterium towards environmental stress [33] and it

has also been found in swine slurry [34]. The band related to *P. pertucinogena* was enriched exclusively during the periods without glycerin and a similar ribotype was found in a microbial study on the anaerobic digestion of cattle manure [35]. *Ruminofilibacter. xylanolyticum*, a rumen bacterium involved in the digestion of xylan, was detected in a full-scale biogas plant fed with maize silage, green rye and liquid manure [36]. This bacterium is also present in energy crops, manure and in grass silage fibres immobilized on zeolite [37], and showed a pronounced hydrolytic xylanase activity. This enzyme might catalyze the degradation of fibers in pig slurries.

In what concerns the archaeal population, DGGE profiles were relatively conserved along time but were clearly distinct when comparing influent and effluent samples (Fig. 2). Population shift occurs at family level concerning *Methanosaetaceae* (*Msae*) and *Methanosarcinaceae* (*Msar*). Except for the first period (i1), *Msar* was the prevalent methanogenic population in influent samples, but the *Msar* population decreased significantly in the effluent, especially at e5, while *Msae* became the predominant methanogenic archaea in all effluent samples. The occurrence of *Methanosarcina* and *Methanosaeta* might be associated to the presence of acetic acid and ammonia in the reactor (Table 2), at concentrations that were always below the known inhibition threshold level for both genera [38].

The DGGE profile reveals that ABP addition (P2 & P3) did not significantly affect the predominant microbial population but, in more accurate quantitative terms, qPCR results show that both bacterial and archaeal populations were affected by changes in substrate composition, especially in effluent samples e3 and e4.

The period P5 was characterized by a second addition of glycerin (e5), which had a clear impact on the microbial community structure in the effluent. The *Msa*r gene content experienced a significant decrease (*Msar/mcrA* ratio of 0.46%) and the band 27 (related

to *Methanosarcina barkeri*, sequence homology 98%) could not be observed in the DGGE profiles. On the contrary, bands 28 and 29 related to *Methanosaeta concillii* were still evident in the DGGE but qPCR results showed low Msae/mcrA ratio (2.31%). Despite of the decrease in *Msar* and *Msae* counts, the total methanogenic population remained quite stable (10^8 *mcrA* gene copy numbers·mL^{-1} fresh sample). This phenomenon could be explained by the enrichment of a methanogenic population related to *Methanospirillum* genus (seen as band 30 in the DGGE, sequence homology 99%). Species in this genus have been reported to use formate or hydrogen and carbon dioxide as substrates for methane formation and growth [39]. The enrichment of *Methanospirillum* could hence be associated to the fermentation of glycerol, which has been reported to result in formate [40].

4. Conclusions

The co-digestion of pasteurized animal by-product with pig manure was improved by the addition of glycerin as carbon source. The best results concerning the methane yield (18.7 m^3_{CH4} ·t^{-1}) were obtained with the highest C/N value of 10.3. The combination of qualitative and quantitative molecular techniques (DGGE/qPCR) proved to be a useful tool for analyzing the microbial community dynamics during the adaptation process. The domain B*acteria* was more diverse and displayed a higher sensitivity towards operational changes than the more conserved *Archaea* domain. This later group was dominated by the genera *Methanosaeta* and *Methanosarcina*. Yet, *Methanospirillum* played a significant role, particularly upon glycerin supplementations and its metabolism via formate.

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