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Acclimatised rumen culture for raw microalgae conversion into biogas: linking microbial community structure and operational parameters in Anaerobic Membrane Bioreactors (AnMBR)

Núria Zamorano-López[1*], *Luis Borrás*[1], *Juan B. Giménez*[1], *Aurora Seco*[1], *Daniel Aguado*[2]

[1]*CALAGUA – Unidad Mixta UV-UPV, Departament d'Enginyeria Química, Universitat de València, Avinguda de la Universitat s/n, 46100 Burjassot, Valencia, Spain. Contact: nuria.zamorano@uv.es, luis.borras-falomir@uv.es, juan.b.gimenez@uv.es, aurora.seco@uv.es.*
[2]*CALAGUA – Unidad Mixta UV-UPV, Institut Universitari d'Investigació d'Enginyeria de l'Aigua i Medi Ambient – IIAMA, Universitat Politècnica de Valencia, Camí de Vera s/n, 46022 Valencia, Spain. Contact: daaggar@hma.upv.es*

*corresponding author is Núria Zamorano-López (nuria.zamorano@uv.es)

Abstract

Ruminal fluid was inoculated in an Anaerobic Membrane Reactor (AnMBR) to produce biogas from raw *Scenedesmus*. This work explores the microbial ecology of the system during stable operation at different solids retention times (SRT). The 16S rRNA amplicon analysis revealed that the acclimatised community was mainly composed of *Anaerolineaceae*, *Spirochaetaceae*, *Lentimicrobiaceae* and *Cloacimonetes* fermentative and hydrolytic members. Overall, the dominance of *Fervidobacterium* and *Methanosaeta* was attributed to the highest biodegradability achieved in the AnMBR (62%). Different microbial community clusters were observed at different SRT conditions. Interestingly, syntrophic bacteria *Gelria* and *Smithella* were enhanced after increasing 2-fold the organic loading rate (OLR) suggesting their importance in continuous systems producing biogas from raw microalgae.

Keywords

anaerobic membrane bioreactor (AnMBR); biogas; microalgae; 16S rRNA gene; rumen

1. Introduction

Natural environments like the stomach cavity of the ruminant living beings are interesting sources of hydrolytic microorganisms (Weimer *et al.*, 2009). Nowadays, methodologies for high-throughput sequencing analysis of rumen (McGovern *et al.*, 2018) are allowing to elucidate the ruminal fluid composition of different sources (Li *et al.*, 2019; Trabi *et al.*, 2019). Application of these microbial communities to complex feedstock conversion into valuable products, including biogas, has attracted the interest of the scientific community since 1980s (Gijzen, 2002). On the basis of a circular economy perspective, microalgae can be included as a post-stage of anaerobic treatment of sewage, being later harvested and finally used as a substrate to produce biogas (González-Fernández *et al.*, 2015; Stiles *et al.*, 2018). Moreover, their carbon dioxide biofixation capacity from the atmosphere can reduce the carbon footprint of future water resource recovery facilities integrating microalgae processes (Seco *et al.*, 2018).

Some of the most common microalgae harvested from sewage and other water bodies have robust cell walls, like *Chlorophyta* belonging genera. Hemicelluloses and celluloses are needed to achieve high disruption values of recalcitrant microalgae (González-Fernández *et al.*, 2012; Mussgnug *et al.*, 2010). Cell walls from *Scenedesmus* (phylum *Chlorophyta*) also contain a recalcitrant and aliphatic compound, algaenan (Baudelet *et al.*, 2017), which difficult their disruption and further conversion into biogas. As a consequence, bioenergy production from microalgae via anaerobic digestion becomes challenging, with an intrinsic energetic and economic cost associated with their disruption (González-Fernández *et al.*, 2015). Several efficient physicochemical pretreatment strategies for microalgae biomass breakdown have been explored and evaluated during the last years (Passos *et al.*, 2015). However, they might not be feasible when operating at industrial scale and thus, alternative strategies with lower energetic demands need to be searched.

Biological strategies have been proposed for microalgae disruption, including commercial enzymatic mixtures, bioaugmentation with hydrolitic cultures and the use of natural hydrolytic consortia (Carrillo-Reyes *et al.*, 2016). Weimer *et al.* (2009) suggested the potential use of ruminal fluids to convert in a single bioengineered system, complex plant-based substrates into high value products like fatty acids or methane. Interestingly, Zhao *et al.* (2016) evaluated the efficiency of a batch system inoculated with cow rumen bacteria for microalgal disruption and found high rates of carboxylic acids production. In terms of biomethanization, another study determined a 58.0% efficiency when co-inoculating rumen from a slaughterhouse with anaerobic sludge to transform lignocellulosic substrates in methane (Deng *et al.*, 2018). Moreover, Barragan-Trinidad *et al.* (2017) reported a methane production of 193 mL $CH_4 \cdot gCOD^{-1}$ in a batch system inoculated with rumen and fed with *Scenedesmus* biomass. Several authors pointed out that adhesion capacity of ruminant-living microorganisms to the plant fibers is crucial for their disruption. Interstingly, high solids retention systems can potentially simulate this environment (Weimer *et al.*, 2009). Indeed, a high retention upflow anaerobic sludge blanket (UASB) has been reported for complex polysaccharide anaerobic digestion (Zhao *et al.*, 2016), as well as anaerobic sequencing batch reactors (Barnes and Keller, 2004) and microalgae digestion at high SRT in AnMBR (Greses *et al.*, 2017).

Some of the studies focused on the use of rumen in anaerobic digesters have partially explored the microbial community developed and determined the relevance of *Firmicutes, Bacteroidetes* or *Proteobacteria*, which are also among the common phyla of anaerobic digesters (McIlroy *et al.*, 2017). Recently, Deng and co-workers (2018) have evaluated a semi-continuous system co-inoculated with rumen and linked the presence of *Bacteroidales*, *Prevotellaceae* and *Rickenellaceae* to a 58% efficiency in terms of methane production. However, this yield decreased

after an overload of lignocellulosic material that disturbed the hydrogenotrophic methanogens population. Therefore, further research is needed prior to up-scale a process for biological disruption of microalgae using ruminant sources. Besides the organic loading rate (OLR) (Deng *et al.*, 2018) or the temperature (Gonzalez-Fernandez *et al.*, 2018), the effect over microbial population of essential operational parameters like solids or hydraulic retention times (SRT and HRT, respectively) remain poorly explored.

To the knowledge of the authors of the present manuscript, this is the first work revealing the 16S rRNA microbial community of a rumen AnMBR system and its associated long-term response to changes in operational conditions. This work has been performed for more than 14 months in a continuous AnMBR operated at high SRT and different OLR, feeding raw microalgae harvested from an outdoor photobioreactor pilot plant. Microbial ecology and bioengineering concepts are here combined to broad our knowledge on complex feedstock degradation through anaerobic digestion using membrane technology and natural hydrolytic communities *i.e.* a rumen inoculum.

2. Materials and methods.

2.1. Anaerobic system configuration and experimental design

Ruminal fluid extracted from a fistulated goat was used as an inoculum source for the anaerobic system. The extracted fluid was directly inoculated into the reactor after removal of coarse material through gauze straining. The volatile solids content in the ruminal fluid had a mass ratio of 0.75 volatile solids per total solids (VS/TS). The system worked at 39°C during more than 14 months since this temperature is close to the optimum for ruminal sources (Giménez *et al.*, 2017) and far from unfavorable conditions for mesophilic microorganisms.

The system was composed of two tanks with the same volume of 14 L (4 L of head-space volume). Tank 1 was used as the main tank of the system whereas Tank 2 was simply used as a

continuous biomass reservoir. This second tank was added to the system after 28 days of operation and it was neither considered for process performance nor to study any operational parameter. The digestate extracted from Tank 1 to control the SRT of the system was thus stored in Tank 2 to preserve a biomass that could be later used as a new inoculum or reintroduced in Tank 1 after a performance failure, for example. A detailed diagram of the anaerobic system can be found in Figure 1.

During period 1 (Figure 1a), the system consisted of two continuous stirred tank reactors (CSTR+CSTR). The influent was firstly degraded in the first CSTR (Tank 1) with an SRT ranging between 7 and 28 days. The digestate extracted from Tank 1 to maintain this SRT was stored in the second CSTR (Tank 2). This second tank was not considered for process performance analysis, as explained before. After 56 days of operation an external ultrafiltration hollow-fiber module was coupled to Tank 1 (transforming the CSTR+CSTR configuration into an AnMBR+CSTR system) to evaluate high SRT influence over the microbial community without increasing the HRT. The new AnMBR+CSTR configuration of the anaerobic system is shown in Figure 1b. During Period 2 the SRT was increased and studied between 70-100 days while maintaining an HRT of 30 days (the rest of the operational conditions remained the same, as can be seen in Table 1). Finally, to evaluate the effect of a higher microalgae load over the microbial population, the HRT was decreased to 15 days in Period 3 to increase 2-fold the OLR of the system. The rest of the operational conditions and the AnMBR+CSTR configuration were maintained.

