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ABSTRACT

For small-scale farms, the development of rustic and cheap psychrophilic anaerobic digestion systems appears as an opportunity to treat manure, mitigate gaseous emissions and promote decentralized renewable energy production. However, the development of such processes is limited by our understanding of their start-up. In this research, we tested the ability of one mesophilic digestate and four different manure to be used as inoculum for the start-up of psychrophilic anaerobic digestion of swine slurry at 13 °C. The most efficient inoculum appeared to be a swine manure that had been stored for 2 months in a pit. After 9 months of acclimation, the corresponding reactor produced a maximum methane yield of 42L of CH_4/kg Volatile $Solide_{substrate}$/day and a CH_4 volume of 125L of CH_4/kg-Chemical Oxygen $Demand_{added}$. The maximum methane production expressed at 13 °C was between 55% and 68% of that obtained at 37 °C. Monitoring of the microbial community dynamics by high throughput 16S rDNA sequencing showed the smooth adaptation of manure microbial species, underlining the transient dominance of the acetogen syntroph candidatus *Cloacimonas* during acclimation and the enrichment in the *Methanosaeta* and *Methanosarcina* methanogens for an efficient methane production.

1. Introduction

Anaerobic digestion (AD) of livestock waste is strongly promoted in Europe since it allows the treatment of manure along with the capture of gaseous emissions (greenhouse gas, ammonia and odors) and the production of both a renewable energy and a digestate used as fertilizer (European Commission, 2018; Martinez et al., 2009). Among all European countries, Germany is the leader with about 11,000 installations at farm scale. Its economic model focuses on biogas production in medium and large mesophilic reactors digesting a mixture of animal manure and several co-substrates among which energy crops. However, in countries where the average size of farms is smaller and the use of energy crops is restricted (as in France), this anaerobic digestion model is often not economically sustainable without incentives or long payback (Bhatt and Tao, 2020; O'Connor et al., 2021; Wilkinson, 2011). There is thus a need to reduce the cost of biogas production in small-scale farm systems.

AD is the biological process of organic matter degradation in the absence of oxygen, leading to the production of a biogas composed mainly of methane (CH_4) and carbon dioxide (CO_2) and of a digestate composed of undegraded residues. During manure storage, AD occurs naturally releasing methane to the atmosphere as well as other gas like ammonia, nitrous oxide and hydrogen sulfide (Holly et al., 2017; Husted, 1994; Im et al., 2020; Masse et al., 2002; Masse et al., 2008). A recent carbon emissions inventory of pig husbandry showed that the production of one pig results in the emission of 1.17 kg of N_2O (Nitrous oxide) and 1.36 kg of CH_4, with about 57% coming from pig waste disposal (Li et al., 2021). Thus, to reduce the impacts of pig production on climate change, the EU-28 promotes the covering of manure storage tanks.

Instead of simply covering manure tanks to prevent gaseous emissions, the idea of changing manure storage tanks into cheap and rustic AD systems has gain growing interest (Surendra et al., 2014). Early in the eighties, Chandler et al. (1983) and Safley and Westerman (1989) proposed to capture biogas emissions from pig slurry storage lagoons. The biogas capture ranged from 0.03 to 0.15 m^3 of biogas/lagoon's m^3/d with 62–70% methane content but production was highly

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dependent on the lagoon's organic load and the ambient temperature, varying between 0.01 and 0.31 m^3 of biogas/m^3/d. Thereafter, Massé et al. (1996, 2003 and 2010) studied biogas production from anaerobic psychrophilic sequencing batch reactors treating swine slurry at temperatures ranging from 10 to 25 °C. Methane production was respectively 0.26, 0.22 and 0.08 L of CH_4/g-COD_{added} at 25, 15 and 10 °C (Masse et al., 2003). Finally, Abou Nohra et al. (2003) introduced the concept of In-Storage-Psychrophilic-Anaerobic-Digestion (ISPAD) of livestock manure which consists in covering slurry storage tanks with an airtight floating membrane allowing in the same time the promotion of AD, collection of biogas, prevention of gaseous emissions, and prevention of manure dilution by rainfall. This system bypasses the need to build an anaerobic digester besides the storage facility but requires the operation of the digestion process at ambient temperature that fluctuates with climatic conditions (Giard et al., 2013).

While AD occurs in nature between 0 and 97 °C, temperature has a strong impact on anaerobic digestion and methane emissions from manure (Im et al., 2020; Kashyap et al., 2003; Keating et al., 2018; Masse et al., 2003; Nozhevnikova et al., 1999; Rajagopal et al., 2019). A linear relation between methane production and manure temperature has been observed in non-inhibitory conditions between 10 and 23 °C, with decreasing temperature inducing decreasing methane production (Cullimore et al., 1985; Safley and Westerman, 1994). However, more complex patterns have also been observed with nonlinear responses and/or long lag phase before methane emissions suggesting the acclimation of manure microbial community to low temperature (Hill et al., 2001; Masse et al., 2003). As examples, more than one year of acclimation was required for a swine slurry to obtain at 10 °C a methane production rate similar to the one observed at 23 °C (Kaufmann et al., 1982; Nozhevnikova et al., 1999; Safley and Westerman, 1994). Sequencing batch reactors showed methane production of 0.3–0.66 L of CH_4/g-VS at 20 °C similar to the one in mesophilic conditions after 77 days of acclimation (Masse et al., 1997). Finally, King et al. (2011) compared the biochemical methane production (BMP) rates at 35, 18 and 8 °C of the pig slurry from a three-year old ISPAD along with the one of fresh slurry and the one of a one-year old storage tank stored slurry. This rate diminished from 44.6 L of CH_4/Kg/d at 35 °C to 9.8 and 8.5 L of CH_4/Kg/d at respectively 18 and 8 °C. However, whatever the temperature, the ISPAD slurry had the highest BMP rate, 3 to 12 times higher than those from the one-year storage tank slurry and 25 to 85 times higher than those of fresh slurry. All these data show that microbial communities from manure can adapt to low temperature and produce methane, but acclimation time is highly variable and may last for weeks, months or even years.

In this context, the objective of this work was to test the ability of different manures to be used as inoculum and promote anaerobic digestion of fresh swine slurry at 13 °C. Thirteen °C is the average annual temperature of Brittany (West of France) that concentrates 60% of French swine production. To do so, one stored dairy manure, three swine slurries (one fresh and two stored over a short or long time) and one digestate from a mesophilic AD plant treating swine slurry were collected in real farms. Each one was used as inoculum to start laboratory reactors that were regulated at 13 °C and gradually filled by the weekly addition of a fresh swine slurry to mimic manure storage tanks filling. The start-up of AD was then assessed by monitoring biogas production, AD metabolic intermediates and the microbial community dynamics. Acclimation was monitored for about 9 months.