2.2. Microalgae source

A photobioreactor pilot plant located in Valencia (Spain) was used as a source of microalgae biomass. This plant is used as a tertiary treatment of sewage in "Carraixet WWTP" (Valencia, Spain) (Viruela *et al.*, 2017). This plant is fed with the anaerobic effluent of an anaerobic

membrane bioreactor treating sewage, which is characterized by high nutrient concentration (Seco et al., 2018). The mixed phototrophic culture is dominated by *Scenedesmus* spp. (99% relative abundance), according to microscopic observations of the phytoplankton. These microalgae grow spontaneously in the plant conditions.

As detailed in Giménez *et al.* (2017), the algae were concentrated up to 6093 g $ST \cdot L^{-1}$ after being harvested within a crossflow ultrafiltration hollow-fiber (CFUHF) membrane unit (Koch Romicon 2″, 0.03 μm pore size). The resulting biomass was stored at 4ºC for no longer than two weeks and daily fed to the system according to the established OLR (Table 1).

2.3. Performance analysis: physicochemical analysis and biogas production

The system performance was evaluated using digestate and effluent samples to determine the concentration of TS, VS, total suspended solids (TSS), total and soluble COD (T-COD and S-COD respectively), sulphate ($S\text{-}SO_4$) and nutrients (ammonium as NH_4-N and phosphate as PO_4-P), according to the standard methods (APHA, 2012). Additionally, carbonate alkalinity and volatile fatty acids were determined following the titration method of the South African Water Research Commission (Moosbrugger *et al.*, 1993).

Biogas was continuously measured using a μflow® gas flow meter (Bioprocess Control, Sweden). The biogas from Tank 1 and Tank 2 headspaces was sampled three times per week. The methane content of the biogas was measured using a gas chromatograph fitted with a flame ionization detector (GC-FID, Agilent Technologies, USA). The gas sample (0.25 mL) was taken using a gas-tight syringe through a sampling point located on the top of each tank. The GC-FID was equipped with a TRACER column (Teknokroma) of 15 m x 0.53 mm x 1 μm dimensions and 40ºC temperature. Helium was chosen as the carrier gas with a flow rate of 5 $mL \cdot min^{-1}$. The

standards for methane quantification were prepared with high pure (99.99% purity) methane gas (Air Products Inc.).

2.4. Biomass collection and 16S rRNA gene sequencing

Digestate samples for microbial analysis were collected from the two main 14.0 L tanks conforming the system (Figure 1) after 0, 8, 42, 92, 106, 155, 169, 190, 339 and 435 days of operation (Table 1). Digestate pellets obtained after 10 minutes centrifugation at 5000 x g were stored at -20°C and later used for nucleic acid extraction within E.Z.N.A DNA Extraction Kit for Soil (Omega-Biotek, USA), according to the manufacturer's protocol. Nucleic acid from the reservoir at collection point 106 days was extracted in duplicate and included as a control of the nucleic acid isolation stage (sample 106-Rb). After fluorometric dsDNA quantification assay with Qubit 2.0 (Thermo Scientific, USA), Illumina amplicon libraries were generated using indexed primers that target the 16S rRNA gene (Takahashi *et al.*, 2014). The 2x300 bp run was performed within an Illumina MiSeq using v.3 chemistry (Illumina, USA) in FISABIO next-generation sequencing service (Valencia, Spain). Collected samples from Tank 1 (day 155) and reservoir Tank 2 (days 106 and 155) were used as a control of the sequencing stage. Nucleic acid from these three samples was sequenced in a different run, using the same conditions for library preparation, Illumina sequencing chemistry and machine. All sequences retrieved were deposited on the NCBI Sequence Reading Archive (SRA) database under bioproject number PRJNA434206 (accession numbers SAMN11567577-96).

2.5. Amplicon sequencing downstream analysis

A downstream high-quality sequencing data analysis based on the fastq-score of each read ($q \geq 30$ threshold) was applied to the sequences retrieved from the Illumina platform as previously described (Zamorano-López *et al.*, 2019). The resulting operational taxonomic units ($OTU_{0.97}$)

were generated in an open-reference clustering step at 3.0% dissimilarity. Taxonomic assignment was performed according to the 16S rRNA-based LTP 128 release of SILVA in QIIME. Phytoplankton related reads (Chloroplast and *Cyanobacteria*) were removed before downstream analysis since they are mainly related to the microalgae feedstock used in this study and might not be functional in an anaerobic system due to the absence of both oxygen and light. Besides, these reads are commonly associated to primer biases. As well, $OTU_{0.97}$ below 0.01% relative abundance percentages were excluded from analysis to reduce the background noise effect of rare reads.

The 16S rRNA gene analysis was performed over rarefied sequences to the minimum depth achieved (17,993-125,892 raw reads) to exclude the effect of differences in the sequencing depth per sample. The microbial community structure was evaluated first calculating the weighted unifrac distances between samples according to the observed species and later analyzing the distance matrix in a principal co-ordinate analysis (PCoA). The different community structures observed were statistically evaluated in an analysis of similarities test (ANOSIM). The link between operational parameters, digestate and feedstock physicochemical characteristics and microbial community dominant members (over 0.5% relative abundance) was performed through sparse partial least square analysis (sPLS). As a result, a relevance network and a pair-wise correlation heatmap were constructed using the retrieved sPLS regression model, showing the correlation between both biological and physicochemical data matrixes (González *et al.*, 2013).

3. Results and Discussion

3.1. Acclimatised biomass from a rumen inocula at high solids retention time improves raw *Scenedesmus* conversion into biogas

Figure 2 shows the relative abundance of the different phyla identified in the AnMBR along the complete experimental period. As can be seen in the figure, the potential for *Scenedesmus*

biomass conversion into biogas was mainly attributed to *Bacteroidetes*, *Chloroflexi*, *Cloacimonetes*, *Euryarchaeota*, *Firmicutes*, *Proteobacteria*, *Spirochaetae* and *Thermotogae* phyla. Most of these groups were not only observed during the first stages of the rumen system, but also remained during the whole experience. The enhance and persistence during the studied period of these groups, especially *Thermotogae* and *Chloroflexi*, might have helped the system to achieve the 62% biodegradability values of raw *Scenedesmus* (Table 2).

The modification of the treatment scheme from a CSTR+CSTR system operated at low solids retention time (7-28 days) in Period 1 to an AnMBR+CSTR system with higher solids retention time (70-100 days) in Period 2 shifted the 16S community composition profiles. Microbial groups with slower growth rates but high hydrolytic potential like *Thermotogae* were then enhanced and remained in the system while maintaining SRT at 100 days and an OLR of 0.2 $g \cdot L^{-1} \cdot d^{-1}$. Interestingly, the biodegradation potential increased in the system 2-fold as the SRT was increased through membrane operation from Period 1 to Period 2. Dominant phyla found during this period ranged as follows: 9.1-27.1% *Thermotoga*, 7.3-11.4% *Bacteroidetes*, 11.2-15.3% *Chloroflexi*, 2.7-13.5% *Cloacimonetes*, 3.1-9.0% *Firmicutes*, 7.3-13.6% *Proteobacteria* and 6.6-12.8% *Spirochaeta* (provided in E-supplementary data of online version). Besides, the higher detection of methanogens was observed after coupling the membrane tank to the system and increasing the SRT (Period 2). Under AnMBR+CSTR configuration the *Euryarchaeota* phylum (where the methanogens found here were classified) accounted for maximum relative abundance values of 5.0%. According to this result, high SRT (over 70 days) allows a good acclimation of the biomass boosting slow-growing microorganisms like potential hydrolyzers and methanogens in the system, establishing a more positive scenario for raw *Scenedesmus* conversion into biogas.

Natural hydrolytic consortia like the ruminal fluid can improve the hydrolysis efficiency of the first stages of anaerobic digestion, triggering the consequent stages and enhancing methane production (Barragán-Trinidad *et al.*, 2017). These authors demonstrated that ruminal fluid taken out from a cow enhanced a 29% the hydrolysis rate, resulting in a 193 mL $CH_4 \cdot gCOD^{-1}$ methane yield in a two-stage anaerobic digestion process. In the present work, the values obtained after rumen acclimation at high SRT accounted for 214 mL $CH_4 \cdot gCOD^{-1}$. This methane yield is very similar to the values reported by Mendez *et al.* (2014), who applied an enzymatic treatment stage to the *Scenedesmus* biomass prior to its anaerobic digestion. In the present work, 305 $mLCH_4 \cdot gVS^{-1}$ were produced from a robust microalga without pretreatments. This methane yield is higher than the ranging values between 127-258 $mLCH_4 \cdot gVS^{-1} \cdot L^{-1}$ summarized by Klassen *et al.* (2016) using untreated *Scenedesmus* biomass under mesophilic conditions. Only the study from Frigon *et al.* (2013) reached a higher value of 397 $mLCH_4 \cdot gVS^{-1}$. However, as pointed by the authors, previous freezing stage due to microalgae transportation could have enhanced the methane yield in the experiment. Hence, it is worth highlighting that the use of the ruminal fluid inoculum in the AnMBR to convert raw *Scenedesmus* in biogas avoids the associated economic cost to the pretreatment stage of the biomass. This strategy should be therefore considered for industrial systems.

3.2. Rumen inoculum role during the early stages of anaerobic digestion

The resulting biomass retained and enhanced in the early stages of the rumen inoculated bioreactor was mainly composed of *Leptospiraceae* (*Spirochaeta* phylum), *Planctomycetaceae* and *Pirellula* (*Planctomycetes*), *Synergistaceae* (*Synergistes*), *Gelria* (*Firmicutes*) and other uncultured members from *Bacteroidetes*; besides WS6 and WWE3 (Figure 3).

Little is known about WS6 and WWE3 phyla, recently proposed as *Candidate Dojkabacteria* and *Ca. Katanobacteria*, respectively. Their potential implication in hydrolytic pathways has been suggested using a metagenomic approach. Pandit *et al.* (2016) determined that both phyla contain encoding genes for degradation of chitin, xylose, cellobiose and hemicellulose. Some of these complex compounds are commonly found in *Scenedesmus* cell bodies (Baudelet *et al.*, 2017). In the present work, the relative abundance of WS6 and WWE3 groups were remarkable only between 0-8 days of operation (19.4% WS6 and 7.2% WWE3 maximum relative abundance values). However, both groups were washed out during the performance at higher SRT, which was increased from 28 days to 70-100 days. During Period 1, only a 32% biodegradability value was reached in the rumen inoculated system. This value is slightly higher than the 22-24% values reported by González-Fernández *et al.* (2015) under mesophilic conditions for raw *Scenedesmus*. However, this is a very low value that corresponds only to 110 mL $CH_4 \cdot gCOD^{-1}$ methane yield (see Period 1 in Table 2). Thus, *Scenedesmus* cell walls and organelles were poorly disrupted when operating between 7-28 days SRT and HRT. This was mainly attributed to the washed-out of the main hydrolytic potential groups like WS6 and WWE3.

The anaerobic digester environment differs from the ruminant cavities. Instead, several groups such as *Bacteroidetes*, *Proteobacteria* and *Firmicutes* trend to be dominant (McIlroy *et al.*, 2017). Despite the presence in the system of interesting groups for microalgae cell wall disruption, not all of them were selected yet they were replaced by others. Moreover, the composition of *Scenedesmus* cells is unique and complex due to the presence of algaenan (Baudelet *et al.*, 2017; Carrillo-Reyes *et al.*, 2016) and might have had a substrate-specific selective effect over rumen dominant microorganisms. Hence, a long-term operation for biomass acclimation to the

characteristics of *Scenedesmus* biomass was required to enhance the performance in terms of biodegradation and consequent energy recovery as biogas.

3.3. Key role of *Fervidobacterium* for *Scenedesmus* disruption at 39°C

After a first acclimation stage of the ruminal fluid in the reactor, microbial groups with potential affinity for *Scenedesmus* disruption were stabilized in the system. The coupling of the membrane tank allowed to increase the SRT up to 70 and 100 days (maintaining the HRT in 30 days). This operational change increased the biodegradability values observed from 32% to 62% in Period 2 (Table 2).

A remarkable change in the population was attributed to the relative abundance of *Thermotoga* phylum, that peaked during Period 2 and reached relative abundance values up to 26.8% in the system. The remarkable *Thermotoga* presence was attributed to one single $OTU_{0.97}$ closely related to a *Fervidobacterium* strain isolated from a full-scale digester located in Arizona, USA (SILVA accession number FJ769489.1.1476). *Fervidobacterium* genus has been found in a mining study for detection of genes and microbial taxa involved in complex biopolymer degradation, like hemicelluloses (Pandit *et al.*, 2016). Also, this genus has been related to a primary fermenting lifestyle, releasing acetate, hydrogen and carbon dioxide end-products (Wushke *et al.*, 2018). However, further research focused on proteomic and metabolomic analysis would be needed to explore the catabolic implications of *Fervidobacterium* during *Scenedesmus* cells decomposition in the present work.

To the current knowledge of the authors of this manuscript, no other studies have reported before the role of *Fervidobacterium* in a similar biological process for microalgae conversion into biogas. This could be related to the temperature fixed in this study (39°C), which differs to other similar studies that are closer to 35°C or 55°C when evaluating mesophilic or thermophilic

conditions, respectively (Gonzalez-Fernandez *et al.*, 2018; Klassen *et al.*, 2016). Nevertheless, the remarkable abundance of this group in the acclimatised rumen system suggests its potential role during raw microalgae anaerobic digestion.

3.4. *Anaerolineaceae, Spirochaetaceae, Lentimicrobiaceae* and *Cloacimonetes* members control raw *Scenedesmus* anaerobic digestion at high SRT

Together with *Fervidobacterium*, members of *Anaerolineaceae*, *Spirochaetaceae*, *Lentimicrobiaceae* and *Cloacimonetes* conformed a unique microbial community structure in the AnMBR+CSTR system operated at high SRT. *Anaerolineaceae* microorganisms have a fermentative metabolism and have been previously related to the degradation of microalgae biomass, including *Scenedesmus* in continuous anaerobic systems at mesophilic temperatures. Interestingly, *Anaerolineaceae* were also observed when degrading raw *Scenedesmus* with an acclimatised mesophilic sludge inoculum at SRT of 100 days reaching 40% relative abundance values (Greses *et al.*, 2017). Furthermore, Sanz *et al.* (2017) determined the dominance of this family (22.6-25.0%) in different CSTR treating a Chlorella biomass at SRT of 15 days . In the present study, an $OTU_{0.97}$ related to *Methanosaeta* was observed in the system ranging 0.4-3.5% relative abundances. These results suggest the relevance of methane producing pathways that are dependent to acetate-producing fermentative partners like *Anaerolineaceae* members. The fermentative metabolism of *Anaerolineaceae* was reported from genomic anotation, while their syntrophic interaction with methanogens like *Methanosaeta* was demonstrated through rRNA fluorescence-in situ hybridation by Mc. Ilroy *et al.* (2017). The authors observed that both *Methanosaeta* and *Anaerolineaceae* members are filamentous and tend to agreggate in anaerobic environments. This association might be enhanced in AnMBR as a result of the biofouling development in the membrane tank through cycle combination of filtration and backwashing.

Besides saccharolytic members of *Chloroflexi*, other uncultured groups related to *Spirochaetaceae* and *Lentimicrobiaceae* were observed. A recent study, focused on bioaugmentation with rumen-related microorganisms for lignocellulose degradation, highlights the potential role of *Spirochaetaceae* uncultured members for volatile fatty acid production in anaerobic digesters (Deng *et al.*, 2018). Values ranging 0.8-12.4% of an $OTU_{0.97}$ related to this family were observed during Period 2 in this work. Bacteroidetes members were mainly attributed to the *Lentimicrobiaceae* member (up to 9.9% presence), that encompasses uncultured bacteria able to degrade complex polysaccharides such as starch at high-loaded waste streams (Sun *et al.*, 2016). Finally, another dominant group related to an uncultured *Cloacimonetes* was found between 1.7-13.5 % relative abundance values. Members belonging to this group are widely extended in anaerobic digestion systems, according to a recent study of 20 mesophilic full-scale bioreactors (Calusinska *et al.*, 2018). Despite of the lack of further metabolic information, the evidences found in the present work suggest their important role for *Scenedesmus* degradation and their enhancement from rumen inoculum.