2. Material and methods

2.1. Sample collection and preparation

Five different types of manure were collected from farms in Brittany (France). Fresh Swine slurry (FSM, less than 2 weeks of storage) and Stored Swine slurry (SSM, about 2 months of storage) were collected from the same weaning-finishing pig farm. Long Time Stored Swine slurry (LTSSM, more than 9 months of storage) was collected from the storage tank of another weaning-finishing pig farm. Bovine manure (BM) was collected from the storage tank of a dairy cattle farm (about 3 months of storage). Finally, the Digested Swine slurry (DSM) was collected from a mesophilic anaerobic digester treating swine slurry. Manure and slurry samples were stored less than 2 weeks at room temperature before to start acclimation. A part of the fresh swine slurry (FSM) was homogenised and frozen to be used as substrate for all experiments. To get comparable results between experiments, manure and slurries were sieved to remove all particles larger than 2 mm and the BM, LTSSM and DSM had to be centrifuged at 4000×*g* for 20 min at 13 °C to concentrate their volatile suspended solids (VSS) towards close levels (Table 1).

2.2. Acclimation of manure inocula at low temperature

Acclimation of manures to low temperature was realized in conditions that simulated the filling of a storage tank. Half a liter of each raw (FSM, SSM) or concentrated (BM, LTSSM, DSM) manure was used as inoculum to start separate batch digesters made of 2.2 L bottles. All digesters were flushed with CO_2 to remove O_2 and facilitate anaerobic digestion start-up. Then digesters were incubated at 13 °C without shaking in a water bath connected to a recirculating cooler unit (Julabo F-250) (Fig. 1). Each experiment was done in triplicate to test reproducibility.

After 6 days of stabilization, 75 mL of FSM thawed at room temperature and diluted twice in tap water was added to each digester weekly making an initial organic loading rate (OLR) of about 1.2 g-COD/L/d. With the increasing volume of slurry in the bottle, this load reached 0.56 g-COD/L/d after 104 days of acclimation. This low OLR was chosen to avoid inhibition of anaerobic digestion. Since biogas production stabilized after 90 days of acclimation, the organic load was increased on day 104 by diluting the FSM feed only 1.5 times in tap water. Because this change of loading did not affected biogas production, 75 mL of undiluted FSM was then added weekly to each digester from day 118 to the end of the experiment (day 222). The resulting OLR was 0.9 g-COD/L/d on day 118 that decreased gradually to reach 0.56 g-COD/L/d again at the end of the experiment.

For each 2.2 L digester the volume of methane produced over time was monitored using an Automatic Methane Potential Test System (AMPTS, Bioprocess Control AB, Lund, Sweden) (Fig. 1) as published in (Badshah et al., 2012). Reproducibility among triplicate was of ±7%. Accidental shut downs of the refrigeration unit occurred on days 18 and 53 leading to a slight increase in methane production as the temperature reached 20 °C for one and two days respectively.

Monthly, a 75 mL sample was collected from each digester to measure pH, volatile fatty acids (VFA), total and soluble chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and ammonia nitrogen (N-NH_4). Four mL samples were centrifuged 20 min at 6000×*g* and 4 °C, the pellets were stored at −80 °C for molecular biology analyses.

2.3. Analytical and biological methods

Biogas composition was determined by gas chromatography (Network GC System 6890N Agilent technologies). Total and soluble COD were measured using the micro-method MERCK. Normalized APHA methods (APHA, 2010) were used to determine total solids (TS), volatile solids (VS) on raw samples and total suspended solids (TSS) and volatile suspended solids (VSS) on pellets obtained after centrifugation of raw samples (6000×*g*, 20 min, 4 °C). The standard deviation for triplicate samples was below 2%. VFA quantification was done on supernatants obtained by centrifugation of digestate samples at 20,000×*g* for 20 min. Supernatants were diluted 10 times in MilliQ® water before analysis using high performance liquid chromatography (Ultimate 3000, Dionex, USA) according to Peu et al. (2004). This method allows identifying six VFAs: acetic acid, propionate, butyrate, isobutyrate, valerate

Table 1
Physico-chemical characteristics of the four manures and digestate used as inoculum and after 222 days of acclimation at 13°C.

Digester	pH		TSS (g/L)		VSS (g/L)		Total COD (g O_2/L)		Soluble COD (g O_2/L)		VFA (g O_2/L)		N-NH_4 (g/L)		CH_4 (%)[b]	*Bacteria* Gene copies 10^{10}/L[c]		*Archaea* Gene copies 10^8/L[c]		% *Archaea*[d]	
	in[a]	fi[a]	In	fi	in	fi	in	fi	in	fi	in	fi	in	fi	fi	in	fi	in	fi	in	fi
Fresh Swine Manure (FSM)	7.4	7.9	76.3	42.0	58.0	30.3	109	82.0	31.0	31.2	18.6	9.3	4.91	4.01	69	3	1.7	3	0.6	0.98	0.33
Stored Swine manure (SSM)	7.4	7.9	83.1	42.8	55.0	29.3	100	71.8	6.6	11.7	0.0	0.2	1.52	3.53	69	46	5.1	140	35.0	2.91	6.42
Bovine Manure (BM)[e]	7.3	7.6	59.0	41.0	51.0	29.2	102	92.6	12.3	18.5	1.4	7.7	0.99	3.54	60	62	22.0	200	26.0	3.14	1.17
Long Time Stored Swine Manure (LTSSM)[e]	7.8	7.7	82.0	41.5	58.6	29.1	85	93.3	4.1	18.9	0.1	5.0	0.94	3.50	64	49	27.0	390	54.0	7.36	1.98
Digested Swine Manure (DSM)[e]	7.5	8.0	83.0	36.2	53.0	23.9	131	88.3	34.6	29.3	15.3	9.5	7.96	4.55	18	120	36.0	9	12.0	0.07	0.33

[a] Values for the inocula at T0 (in) and for the digestates at the end of acclimation (fi)
[b] CH_4 content of biogas at the end of the acclimation
[c] Copies of 16S rDNA / L of manure as measured by real time qPCR
[d] Calculated as: (copies of archaeal 16S rDNA / (copies of bacterial 16S rDNA + copies of archaeal 16S rDNA)) x 100
[e] These inocula have been concentrated as described in M&M to get similar VSS for all tests at the beginning of the experiment

and isovalerate.

Biochemical Methane Potential (BMP) assays were performed using digestate from a WWTP sludge mesophilic digester as inoculum and an inoculum/substrate ratio of 3. Endogenic biogas production was measured using the inoculum alone. Each set comprised three batches incubated at 37 °C. Biogas production was monitored until production ceased (41 days). Before each change of OLR (4 and 8 months), the methane production obtained over 1 week at 13 °C was compared to the FSM methane potential determined at 37 °C.

2.4. Microbial communities' analysis

Total genomic DNA was extracted from 1.3 to 0.6 g of frozen sample using the PowerSoil™ DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, USA) as recommended by the manufacturer. Quality and concentration of the extracted DNA were examined by agarose gel (TAE 1X, 0.8% agarose w/v) electrophoresis and spectrophotometry (Nanodrop ND 1000, Thermo Scientific). The DNA was stored at −20 °C for further analyses. All DNA extraction and analysis were performed in triplicate.