After inoculating the present anaerobic system with ruminal fluid, several microbial groups were retained and gradually enhanced as the SRT was being increased up to 100 days SRT, developing an efficient acclimatised biomass for raw *Scenedesmus* disruption. Gimenez *et al.* (2017) previously demonstrated the favorable effect of high solids retention over the biodegradability capacity of the system. Now, the microbial analysis here reported reveals the composition of the resulting AnMBR microbial community. The presence of microbial groups capable of perform the hydrolysis of complex polysaccharides as *Fervidobacterium*, *Anaerolineaceae*, *Lentimcirobiaceae*, *Spirochaetaceae* and *Cloacamonas* also supports the favorable effect of high SRT achieved in the AnMBR for boosting biomethanization of

microalgae. The configuration of the reactor should also be carefully considered, as biofouling of membrane systems promotes substantial changes in microbial communities and stimulate methanogenic-niche generation (Smith *et al.,* 2015).

Biofouling in AnMBR systems is still poorly understood from a microbial ecology perspective. However, the importance of direct interspecies electron transfer (Lovley, 2017) in these bioreactors should be considered when degrading microalgae. As pointed out by several authors, adhesion capacity of microorganisms to the plant fibers is crucial for their disruption (Yue *et al.*, 2013). Hence, biofouling in the AnMBR might have promoted aggregation between *Scenedesmus* cell-bodies and microbial groups with cellular attachment capacity like *Anaerolineaceae* (Xia *et al.*, 2016). In fact, this group was enhanced in the digester in Period 2 from 6.7% to 15.3% relative abundance. Finally, cellular adhesiveness might have facilitated the transference of metabolites between hydrolytic and primary fermenters to other groups involved in later stages of anaerobic digestion such as syntrophic-oxidizing bacteria and methanogens.

3.5. High solids retention time achieved in the AnMBR shaped the microbial community structure.

High SRT with a maximum of 100 days was achieved in the AnMBR in Periods 2 and 3. The effect of this important parameter over the rumen digester microbial community structure was evaluated through beta diversity ecological analysis (Figure 4).

The first two PCoA components explain the 78% of the variability between the rumen system samples analyzed. The system configuration significantly shaped the microbial community structure, as three different clusters were observed (ANOSIM statistic R 0.9762; $p<0.001$). The analysis of the biomass reservoir samples reveals the stability of the community structures observed in the three periods. As can be seen in the PCoA, samples taken from the reservoir show

the same community structure changes than those collected from the main tank among periods. Slight differences observed between these samples in Period 2 might be related to the higher retention times of this reservoir tank than the main tank since the membrane tank was not included.

Presumably, microbial population was shaped by the synergistic effects of biomass acclimation to microalgae composition and SRT over 70 days. Microorganisms selection when long-term degrading a specific substrate is thus an important parameter that shapes biogas producing microbial communities. However, other relevant parameters like the OLR have a secondary effect over these microbial structures as a different structure was observed in Period 3 despite maintaining the SRT at 100 days in the AnMBR+CSTR configuration. The absence of the key microorganism *Fervidobacterium* and the increase of key *Anaerolineaceae* and *Spirochaetaceae* members are the responsible for this structural change. As reported by Muñoz-Sierra *et al.* (2018), the use of AnMBR to adapt anaerobic biomass to specific and complex compounds promotes strengthened microbial structures and end up in process optimization. In fact, these community structures are robust over-time. This can also be concluded in the present study, as no diversity differences have been found between the samples taken from the pseudo-steady periods studied.

3.6. Linking microbial community and operational parameters during *Scenedesmus* biomethanization

The sPLS analysis allowed the elucidation of a relevance network based on the performance data retrieved from the system during the studied periods and the $OTU_{0.97}$ relative quantification (Figure 5).

The sPLS regression model was constructed using the first two components extracted (38.0% and 31.0% of explained variance). Similarity between the samples distribution based on sPLS and PCoA analysis highlights the importance of the community structure for the better performance of

the digester found during Period 2. A negative correlation between several groups and the COD removal variable reveals those members that were not selected for *Scenedesmus* conversion, *Ca. Dojkabacteria* and *Katanobacteria* among others. In contrast, a positive correlation is shown in the analysis between *Leptolinea* (phylum *Chloroflexi*, family *Anaerolineaceae*) and the methane yield determined during the experience. This parameter and the SRT have a close distance in the network analysis, showing the relationship between the favourable effect of high SRT and system performance in terms of methane production. Interestingly, a very high correlation was elucidated from the sPLS analysis between *Fervidobacterium*, a *Lentimicrobiaceae* member and HRT. Both $OTU_{0.97}$ were outcompeted and washout from the system when decreasing the HRT from 30 to 15 days and enhancing 2-fold the OLR. A progressive increase of the OLR could have mitigated the effect of a feedstock overload over these groups. On the other hand, the network analysis shows a positive correlation between *Smithella*, *Gelria* and *Methanolinea* and the OLR. Although correlation does not necessarily indicate causation, these results suggest the potential role of these groups during the system response to a *Scenedesmus* feedstock overload.

3.6.1. Dominance of acetoclastic methanogens during raw Scenedesmus biomethanization

After *Scenedesmus* hydrolytic disruption, released components are converted into methanogenic substrates such as hydrogen, carbon dioxide and fatty acids (mainly acetate). A fast dominance of the *Methanosarcinales* group was detected in the system, reaching relative abundance values up to 5.0% (E-supplementary data). Acetoclastic capacity for methane production is specifically attributed to different members of this group such as *Methanosarcina* and *Methanosaeta* (Schmidt *et al.*, 2016).

Methanosaeta, the dominant methanogen observed in this work, has been also identified as the main methane producer from acetate in similar studies degrading microalgae under mesophilic

conditions (Greses *et al.*, 2017; Klassen *et al.*, 2016; Zamalloa *et al.*, 2012). In this work, the biodegradability experimented a 2-fold increase from Period 1 to Period 2, suggesting that acetate released from the fermented *Scenedesmus* hydrolyzed compounds was quickly cleaved by *Methanosaeta* into methane and carbon dioxide. The upward trend of this $OTU_{0.97}$ seems to be positively correlated with the biomethanization enhance observed (E-supplementary data), until reaching its maximum value of 214 mL $CH_4 \cdot gCOD^{-1}$. Hence, the importance of the acetoclastic pathways for methane production can be suggested from the 16S rRNA gene sequencing findings in this work. Finally, this is in accordance with the findings from Venkiteshwaran *et al.* (2015), that reported most of the methane produced in high solids retention systems (such as the municipal full digesters) comes from acetate.

3.6.2. Syntrophic-microorganisms response against a feedstock overload

Syntrophic acetogens play an important role during anaerobic digestion as they can convert intermediate products such as butyrate, propionate, lactate and ethanol in methanogenic substrates i.e. acetate, hydrogen, carbon dioxide and methyl compounds (Leng *et al.*, 2018). In the present work, the acetoclastic pathway was the main methanogenic reaction suggested according to the dominance of *Methanosaeta*. However, after increasing the OLR during Period 3 lower values of this methanogen were observed (from 2.0% to values below 0.5%).

The methane yield obtained during Period 3 was lower than in Period 2 (177 vs 214 $mLCH4 \cdot gCOD^{-1}$ respectively). The lack of the potential acetate-producing bacteria found in this work, *Fervidobacterium*, resulted in a more complex microbial network for methane production. In this period up to 9.5% *Smithella* (δ-*Proteobacteria*) and 13.2% *Gelria* (*Firmicutes*) syntrophic bacteria were observed in the system. Both $OTU_{0.97}$ had been observed during the whole

experience, at relative abundance values below 5.0%. However, they showed up a fast response against the higher load of *Scenedesmus* fed to the AnMBR in Period 3 (OLR 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$).

Smithella is involved in the conversion of butyrate and propionate into acetate (Narihiro *et al.*, 2018). *Methanosaeta* could probably remain in the system as a result of the *Smithella* role in fatty acids transformation into acetate. The role of *Gelria* in anaerobic environments is less understood compared to *Smithella*. Up to date, no isotope-probing confirmation has been found that reveals its suggested metabolic implication. However, previous metaproteomic analysis proposed its role as a syntrophic hydrogen-producing bacteria during cellulose biomethanization (Lu *et al.*, 2014). Moreover, a recent transcriptomic study of municipal co-digesters also hypothesized its implication in syntrophic acetate oxidation of fatty acids. Although the biodegradability obtained in Period 3 did not reach the higher values previously found, the viability of continuous conversion of raw microalgae into biogas was still observed, accounting for 49% raw microalgae biodegradation.

4. Conclusions

High anaerobic biodegradability of raw *Scenedesmus* (62%) was reached using an acclimatised rumen inoculum. The importance of *Fervidobacterium* for microalgae disruption besides the release of intermediate products by *Anaerolineaceae*, *Spirochaetaceae*, *Lentimicrobiaceae* and *Cloacimonetes* was here highlighted. Acetoclastic *Methanosaeta* and syntrophic groups thrived in the system allowing a good flux of acetate conversion into methane (305 $mLCH_4 \cdot gVS^{-1}$). The stabilization of the microbial structure and its hydrolytic potential supports the use of membrane technology in anaerobic systems to overcome operational limitations and benefit from the favorable effect of high solids retention time during anaerobic digestion of complex substrates.

Appendix A. Supplementary data

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

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References

1. APHA, APHA/AWWA/WEF, 2012. Standard Methods for the Examination of Water and Wastewater. Stand. Methods 541. doi.org/ISBN 9780875532356
2. Barnes, S.P., Keller, J., 2004. Anaerobic rumen SBR for degradation of cellulosic material. Water Sci. Technol. 50, 305–311. doi.org/10.2166/wst.2004.0664
3. Barragán-Trinidad, M., Carrillo-reyes, J., Buitrón, G., 2017a. Hydrolysis of microalgal biomass using ruminal microorganisms as a pretreatment to increase methane recovery. Bioresour. Technol. 244, 100–107. doi.org/10.1016/j.biortech.2017.07.117
4. Baudelet, P.H., Ricochon, G., Linder, M., Muniglia, L., 2017. A new insight into cell walls of Chlorophyta. Algal Res. 25, 333–371. doi.org/10.1016/j.algal.2017.04.008
5. Calusinska, M., Goux, X., Fossépré, M., Muller, E.E.L., Wilmes, P., Delfosse, P., 2018. Biotechnology for Biofuels A year of monitoring 20 mesophilic full - scale bioreactors reveals the existence of stable but different core microbiomes in bio - waste and wastewater anaerobic

digestion systems. Biotechnol. Biofuels 1–19. doi.org/10.1186/s13068-018-1195-8

6. Carrillo-Reyes, J., Barragán-Trinidad, M., Buitrón, G., 2016. Biological pretreatments of microalgal biomass for gaseous biofuel production and the potential use of rumen microorganisms: A review. Algal Res. 18, 341–351. doi.org/10.1016/j.algal.2016.07.004
7. Deng, Y., Huang, Z., Ruan, W., Miao, H., Shi, W., Zhao, M., 2018a. Enriching ruminal polysaccharide-degrading consortia via co-inoculation with methanogenic sludge and microbial mechanisms of acidification across lignocellulose loading gradients. Appl. Microbiol. Biotechnol. 102, 3819–3830. doi.org/10.1007/s00253-018-8877-9
8. Frigon, J.C., Matteau-Lebrun, F., Hamani Abdou, R., McGinn, P.J., O'Leary, S.J.B., Guiot, S.R., 2013. Screening microalgae strains for their productivity in methane following anaerobic digestion. Appl. Energy 108, 100–107. doi.org/10.1016/j.apenergy.2013.02.051
9. Gijzen, H.J., 2002. Anaerobic Digestion for Sustainable Development. Water Sci. Technol. 45, 321–328. doi.org/10.2166/wst.2002.0364
10. Giménez, J.B., Bouzas, A., Seco, A., Aguado, D., Ferrer, J., Bouzas, A., Ferrer, J., Seco, A., 2017. Use of rumen microorganisms to boost the anaerobic biodegradability of microalgae. Algal Res. 24, 309–316. doi.org/10.1016/j.algal.2017.04.003
11. González-Fernández, C., Sialve, B., Bernet, N., Steyer, J.P., 2012. Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production. Biofuels, Bioprod. Biorefining. doi.org/10.1002/bbb.337
12. González-Fernández, C., Sialve, B., Molinuevo-Salces, B., 2015. Anaerobic digestion of microalgal biomass: challenges, opportunities and research needs. Bioresour. Technol. 198, 896–906. doi.org/10.1016/j.biortech.2015.09.095
13. Gonzalez-Fernandez, C., Vescovo, S.B., Godos, I. De, Fernandez, M., Zouhayr, A.,

Ballesteros, M., Gonzalez-Fernandez, C., Barreiro-Vescovo, S., de Godos, I., Fernandez, M., Zouhayr, A., Ballesteros, M., 2018. Biochemical methane potential of microalgae biomass using different microbial inocula. Biotechnol. Biofuels 11, 184. doi.org/10.1186/s13068-018-1188-7

14. González, I., Lê Cao, K.-A.A., Davis, M., Déjean, S., 2013. Insightful graphical outputs to explore relationships between two 'omics' data sets. BioData Min. 5, 19.

15. Greses, S., Gaby, J.C., Aguado, D., Ferrer, J., Seco, A., Horn, S.J., 2017. Microbial community characterization during anaerobic digestion of Scenedesmus spp. under mesophilic and thermophilic conditions. Algal Res. 27, 121–130. doi.org/10.1016/j.algal.2017.09.002

16. Klassen, V., Blifernez-klassen, O., Wobbe, L., Schlüter, A., Kruse, O., Mussgnug, J.H., 2016. Efficiency and biotechnological aspects of biogas production from microalgal substrates. J. Biotechnol. 234, 7–26. doi.org/10.1016/j.jbiotec.2016.07.015

17. Leng, L., Yang, P., Singh, S., Zhuang, H., Xu, L., Chen, W.H., Dolfing, J., Li, D., Zhang, Y., Zeng, H., Chu, W., Lee, P.H., 2018. A review on the bioenergetics of anaerobic microbial metabolism close to the thermodynamic limits and its implications for digestion applications. Bioresour. Technol. 247, 1095–1106. doi.org/10.1016/j.biortech.2017.09.103

18. Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., 2019. Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. Microbiome 1–17. doi.org/10.1186/s40168-019-0699-1

19. Lovley, D.R., 2017. Syntrophy Goes Electric: Direct Interspecies Electron Transfer. Annu. Rev. Microbiol. 71, annurev-micro-030117-020420. doi.org/10.1146/annurev-micro-030117-020420

20. Lu, F., Bize, A, Guillot, A, Monnet, V., Madigou, C., Chapleur, O., Mazeas, L., He, P., Bouchez, T., 2014. Metaproteomics of cellulose methanisation under thermophilic conditions reveals a surprisingly high proteolytic activity. Isme J 8, 88–102. doi.org/10.1038/ismej.2013.120

21. McGovern, E., Waters, S.M., Blackshields, G., McCabe, M.S., 2018. Evaluating established methods for Rumen 16S rRNA amplicon sequencing with mock microbial populations. Front. Microbiol. 9, 1–14. https://doi.org/10.3389/fmicb.2018.01365

22. McIlroy, S.J., Kirkegaard, R.H., Dueholm, M.S., Fernando, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. Culture-independent analyses reveal novel anaerolineaceae as abundant primary fermenters in anaerobic digesters treating waste activated sludge. Front. Microbiol. 8. doi.org/10.3389/fmicb.2017.01134

23. Mcilroy, S.J., Kirkegaard, R.H., McIlroy, B., Nierychlo, M., Kristensen, J.M., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. MiDAS 2.0: An ecosystem-specific taxonomy and online database for the organisms of wastewater treatment systems expanded for anaerobic digester groups. Database 2017, 1–9. doi.org/10.1093/database/bax016

24. Mendez, L., Mahdy, A., Ballesteros, M., González-Fernández, C., 2014. Methane production of thermally pretreated Chlorella vulgaris and Scenedesmus sp. biomass at increasing biomass loads. Appl. Energy 129, 238–242. doi.org/10.1016/j.apenergy.2014.04.110

25. Moosbrugger, R.E., Wentzel, M.C., Ekama, G.A., Marais, G. V, 1993. Alkalinity Measurement .2. a 4-Ph Point Titration Method To Determine the Carbonate Weak Acid-Base in Aqueous-Solutions Containing Other Weak Acid Bases of Known Concentrations. Water Sa 19, 23–28. doi.org/10.2166/wst.1993.0112

26. Muñoz Sierra, J.D., Oosterkamp, M.J., Wang, W., Spanjers, H., van Lier, J.B., 2018. Impact of long-term salinity exposure in anaerobic membrane bioreactors treating phenolic wastewater: Performance robustness and endured microbial community. Water Res. 141, 172–184. doi.org/10.1016/j.watres.2018.05.006