Bacterial and archaeal communities of samples were quantified by 16S rRNA gene-targeted real-time PCR (qPCR) using generic primers and the iQ SYBR Green Supermix 2X (Bio-Rad) on a CFX96 thermocycler (Bio-Rad) as described in Zeng et al. (2012). PCR reactions were done in triplicate with 2 μL of DNA diluted 10 fold in water. Standard DNA was diluted 10 fold in series ranging from 10 to 10^8 gene copies/μL.

Microbial communities dynamics was investigated by high throughput DNA sequencing using Ion Torrent Personal Genome Machine methods and technologies (Life Technologies, USA) as described in Madigou et al. (2019) with a few adaptations. The analysis targeted the V4-V5 hypervariable regions of the bacterial and archaeal 16S rRNA genes using PCR amplification (Platinum SuperFi PCR protocol from Life Technologies) and fusion primers 515F (5′- Ion A adapter–Barcode–GTGYCAGCMGCCGCGGTA-3′) and 928R (5′-Ion trP1 adapter–CCCCGYCAATTCMTTTRAGT-3′) (Wang and Qian, 2009) which include a barcode and sequencing adapters. Resulting amplicons were purified and quantified according to manufacturer's instructions using respectively the Agencourt AMPure XP magnetic beads (Beckman Coulter) and the DNA 1000 kit and 2100 Bioanalyzer (Agilent Technologies). Template preparation for emulsion PCR and subsequent sequencing were performed using the Ion PGM Hi-Q View OT2 Kit and Ion PGM Hi-Q View Sequencing kit (Life Technologies) as described in Madigou et al. (2019). The high-throughput DNA sequencing produced an average of 28,268±6454 sequence reads of about 380 base pairs length for each sample.

These sequences were processed with the FROGS pipeline (Escudié et al., 2018) following the authors' recommendations on the MIGALE Galaxy instance (INRAE, Jouy-en-Josas, France): primers trimming, sequences clustering, chimera and singleton removal, low abundance OTU filtering, and taxonomic affiliation of the OTUs with blastn and 16S_SILVA_Pintail100_138 and Ribosomal Database Project II database (https://rdp.cme.msu.edu/). The OTUs abundance, microbial community diversity indices calculations and principal component analysis of microbial community structures were performed using Easy16S (https://shiny.migale.inrae.fr/app/easy16S) a shiny web interface based on the phyloseq R package (McMurdie and Holmes, 2013).

3. Results and discussion

3.1. Characteristics of the manures and digestate used as inoculum

Physicochemical and microbiological characteristics of the manures and digestate used as inoculum are presented in Table 1. The different inocula have similar neutral pH values between 7.3 and 7.8, a VSS content between 51 and 58.6 g/L, and a total COD ranging from 85 to 109 g/L. The composition of FSM is in agreement with previous

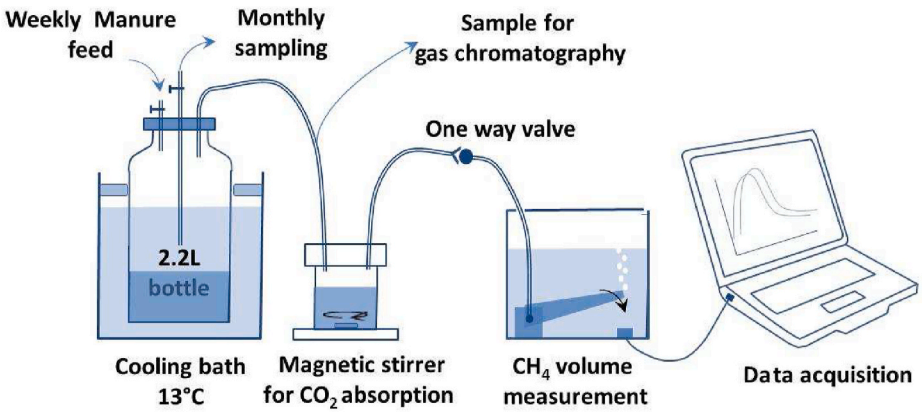

Fig. 1. Description of the automatic methane measurement system (AMPTS, Bioprocess Control AB, Lund, Sweden). Biogas produced in temperature controlled bottles (13 °C) enter a scrubbing solution unit (NaOH 3M) continuously stirred where CO_2 is fixed while methane pass through a one way valve and continues to the sensor chamber. CH_4 volume is recorded by the software.

publications (Bhatt and Tao, 2020) suggesting it contains about 60% of fermentable components. This fresh swine manure (FSM) and the digested swine manure (DSM) differ from the other inocula by their high concentration in soluble COD, VFA and ammonia. They also differ by the low proportion of *Archaea* (putative methanogens) in their microbial community, respectively 0.98% and 0.07% of *Archaea* for FSM and DSM. If this low archaeal proportion is expected in fresh pig manure since it is a monogastric animal (Gresse et al., 2019), it is a little surprising for the mesophilic manure digestate that usually contains between 1 and 10% of *Archaea* (Kim et al., 2015; Pampillón-González et al., 2017). Comparison of the FSM and SSM swine manures, collected in the same farm, suggests that the proportion of *Archaea* in microbial communities increased during manure storage.

3.2. Methane production under psychrophilic conditions

3.2.1. Cumulated methane production

Fig. 2 shows the average cumulative methane production of each digester during its acclimation to 13 °C. After a slight latency period from days 7–13, methane production started slowly for all digesters except for the one inoculated with the digested swine manure (DSM). This latter did not produce biogas for the 8 months of the experiment.

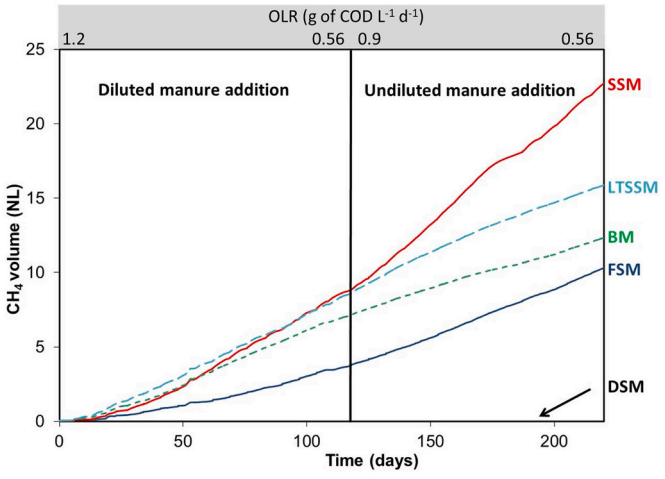

Fig. 2. Average cumulative methane production (NL) during anaerobic digestion of fresh swine manure in laboratory reactors incubated at 13 °C and inoculated by five different manures: FSM, fresh swine manure; SSM, stored swine manure; BM, bovine manure, LTSSM, long time stored swine manure and DSM, digested swine manure. Vertical bar represents the OLR change on day 118. Average of three replicates with 7% reproducibility.