27. Mussgnug, J.H., Klassen, V., Schlüter, A., Kruse, O., 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. J. Biotechnol. 150, 51–56. doi.org/10.1016/j.jbiotec.2010.07.030

28. Narihiro, T., Nobu, M.K., Bocher, B.T.W., Mei, R., Liu, W.T., 2018. Co-occurrence network analysis reveals thermodynamics-driven microbial interactions in methanogenic bioreactors. Environ. Microbiol. Rep. 00. doi.org/10.1111/1758-2229.12689

29. Pandit, P.D., Gulhane, M.K., Khardenavis, A.A., Purohit, H.J., 2016. Mining of hemicellulose and lignin degrading genes from differentially enriched methane producing microbial community. Bioresour. Technol. 216, 923–930. doi.org/10.1016/j.biortech.2016.06.021

30. Passos, F., Gutiérrez, R., Brockmann, D., Steyer, J.P., García, J., Ferrer, I., 2015. Microalgae production in wastewater treatment systems, anaerobic digestion and modelling using ADM1. Algal Res. 10, 55–63. doi.org/10.1016/j.algal.2015.04.008

31. Sanz, J.L., Rojas, P., Morato, A., Mendez, L., Ballesteros, M., González-Fernández, C., 2017. Microbial communities of biomethanization digesters fed with raw and heat pre-treated microalgae biomasses. Chemosphere 168, 1013–1021. doi.org/10.1016/J.CHEMOSPHERE.2016.10.109

32. Schmidt, O., Hink, L., Horn, M.A., Drake, H.L., 2016. Peat: Home to novel syntrophic species that feed acetate- and hydrogen-scavenging methanogens. ISME J. 10, 1954–1966.

doi.org/10.1038/ismej.2015.256

33. Seco, A., Aparicio, S., González-Camejo, J., Jiménez-Benítez, A., Mateo. O., Mora, J.F., Noriega-Hevia, G., Sanchis-Perucho, P., Serna-García, R., Zamorano-López, N., Giménez, J.B., Ruiz-Martínez, A., Aguado, D., Barat, R., Borrás, L., Bouzas, A., Martí, N., Pachés, M., Ribes, J., Robles, A., Ruano, M. V., Serralta, J., Ferrer, J., 2018. Resource recovery from sulphate-rich sewage through an innovative anaerobic-based water resource recovery facility (WRRF). Water Sci. Technol. 78, 1925–1936. doi.org/10.2166/wst.2018.492

34. Smith, A.L., Skerlos, S.J., Raskin, L., 2015. Membrane biofilm development improves COD removal in anaerobic membrane bioreactor wastewater treatment. Microb. Biotechnol. 8, 883–894. doi.org/10.1111/1751-7915.12311

35. Stiles, W.A. V, Styles, D., Chapman, S.P., Esteves, S., Bywater, A., Melville, L., Silkina, A., Lupatsch, I., Fuentes, C., Lovitt, R., Chaloner, T., Bull, A., Morris, C., Llewellyn, C.A., 2018. Using microalgae in the circular economy to valorise anaerobic digestate: challenges and opportunities. Bioresour. Technol. 267, 732–742. doi.org/10.1016/j.biortech.2018.07.100

36. Sun, L., Toyonaga, M., Ohashi, A., Tourlousse, D.M., Matsuura, N., Meng, X.Y., Tamaki, H., Hanada, S., Cruz, R., Yamaguchi, T., Sekiguchi, Y., 2016. Lentimicrobium saccharophilum gen. nov., sp. nov., a strictly anaerobic bacterium representing a new family in the phylum bacteroidetes, and proposal of lentimicrobiaceae fam. nov. Int. J. Syst. Evol. Microbiol. 66, 2635–2642. doi.org/10.1099/ijsem.0.001103

37. Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., Nishijima, M., 2014. Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing. PLoS One 9. doi.org/10.1371/journal.pone.0105592

38. Trabi, E., Seddik, H. Eldin, Xie, F., Lin, L., Mao, S., 2019. Comparison of the rumen

bacterial community, rumen fermentation and growth performance of fattening lambs fed low-grain, pelleted or non-pelleted high grain total mixed ration. Anim. Feed Sci. Technol. 253, 1–12. https://doi.org/10.1016/j.anifeedsci.2019.05.001

39. Venkiteshwaran, K., Bocher, B., Maki, J., Zitomer, D., 2015. Relating Anaerobic Digestion Microbial Community and Process Function. Microbiol. Insights 8, 37–44. doi.org/10.4137/MBI.S33593

40. Viruela, A., Robles, Á., Ruano, M.V., Ferrer, J., Barat, R., Durán, F., Seco, A., Viruela, A., 2017. Performance of an outdoor membrane photobioreactor for resource recovery from anaerobically treated sewage. J. Clean. Prod. 178, 665–674. doi.org/10.1016/j.jclepro.2017.12.223

41. Weimer, P.J., Russell, J.B., Muck, R.E., 2009. Lessons from the cow: What the ruminant animal can teach us about consolidated bioprocessing of cellulosic biomass. Bioresour. Technol. 100, 5323–5331. doi.org/10.1016/j.biortech.2009.04.075

42. Wushke, S., Fristensky, B., Zhang, X.L., Spicer, V., Krokhin, O. V., Levin, D.B., Stott, M.B., Sparling, R., 2018. A metabolic and genomic assessment of sugar fermentation profiles of the thermophilic Thermotogales, Fervidobacterium pennivorans. Extremophiles 22, 965–974. doi.org/10.1007/s00792-018-1053-4

43. Xia, Y., Wang, Y., Wang, Y., Chin, F.Y.L., Zhang, T., 2016. Cellular adhesiveness and cellulolytic capacity in Anaerolineae revealed by omics-based genome interpretation. Biotechnol. Biofuels 9, 111. doi.org/10.1186/s13068-016-0524-z

44. Yue, Z.B., Li, W.W., Yu, H.Q., 2013. Application of rumen microorganisms for anaerobic bioconversion of lignocellulosic biomass. Bioresour. Technol. 128, 738–744. doi.org/10.1016/j.biortech.2012.11.073

45. Zamalloa, C., De Vrieze, J., Boon, N., Verstraete, W., 2012. Anaerobic digestibility of marine microalgae Phaeodactylum tricornutum in a lab-scale anaerobic membrane bioreactor. Appl. Microbiol. Biotechnol. 93, 859–869. doi.org/10.1007/s00253-011-3624-5

46. Zamorano-López, N., Greses, S., Aguado, D., Seco, A., Borrás, L., 2019. Thermophilic anaerobic conversion of raw microalgae: Microbial community diversity in high solids retention systems. Algal Res. 41, 101533. https://doi.org/10.1016/j.algal.2019.101533

47. Zhao, B., Liu, J., Frear, C., Holtzapple, M., Chen, S., 2016. Consolidated bioprocessing of microalgal biomass to carboxylates by a mixed culture of cow rumen bacteria using anaerobic sequencing batch reactor (ASBR). Bioresour. Technol. 222, 517–522. doi.org/10.1016/j.biortech.2016.09.12

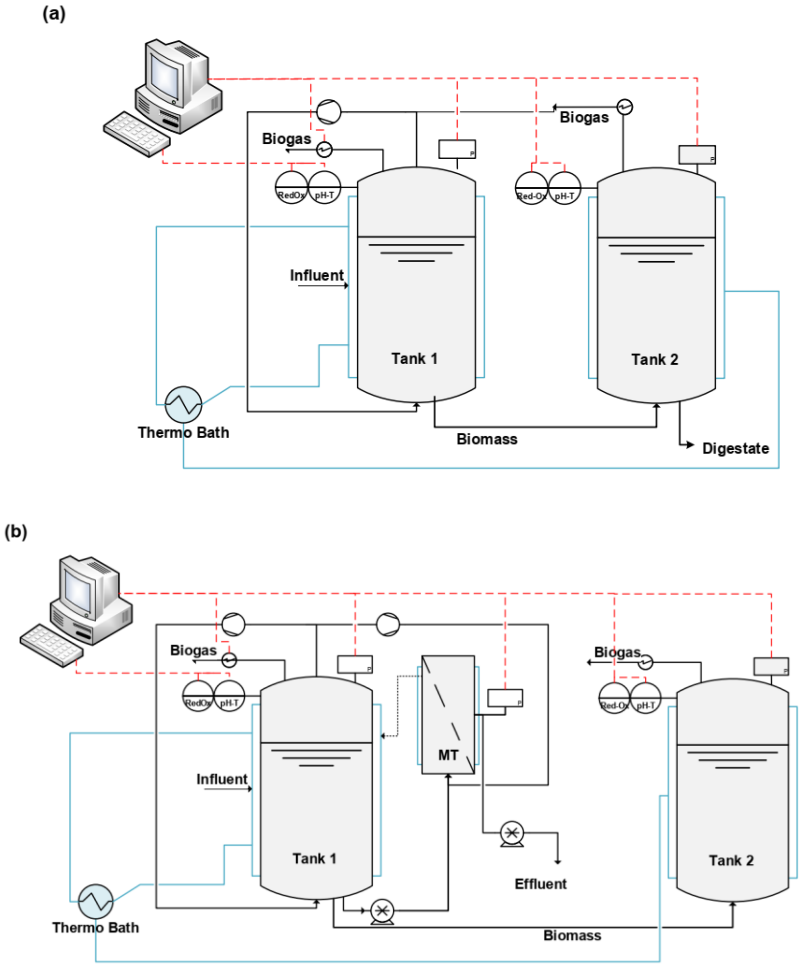

Figure 1. Anaerobic system layout: (a) CSTR+CSTR and (b) AnMBR+CSTR configuration. In figure b the system is composed of a main tank (Tank 1) and a coupled membrane tank (MT) (AnMBR) plus the reservoir (Tank 2, CSTR).

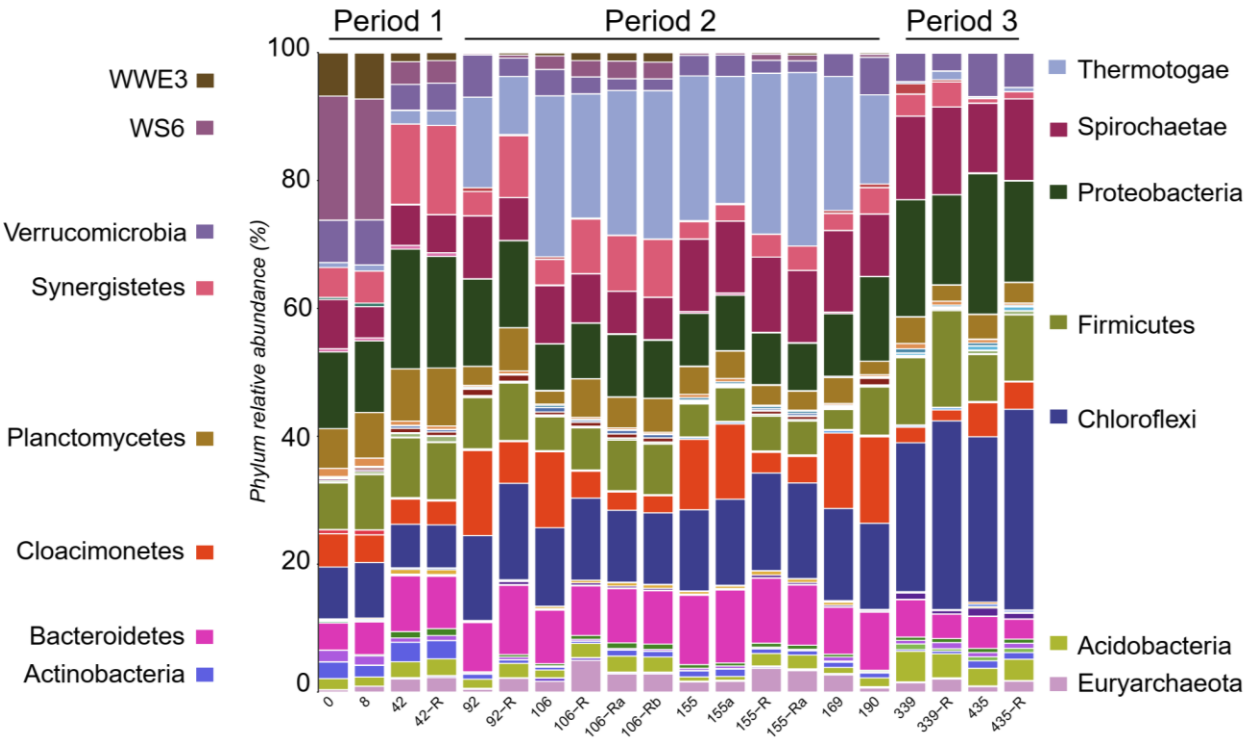

Figure 2. Relative abundances of the different phyla identified in the AnMBR. Samples collected from the biomass reservoir are indicated as "-R". Samples collected after 106 and 155 days were duplicated to be used as control between different Illumina runs (labels 106-Ra, 155a and 155-Ra). Reservoir sample collected after 106 days was extracted twice and included as a control of the nucleic acid isolation, library preparation and 16S rRNA sequencing (label 106-Rb).

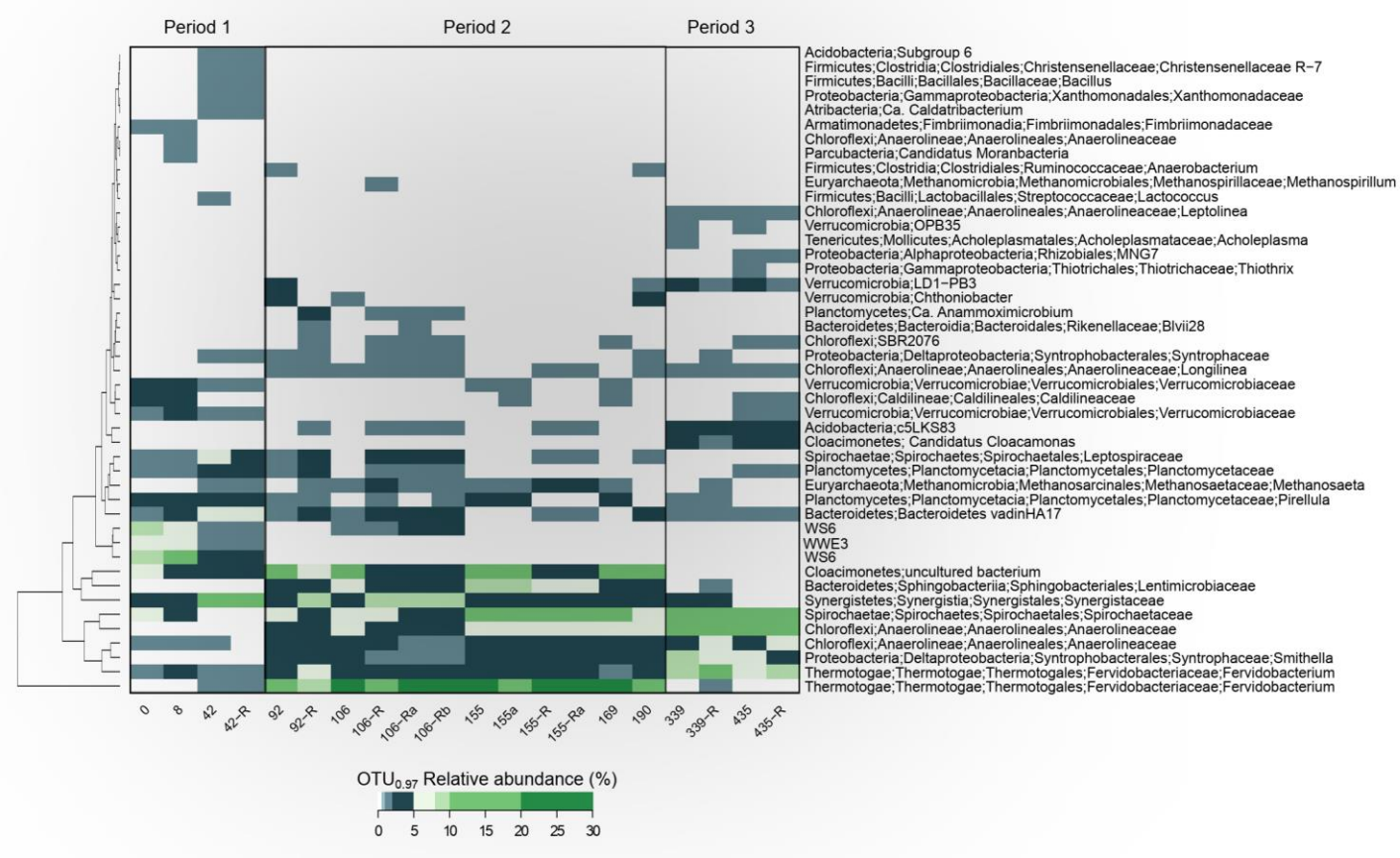

Figure 3. Relative abundance of the main $OTU_{0.97}$ identified during performance of the rumen-inoculated system. A blue palette has been used to differentiate minor groups (0.5-5.0% relative abundances) from dominant $OTU_{0.97}$ which are represented in greens (5.0-30.0%). Sample label indicates the collection day according to the continuous performance and samples taken from the reservoir are indicated as -R. Left-side cluster indicates similar patterns of relative abundances. On the right side appears the corresponding taxonomy from phyla to the minimum taxonomic level assigned to each $OTU_{0.97}$.