For the first 50 days of acclimation, methane production was slightly higher for the digesters inoculated with the long-time stored swine manure (LTSSM) or the bovine manure (BM). However, after this period, methane production from the digester inoculated with the 2-months stored swine manure (SSM) increased and overpassed all the other digesters after day 100. Increasing the OLR on day 118 further favoured this digester compared to the others. At the end of the experiment on day 222, the average cumulated methane production was SSM (22.3 NL) > LTSSM (15 NL) > BM (11.7 NL) > FSM (10 NL). Except for the mesophilic digestate that did not acclimate at all, methane production from the digester inoculated with the fresh swine slurry inoculum was thus the least efficient in comparison to the other ones.

3.2.2. Methane production rates and expressed Biochemical Methane Potential

During the first 90 days of acclimation, the methane production rate of all digesters but DSM increased gradually to eventually stabilize from days 90–118. During this period, the values reached 71.5, 107, 126.5 and 156 NL-CH_4/kg-COD_{added} for respectively FSM, BM, LTSSM and SSM digesters (Table 2). Thereafter, the OLR was increased to 0.9 g-COD/L/d, starting a second period of acclimation for the microbial communities. The methane production rate of FSM and SSM digesters decreased a little to reach values equal to respectively 60 and 125 NL-CH_4/kg-COD_{added}. The BM and LTSSM digesters showed stronger decrease ending around respectively 45 and 50 NL-CH_4/kg-COD_{added}.

The highest CH_4 production of 125 NL-CH_4/kg-COD_{added} observed for the digester inoculated with SSM is within the range of the one published by Masse et al. (2003, 2010) for swine manure treated in anaerobic sequencing batch reactors. They observed methane production rates of 80 L-CH_4/kg-COD_{added} at 10 °C and between 130 and 220 L-CH_4/kg-COD_{added} at 15 °C. Even if the OLR of 1.2 to 0.56 g-COD/L/d applied in this study is relatively low compared to conventional mesophilic digesters fed with animal manure, it is close to the ones used in

Table 2
Expressed methane production of digesters after 4 and 8 months acclimation.

Digesters	4th month: Methane production (NL-CH_4/kg-COD_{added})	Methane Potential expressed (%)[a]	8th month: Methane production (NL-CH_4/kg-COD_{added})	Methane Potential expressed (%)[a]
FSM	71.5	31.2	60.0	26.2
SSM	156.0	68.0	125.0	54.5
BM	107.0	46.7	44.8	19.5
LTSSM	126.5	55.2	50.0	21.8

[a] Compared to BMP test done at 37 °C.

previous studies in stable running conditions. In 1996, Masse et al. published that stable methane production from swine manure treated at 20 °C in anaerobic sequencing batch reactors requires OLR below 1.6 g-COD/L/d (Masse et al., 1996). In 2013, they also showed that a compartmentalized bioreactor treating pig manure operating with an OLR of 2.4 g-COD/L/d at 25 °C could reach specific methane yields ranging from 148.6 to 171.4 L-CH_4/kg-COD (Masse et al., 2013).

The efficient acclimation of the SSM was confirmed by the determination of the methane production expressed by each digester (Table 2). The BMP of the substrate alone (FSM) at 37 °C was equal to 25 NL-CH_4/L of added manure corresponding to 229.3 NL-CH_4/kg-COD_{added}. This value must be compared with the 350 NL-CH_4/kg-COD usually observed for swine manure at 37 °C. The maximum methane production expressed by the digesters at 13 °C was observed for SSM on day 118 with 68% of the methane production that would be obtained by injecting the same amount of FSM at 37 °C. Its value remained at about 55% until the end of the experiment while it was at least twice less for the other digesters. This result is comparable to the one of the Canadian ISPAD which was shown to release 65% of its manure methane potential (King et al., 2011). It is also on line with the expected 60% of fermentable components present in FSM. Hence, for anaerobic digestion under non-optimal temperatures, relatively large volumes of biogas were produced notably with the digester inoculated with stored swine manure SSM.

3.3. Evolution of volatile fatty acids and ammonia concentrations in digestates during acclimation to psychrophilic conditions

The evolution of VFA and ammonia concentrations in all digesters during acclimation is shown in Fig. 3. The soluble COD and TKN variations were directly correlated to respectively VFA and ammonia concentrations and are not shown.

Evolution of the ammonia concentration is particularly interesting since its increase in anaerobic conditions is linked to incoming ammonia and organic matter biodegradation while its decrease is only linked to inoculum dilution by the feeding. Thus for DSM and FSM digesters that started with high concentrations of ammonia, the decreasing concentration observed during the first 118 days of incubation (months 1–4) reflects essentially manure dilution by feeding that exceeds a very low biodegradation activity. On the opposite, the increasing ammonia concentration observed for the other manures show a real organic matter biodegradation activity. This is in agreement with methane productions shown in Fig. 2 and Table 2. During the four last months, all ammonia concentrations slowly converged to the same value of about 4 N-NH_4 g/L and a NH_4^+/NTK ratio around 70% (Table 1). It confirms that ammonia concentration observed for all digesters at the end of the experiment results from an equilibrium between feeding and dilution. The high ammonia concentration of about 8 g N-NH_4/L observed for the DSM digester on day 0 is potentially inhibitor for anaerobic digestion (Chen et al., 2008) and may partly explain the absence of acclimation of this inoculum.

Concerning VFA (Fig. 3), their production and accumulation depends upon organic matter anaerobic biodegradation and methane production. The high VFA concentration observed in the DSM digester, composed in totality of propionate at about 15.3 g-COD/L on day 0 (Table 1), is another possible inhibitory compound explaining the absence of acclimation of this inoculum (Chen et al., 2008). The evolution of VFA, propionate and acetate concentrations in this reactor reflects principally the dilution of propionate and input of acetate by the feeding. The slow decrease of acetate concentration on the last month of experiment may suggest a starting low activity. It is not clear if the inoculum was already degraded at its collection or if it is the strong and rapid shift from 37 °C to 13 °C that provoked this situation.

The FSM digester that also started with a high VFA concentration of 18.6 g-COD/L on day 0 behaved differently since a majority of its VFA was acetate whose concentration decreased slowly during the acclimation. However, this digester conserved a relatively high level of propionate between 4.2 and 7.8 g-COD/L during all experiment, which may have limited anaerobic digestion efficiency. BM and LTSSM digesters, that rapidly and constantly produced methane throughout the experiment (Fig. 2), did not accumulate any VFA during the first four months of acclimation but started to accumulate acetate after increasing the OLR on day 118 (month 4). Finally, the SSM digester that gave the highest methane production did not accumulate any VFA during all acclimation.

Compared to the inoculum characteristics, data obtained from digestate at the end of the experiment showed slightly higher pH values between 7.7 and 8.0 for all of them (Table 1). TSS, VSS and soluble COD values were relatively close for all digestates except for DSM suspended solids and SSM total COD that showed lower values at the end of the

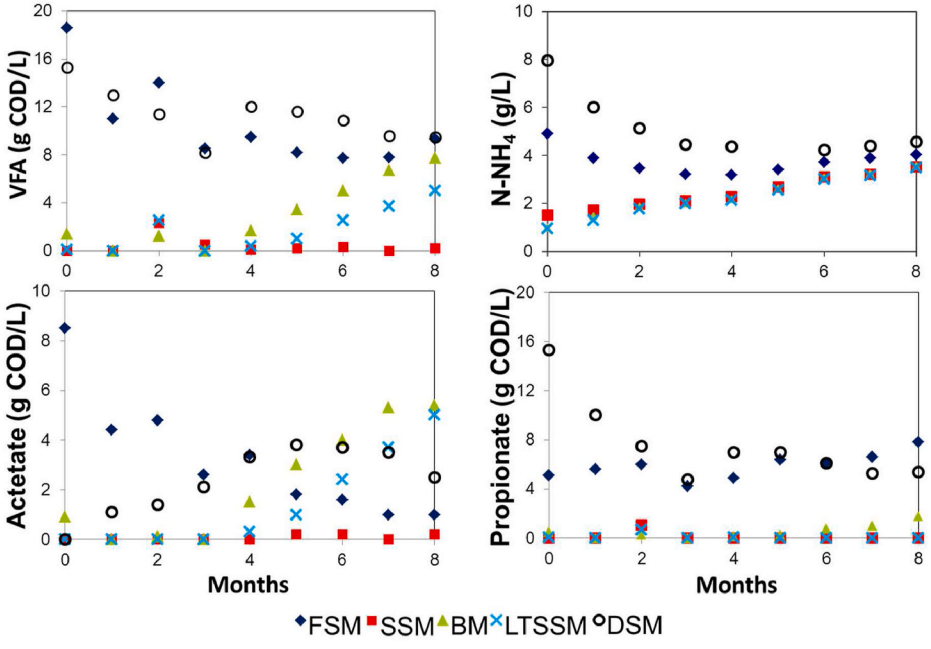

Fig. 3. Average VFA and ammonia concentrations over time for all digestates.

experiment. Both Total and soluble COD were lower for SSM compared to other manures showing that SSM was the most adapted to convert organic matter into methane at 13 °C. Because VFA are intermediate precursors of CH_4 production, the presence of high VFA concentrations for FSM, BM and DSM indicates a limited methanogenic activity compared to acidogenesis. Conversely, the SSM's low VFA value (200 mg O_2/L) indicates that all VFA produced were rapidly consumed, suggesting robust psychrophilic anaerobic digestion in this ecosystem.

3.4. Dynamics of digester microbial communities during acclimation to psychrophilic conditions

Real-time PCR quantification of the total *Bacteria* and total *Archaea* in the digesters during acclimation showed only global trends (Fig. 4). Bacterial concentrations differed by about two Log at the beginning of acclimation with the lowest and highest values of 3.4 10^7 and 1.3 10^9 copies of 16S rRNA gene (rDNA)/mL of digestate for respectively the FSM and DSM digesters. These concentrations stabilized for the first four months of acclimation at about 2 10^8 copies of 16S rDNA/mL. The organic load increase on day 118 (month 4) induced a new graduation of bacterial concentrations from about 6.9 10^6 to 1.5 10^8 for respectively FSM and DSM until the end of acclimation.

Archaeal concentrations were relatively stable for the SSM, BM and LTSSM digesters for the first four months of acclimation at about 10^7 copies of 16S rDNA/mL of digestate. The FSM and DSM digestates had lower initial archaeal concentration of respectively 3.2 10^5 and 8 10^5 16S rDNA/mL that increased for FSM and decreased for DSM. The increased OLR on month four induced variable archaeal concentration decreases, the strongest being for the FSM digester, but they all increased again until the end of the experiment.

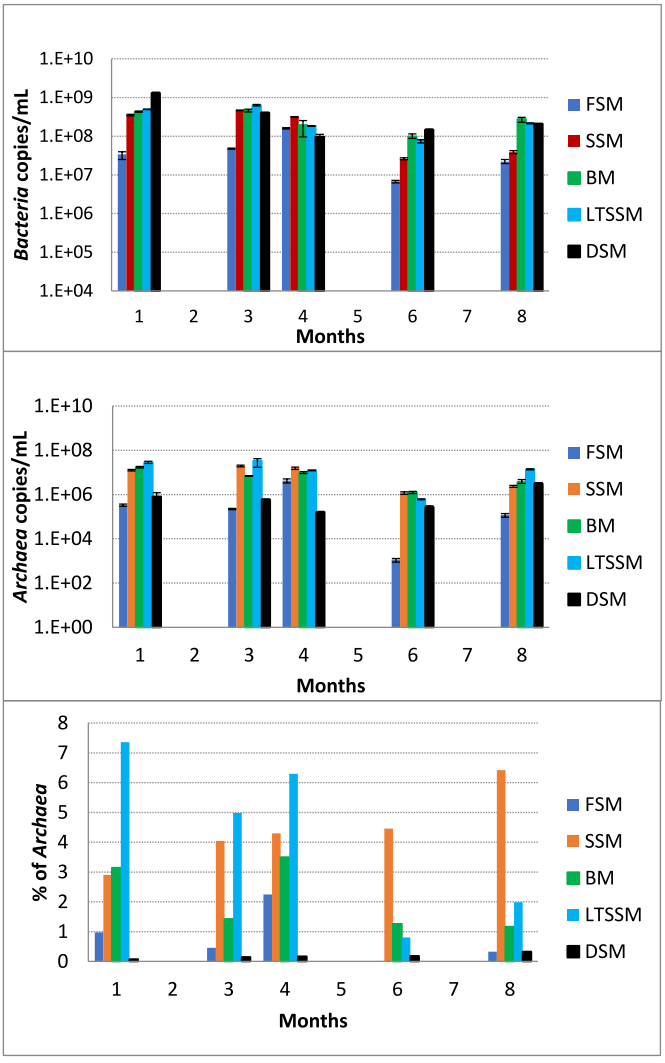

Fig. 4. Quantification of total *Bacteria*, total *Archaea* and % of total *Archaea* in the digester microbial communities during the experiment, in 16S rDNA copies mL^{-1} of digestate.

Overall, it is difficult to link the bacterial and archaeal concentration dynamics with methane productions described in Fig. 2 and Table 2. The evolution of the archaeal relative abundance within the community (Fig. 4) appears more interesting. The LTSSM digester that had the highest methane production during the first four months of acclimation has the highest *Archaea*/total microorganisms ratio. The DSM reactor that did not show methane production during all the experiment has the lowest ratio. The SSM digester that best supported acclimation shows a constant increase in this ratio.

3.5. Characterization of digester microbial communities during acclimation to psychrophilic conditions

3.5.1. Global figures and diversity indices

High throughput DNA sequencing of 16S rRNA genes was used to investigate the microbial community structure and dynamics of the inocula and digestate during acclimation (Table S1 supplementary material). Between 7,640 and 22,274 clean DNA sequences were obtained per sample analysed with the extremes being respectively FSM and SSM on the 4th month of acclimation (T4). The coverage percent of sequencing calculated for each digester was between 58 for BM and 62% for DSM and LTSSM. It is relatively low and suggests a high microbial diversity.