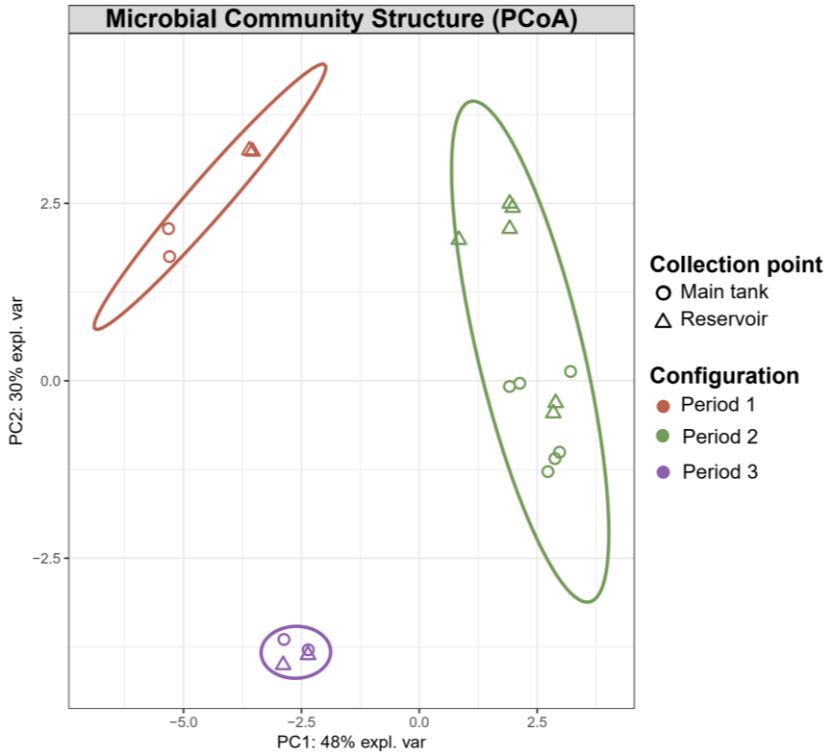

Figure 4. Principal Co-ordinate Analysis (PCoA) ordination plot of the weighted unifrac distances observed between microbial community members of the rumen system. The first two components plotted explain 78% of the variability among samples. Collection points are differentiated using circles (Tank 1, main tank) and triangles (Tank 2, reservoir tank). Ellipses show 0.95 confidence areas estimated through a multivariate t-distribution of the data (ANOSIM statistic R: 0.9762; Significance: $p<0.001$).

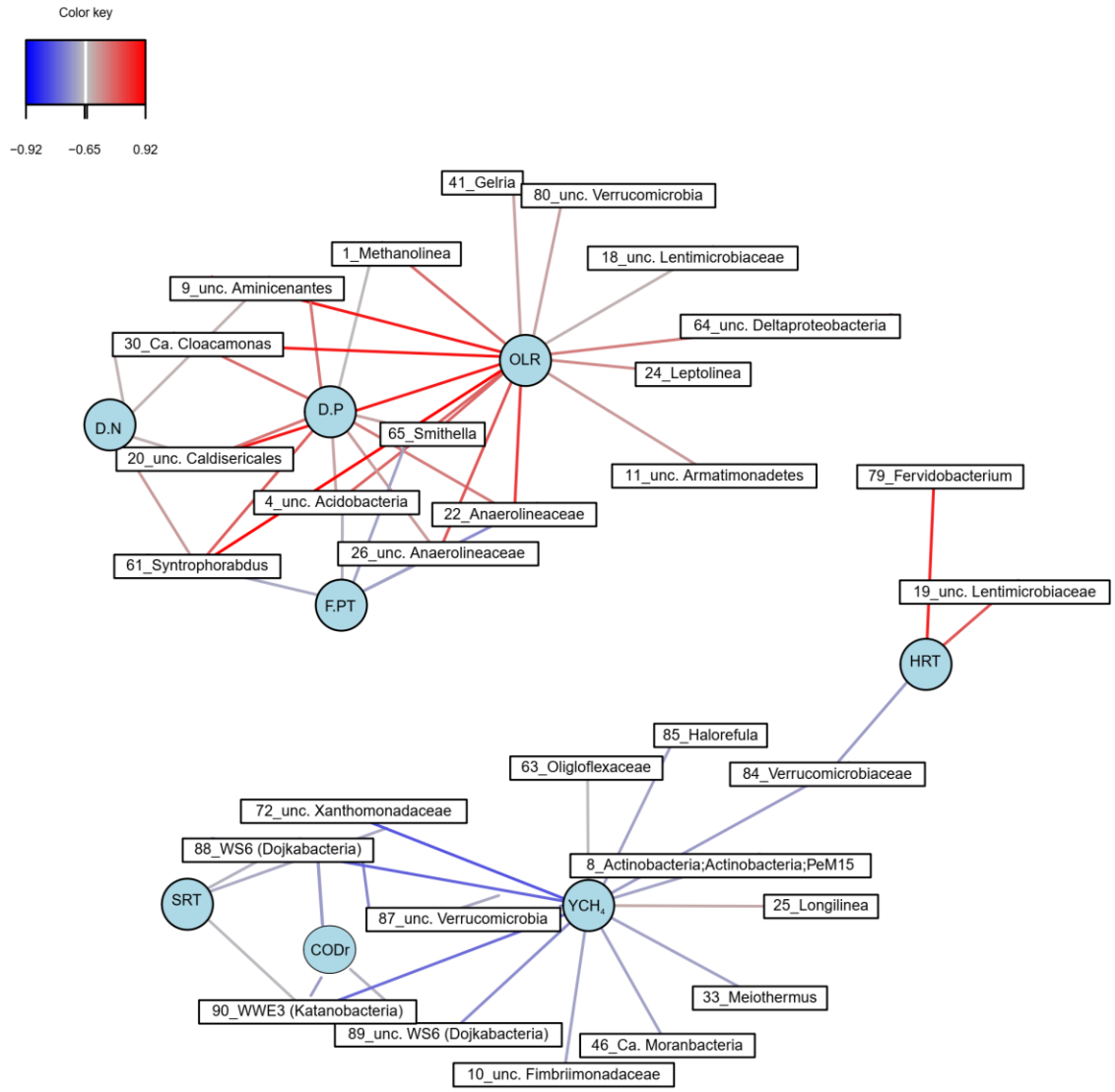

Figure 5. Relevance network from sPLS analysis. Threshold value is 0.65. Positive and negative correlation is shown through red and blue lines between nodes, respectively. The higher intensity of these colors, the higher correlation value. Circle-nodes are significant operational parameters like organic loading rate (OLR), hydraulic retention time (HRT), solids retention time (SRT); or quantitative measurements of Total Nitrogen and Phosphorous from digestate (D.N and D.T) and feedstock (F.PT), methane yield (YCH_4), and COD removal (CODr). Boxes contain the significant $OTU_{0.97}$ selected by the sPLS regression model.

Table 1. Operational conditions of the rumen inoculated bioreactor.

	Period 1	**Period 2**	**Period 3**
Reactor configuration	CSTR+CSTR	AnMBR+CSTR	AnMBR+CSTR
OLR (g COD·L^{-1}·d^{-1})	0.2	0.2	0.4
HRT (d)	7-28	30	15
SRT (d)	7-28	70-100	100
Duration (d)	56	149	231
Biomass collection days (d)	0, 8, 42	92, 106, 155, 169, 190	339, 435

Table 2. Performance mean and standard deviation values of the rumen inoculated system.

		Period 1	**Period 2**	**Period 3**
Biodegradability*	%	32±4	62±4	49±3
COD removal	%	36.1±8.8	70.1±10.7	57.2±1.4
Methane Yield	$mLCH_4 \cdot gCOD^{-1}$	110±24	214±15	177±11
Methane Yield	$mLCH_4 \cdot gVS^{-1}$	185±45	360±52	305±16

*Calculated based on by-product COD over total influent COD, as detailed in Giménez *et al.*, 2017