The bacterial richness (number of OTUs) of the digesters ranged from the relatively low mean value of 271±67 OTUs for DSM to the high value of 539±47 OTUs for LTSSM. The other digesters had mean OTU numbers between 300 and 400 OTUs. The archaeal richness was low with between 3 and 22 OTUs for respectively DSM on month 6 (T6) and LTSSM on months 3 and 4 (T3 and T4). The number of archaeal sequences represented between 0.12% and 6.72% of the total sequences of each community. These ratios are slightly different from the ones calculated previously using qPCR but they follow the same trend with the higher ratios for LTSSM and SSM followed by decreasing values for BM, FSM and DSM.

The α-diversity indices that represent the microbial diversity within each sample are very similar and stable during acclimation suggesting a smooth adaptation of the microbial communities (Table S1). The highest richness and diversity are observed after 4 months of acclimation (T4), just before the increase in organic load on day 118.

3.5.2. Taxonomic identification

Taxonomic identification of the OTUs shows the sequences belonged to 39 bacterial and 4 archaeal phyla. Whatever the inoculum, the dominant phyla in all digesters were the *Firmicutes* and the *Bacteroidetes* that contain between 53% and 86% of the total sequences of each sample (Fig. 5). Then, a third dominant phylum was the *Proteobacteria* for the digested swine manure (DSM) and the *Cloacimonetes* for the stored and long-term stored swine manure (SSM and LTSSM respectively). The dominant archaeal phylum was the *Euryarchaeota* that encompasses all the methanogenic species.

A principal component analysis (PCA) of the microbial community structures clearly separates the DSM, FSM and SSM microbial communities from the BM and LTSSM communities that appear more closely related (Fig. S1). It also highlights several OTUs that participate in the differentiation of these communities from one to another. This PCA analysis, along with the dynamics of the bacterial and archaeal dominant Families (Figs. 6 and 7) allows proposing different metabolic pathways for microbial communities' acclimation.

The DSM digester, which did not produce methane during all the

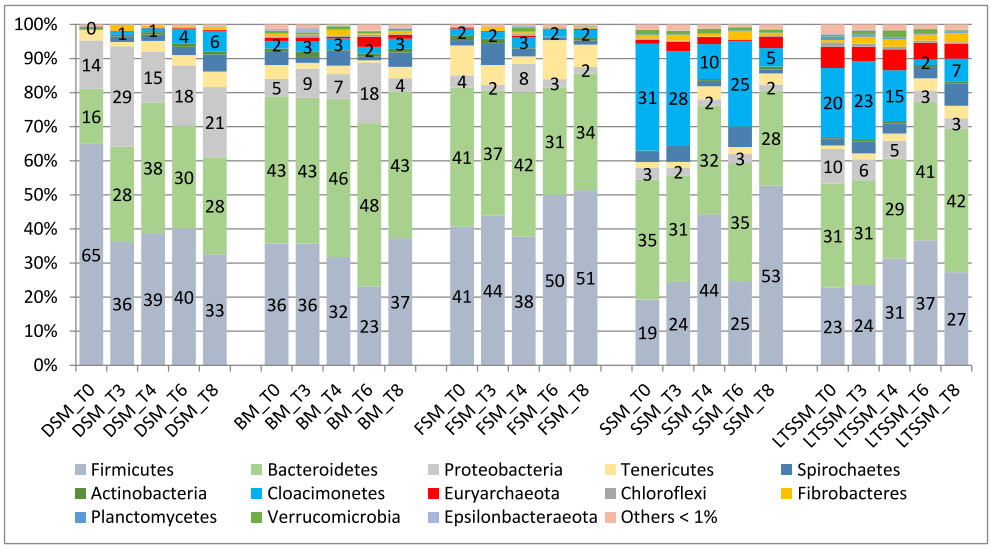

Fig. 5. Relative abundance of the different phyla containing more than 1% of the total sequences during the 8 months of acclimation (T0 to T8). Values are given (in %) for the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Cloacimonetes*.

Kingdom	Phylum	Family	DSM					FSM					BM					SSM					LTSSM				
			T0	T3	T4	T6	T8	T0	T3	T4	T6	T8	T0	T3	T4	T6	T8	T0	T3	T4	T6	T8	T0	T3	T4	T6	T8
Bacteria	Bacteroidetes	Dysgonomonadaceae	6	17	29	22	18	26	21	7	24	21	6	5	6	8	5	10	7	10	8	9	4	4	6	6	5
Bacteria	Bacteroidetes	Rikenellaceae	0	2	2	4	5	7	7	15	4	5	15	24	17	24	9	4	4	7	6	9	7	6	5	7	6
Bacteria	Bacteroidetes	Marinilabiliaceae	1	6	4	1	1	2	4	7	1	3	6	2	7	1	6	14	10	6	9	4	3	6	6	7	7
Bacteria	Bacteroidetes	Prolixibacteraceae	0	0	0	0	0	0	0	5	0	1	7	3	6	6	13	2	4	4	4	3	1	2	2	10	12
Bacteria	Bacteroidetes	Lentimicrobiaceae	0	0	0	0	0	0	0	2	0	0	2	1	2	1	3	1	1	1	2	1	4	4	4	5	4
Bacteria	Bacteroidetes	Bacteroidetes vadinHA17	0	0	0	0	0	0	0	3	0	0	2	1	2	2	1	2	2	1	2	1	4	3	3	2	1
Bacteria	Bacteroidetes	Bacteroidaceae	9	2	2	1	1	4	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria	Cloacimonetes	Cloacimonadaceae W27	0	1	1	4	6	2	2	1	2	2	1	1	1	1	2	30	26	9	23	5	1	1	1	1	2
Bacteria	Cloacimonetes	Cloacimonadaceae W5	0	0	0	0	0	0	0	2	0	0	1	2	2	1	1	2	2	1	2	0	19	22	14	1	5
Bacteria	Firmicutes	Clostridiaceae 1	3	13	15	20	13	12	19	7	4	19	9	6	7	0	16	6	10	13	8	20	5	8	8	11	9
Bacteria	Firmicutes	Ruminococcaceae	3	6	8	6	4	8	7	6	17	8	7	5	6	6	5	5	5	13	6	11	5	5	8	9	7
Bacteria	Firmicutes	Clostridiaceae Family XI	4	3	4	3	2	5	5	11	11	6	8	9	8	7	5	2	2	3	3	5	2	2	4	4	3
Bacteria	Firmicutes	Peptostreptococcaceae	0	2	1	2	1	1	2	3	1	3	3	6	3	3	4	0	1	1	1	2	3	2	2	2	2
Bacteria	Firmicutes	Lachnospiraceae	3	3	3	1	1	4	2	1	5	3	2	1	1	0	1	1	1	3	1	3	1	1	2	2	2
Bacteria	Firmicutes	Erysipelotrichaceae	8	2	2	2	2	2	2	1	1	2	1	1	1	1	2	1	1	1	1	2	2	1	1	1	1
Bacteria	Firmicutes	Unknown DTU014	27	0	1	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria	Firmicutes	Christensenellaceae	0	0	0	0	0	1	0	2	1	1	2	2	2	2	2	2	1	2	1	2	3	2	2	2	1
Bacteria	Firmicutes	Syntrophomonadaceae	1	1	1	1	0	0	0	2	1	1	1	3	1	1	1	1	1	3	1	3	0	0	1	1	1
Bacteria	Proteobacteria	Pseudomonadaceae	12	25	11	14	18	2	0	6	0	1	4	7	6	16	3	2	1	1	1	0	2	1	1	1	1
Bacteria	Spirochaetes	Spirochaetaceae	0	2	2	2	5	2	6	2	1	1	4	1	4	1	4	3	5	1	6	1	1	3	3	3	6
Bacteria	Tenericutes	Unknown Mollicutes RF39	0	1	2	1	1	7	4	2	11	5	3	1	2	0	2	1	1	3	1	3	1	1	2	3	2
Archaea	Euryarchaeota	Methanosaetaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	2	1	0	2	4	2	4	3	2

30
20
10
0

Fig. 6. Relative abundance (in %) and taxonomic identification of the bacterial and archaeal Families that contain more than 1% of the total sequences during the 8 months of acclimation (T0 to T8).

Family	Genus	Metabolism	DSM					FSM					BM					SSM					LTSSM				
			T0	T3	T4	T6	T8	T0	T3	T4	T6	T8	T0	T3	T4	T6	T8	T0	T3	T4	T6	T8	T0	T3	T4	T6	T8
Methanosaetaceae	Methanosaeta	Low [acetate] / methyl / H_2+CO_2	0	3	0	0	9	0	0	8	0	2	44	20	3	29	43	69	72	68	17	62	59	55	70	54	39
Methanospirillaceae	Methanospirillum	H_2+CO_2 / formate and syntrophe	0	3	0	0	2	0	0	4	0	0	6	3	3	0	12	7	9	12	37	3	23	35	23	40	51
Methanocorpusculaceae	Methanocorpusculum	H_2+CO_2 / formate and syntrophe	0	0	0	0	11	2	0	47	0	0	17	49	63	53	13	1	1	0	1	0	6	1	1	1	1
Methanobacteriaceae	Methanosphaera Methanobrevibacter	H_2+CO_2 / formate and syntrophe	14	73	63	64	28	91	79	39	87	72	18	15	22	6	12	2	4	7	13	12	2	2	2	2	3
Methanosarcinaceae	Methanosarcina	High [acetate] / methyl / H_2+CO_2	0	0	0	0	0	0	0	0	0	0	8	5	0	10	14	18	12	9	0	23	3	2	0	1	1
Unknown Bathyarchaeia	nd	Putative methyl	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	6	3	2	1	1
Methanomethylophilaceae	Candidatus Methanoplasma	H_2+CO_2 / formate / methyl	32	5	11	14	33	4	21	1	0	4	2	5	3	2	4	0	0	0	4	0	0	0	0	0	1
Methanomicrobiaceae	Methanoculleus	H_2+CO_2 / formate and syntrophe	55	16	26	21	16	2	0	0	13	22	1	0	0	0	0	2	1	3	4	0	0	0	0	0	0
Unknown Woesearchaeia	nd	non methanogen	0	0	0	0	0	0	0	0	0	0	3	0	2	0	0	1	0	0	9	0	1	0	0	0	1
Unknown Micrarchaeia	nd	non methanogen	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	14	0	0	0	0	1	2
Methanofastidiosaceae	Candidatus Methanofastidiosum	H_2+CO_2 / methylated thiol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Unknown Methanomicrobiales	nd	H_2+CO_2 / formate and syntrophe	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Unknown Lainarchaeales	nd	non methanogen	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0
Unknown Aenigmarchaeales	nd	non methanogen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

100
80
60
40
20
0

Fig. 7. Relative abundance (in %), taxonomic identification and putative metabolic substrates of the archaeal Families during the 8 months of acclimation (T0 to T8).

experiment, was the only one to exhibit a strong difference of microbial community structure between its inoculum at T0 and its other times of the experiment (Fig. 6). At T0, the community was dominated by the syntrophic acetate oxidizing *Clostridia* DTU014 and the hydrogenotrophic methanogen *Methanoculleus* along with the methylotrophic methanogen Candidatus Methanoplasma (Figs. 6 and 7). Members of the uncultured DTU014 Family are common in anaerobic digesters where they are suspected to degrade acetate in association

with H_2 scavenger organisms such as the hydrogenotrophic archaea (Mosbæk et al., 2016). Its dominance at T0 suggests that the major metabolic pathway for methane production in the DSM inoculum was syntrophic hydrogenotrophic methanogenesis. After 3 months of acclimation at 13 °C, the community was dominated by bacterial species from the *Dysgonomonadaceae* (Genus *Proteiniphilum*), the *Clostridiaceae* and the *Pseudomonadaceae*. At this time, the dominant *Archaea* were the hydrogenotrophic methanogens *Methanosphaera* and *Methanobrevibacter*. The brutal change in temperature, possibly associated with high ammonium and VFA concentrations described before, favoured the enrichment in fermentative bacteria members of the *Dysgonomonadaceae*, *Clostridiaceae* and *Ruminococcaceae* families and limited the growth of the archaeal methanogens whose concentration remained below 0.3% of the total community. The abundance of *Pseudomonadaceae* may appear unusual in anaerobic digesters since they are usually associated to aerobic metabolism. However, several species can grow in anaerobic condition through denitrification and others can adapt to cold temperature. Actually, the closest relative of the *Pseudomonadaceae* sequence identified in this study was a sequence retrieved from a swine effluent holding pit (acc. number DQ337540).

The fresh swine manure digester (FSM) community structure presented the same dominant bacterial families that the DSM digester at 13 °C but the *Pseudomonadaceae* were not within the dominants (Fig. 6). The proportion of *Archaea* was also low between 0.2 and 0.5% but concentrated on the hydrogenotrophic *Methanobrevibacter* genus, either alone or with another hydrogenotrophic genus, the *Methanocorpusculum* on T4 (Fig. 7) suggesting that methane production arose primarily by the hydrogenotrophic pathway. This is not surprising since methanogenesis is not a major pathway of pig gastrointestinal tract. This digester showed relatively high ammonium and VFA content at the beginning of the experiment that may explain the absence of enrichment in methanogenic *Archaea*.

The bovine manure digester (BM) bacterial community was slightly different since dominated by the *Rikenellaceae* before the *Clostridiaceae*, the *Ruminococcaceae* and the *Dysgonomonadaceae*. *Pseudomonadaceae* were also observed. Members of the *Rikenellaceae* are often found in the gastrointestinal tract of animals and came certainly with the inoculum. They are fermentative bacteria that degrade carbohydrates into propionic and succinic acids as major end products. Interestingly, the dominant archaeal family at T0 was the versatile methanogen *Methanosaeta* that can produce methane by both acetotrophic and hydrogenotrophic pathways. The incubation in psychrophilic conditions induced a transient shift to hydrogenotrophic methanogenesis with the dominance of *Methanocorpusculum* from T3 to T6. This period corresponds to the transient increase in the total *Archaea* up to about 3% of the total community observed by qPCR in Fig. 4.

Finally, the stored and long term stored swine manure digester communities (respectively SSM and LTSSM) present the particularity of being dominated by two different genera of the uncultured *Cloacimonadaceae* whose relative abundance decreased along acclimation. Members of the *Cloacimonadaceae* have been observed in many mesophilic anaerobic digesters (Goux et al., 2015; Regueiro et al., 2016; Regueiro et al., 2015). Analysis of the genome of Candidatus *Cloacimonas* suggests it is a syntrophic bacterium that can degrade several carbohydrate, amino acids and VFA (and more particularly propionate) into acetate and CO_2 in the presence of H_2 scavengers (Pelletier et al., 2008). Its presence in the SSM and LTSSM digesters may have prevented the accumulation of propionate in the digestate and supported acetotrophic and hydrogenotrophic methanogens, allowing a more efficient acclimation of the community. However, its decline with time suggests it was outcompeted by other acidogenic bacteria such as the *Clostridiaceae 1* and the *Ruminoccoccaceae*. In the literature, the relative abundance of *Cloacimonadaceae* has been positively correlated with protein rich effluent (Solli et al., 2014) and the dominance of *Clostridiales* (De Vrieze et al., 2015). Interestingly, Regueiro et al. (2014) showed its constant decrease in an anaerobic digester shifted gradually from 37 °C to 17 °C while it accumulated in the case of an abrupt temperature decrease. The archaeal community of the SSM digester made up to about 1–3.4% of the total sequences in the NGS characterisation and up to about 6% of the community as measured by qPCR (Fig. 4). It was dominated at T0 by the two acetotrophic and hydrogenotrophic versatile methanogens *Methanosaeta* (69% of the *Archaea*) and *Methanosarcina* (18%). However, the *Methanosarcina* relative abundance decreased from T0 to T6 while in the same time the one of the hydrogenotrophic methanogens *Methanospirillum* and *Methanobrevibacter* increased. These data are in agreement with Regueiro et al. (2014) that showed an increasing proportion of hydrogenotrophic methanogens after a drop in temperature. Moreover, it is well known that the *Methanosaeta* genus is favoured over *Methanosarcina* when acetate concentrations are lower than 1 mM (Kallistova et al., 2017). Both *Methanosaeta* and *Methanosarcina* became dominant again in the digester by the end of the experiment. The ability of these *Archaea* to produce methane from acetoclastic, methylotrophic and hydrogenotrophic pathways allows a better adaptation of these groups to fluctuant environmental conditions (De Vrieze et al., 2015; Garcia et al., 2000; Lee et al., 2018; Regueiro et al., 2015). Compared to the BM and SSM communities, the LTSSM archaeal community appeared very stable along the experiment being able to perform acetotrophic and hydrogenotrophic methanogenesis with the versatile *Methanosaeta* and the hydrogenotrophic *Methanospirillum*. However, the *Archaea* relative abundance estimated at about 7% by both NGS and qPCR on T0 diminished after 4 months to end up at about 4% or 2% by respectively NGS and qPCR. This observation corresponds to the CH_4 production observed on Fig. 2.

3.5.3. Searching for species adapted to low temperatures

In order to improve our understanding of the microbial community adaptation to low temperature we finally looked for the source of the closest relatives of the 17 most dominant OTUs underlined by the PCA (Fig. S1) and Figs. 6 and 7. These relatives were in majority coming from either anaerobic digestion processes or manure not related to low temperature (Table S2). Only the OTU V3 and V8 identified respectively as Candidatus *Cloacimonas* W5 and *Pseudomonas* had relatives from cold environments. The sequence GQ42386 related to OTU V3 was retrieved from a process treating sewage wastewater at 15 °C. The sequence KM870250 related to OTU V8 was obtained from the cold zone of a landfill leachate. However, these sequences had also relatives coming from mesophilic environments suggesting that our enrichment did not selected specific psychrophilic new species. The same was observed for the *Archaea*. Cluster 13 identified as *Methanosaeta* has relatives retrieved from thermophilic (KX063114), mesophilic (KP231448) and psychrophilic (AY570662) conditions. These observations suggest that the applied conditions did not select specific psychrophilic microbial species but rather induced the adaptation of ubiquitous species to a cold environment. However, this remains a hypothesis that we cannot confirm by simply sequencing the species 16S rDNA. A clear demonstration would require either a complete sequencing of the genomes and/or the isolation of the selected strains.

On the opposite, the very low abundance of the *Clostridia* DTU014 (OTU V6) in all digesters at 13 °C, while it dominated the DSM inoculum, may suggest that this Family is strongly disfavoured by psychrophilic conditions. Actually, close relatives of this OTU were observed previously in mesophilic (accession number EU219951 and MH147624) and thermophilic (acc. number EU638960) anaerobic digesters only.

4. Conclusion

The development of small-scale AD systems to treat livestock manure appears as an interesting solution to mitigate gaseous emissions and produce a local renewable energy. When it is mandatory to cover manure storage pits, it could be interesting for small farms to go for a simple, rustic and cheap device changing the pit in an AD reactor allowing a local recovery of biogas in a boiler producing heat for the

farm. Two limitations being the start-up of the AD system and the biogas production at low temperature, this study shows the ability of several manures to acclimate to temperature as low as 13 °C and produce up to 125 NL-CH_4/kg-COD_{added} within 4–8 months. The shortly stored swine slurry (with an average storage time of 2 months) gave the best result as inoculum, allowing a maximum methane production of 55%–68% of that obtained at 37 °C and showing a smooth adaptation of its microbial community to low temperature. Our results suggest that the psychrophilic AD system could be started with a shortly stored exogenous inoculum or an acclimated inoculum, which allows a faster start-up of the installation and a better methane production. A study at real (or pilot) scale should be done to calculate the cost and benefits of the existing technologies compared to the installation of a true AD system.

E-supplementary data of this work can be found in online version of the paper.

CRediT authorship contribution statement

Thomas Lendormi: Conceptualization, Field experiments, data generation, Supervision, Validation, Writing – review & editing, Funding acquisition. **Kaïs Jaziri:** Field experiments, data generation AD process, Writing – original draft. **Fabrice Béline:** Conceptualization, Formal analysis, Validation, Writing – review & editing, Funding acquisition. **Sophie Le Roux:** Field experiments, data generation Molecular biology, Formal analysis. **Chrystelle Bureau:** Data generation & analysis NGS. **Cédric Midoux:** Software, Formal analysis, bioinformatics NGS. **Suzelle Barrington:** Conceptualization, Writing – review & editing, Funding acquisition. **Patrick Dabert:** Project administration, Conceptualization, Supervision, Field experiments, data generation, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2022.130772.

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