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***Methanosarcina* plays a main role during methanogenesis of high-solids food waste and cardboard**

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Abstract

Anaerobic digestion of food waste is a complex process often hindered by high concentrations of volatile fatty acids and ammonia. Methanogenic archaea are more sensitive to these inhibitors than bacteria and thus the structure of their community is critical to avoid reactor acidification. In this study, the performances of three different inocula were compared using batch digestion tests of food waste and cardboard mixtures. Particular attention was paid to the archaeal communities in the inocula and after digestion. While the tests started with inocula rich in *Methanosarcina* led to efficient methane production, VFAs accumulated in the reactors where inocula initially were poor in this archaea and no methane was produced. In addition, higher substrate loads were tolerated when greater proportions of *Methanosarcina* were initially present in the inoculum. Independently of the inoculum origin, *Methanosarcina* were the dominant methanogens in the digestates from the experiments that efficiently produced methane. These results suggest that the initial archaeal composition of the inoculum is crucial during reactor start-up to achieve stable anaerobic digestion at high concentrations of ammonia and organic acids.

Keywords

Biogas; solid-state anaerobic digestion; methanogenesis; free ammonia

1. Introduction

Novel technologies for treatment and valorization of the organic fraction of municipal solid waste (OFMSW) must be developed to deal with an increasing production and new international regulations. Anaerobic digestion (AD) is a well-known process used for efficient treatment of organic waste with high total solids (TS) contents ($\geq 20\%$), converting them into biogas and digestate, both added-value end-products. However, AD of highly biodegradable substrates such as food waste (FW), which is a major component of OFMSW, is often associated with accumulation of volatile fatty acids (VFAs), which are detrimental to the AD process. In addition, FW is rich in organic nitrogen, which is reduced to ammonia during AD, leading to high concentrations of total ammonia nitrogen (sum of NH_3 and NH_4^+ ; TAN) in the digesters (L. Zhang et al., 2012). Accumulation of both VFA and/or TAN might lower the methane yields and can even lead to failure of the AD process (Banks et al., 2008). The reactors are particularly vulnerable to these inhibitions during the start-up period (Fernández et al., 2001). This occurs because the microbial communities are not adapted to the stressful conditions imposed by the substrates and the operational parameters (*i.e.* high organic loading rates). Therefore, to achieve efficient methane yields and productivities with FW as substrate, it is crucial to have well-adapted microbial communities in the digesters, which are resistant to high VFA and free ammonia nitrogen (NH_3 ; FAN) concentrations. Methanogenic archaea are generally more sensitive to inhibitors than bacteria and thus methanogenesis is usually the first process affected by common inhibitors, such as FAN or VFAs (De Vrieze et al., 2012). Nonetheless, not all methanogenic archaea have the same

resistance to these inhibitors and thus the composition of the archaeal microbial community varies according to the operating conditions (Abbassi-Guendouz et al., 2013). Due to their high substrate affinity, acetotrophs such as *Methanosaeta* are generally predominant under unstressed conditions and thus acetotrophic methanogenesis is the predominant pathway for methane production. On the other hand, under stressful AD conditions, these methanogens are preferentially inhibited and mixotrophic microorganisms (*i.e.* able to consume acetate and hydrogen to produce methane), such as *Methanosarcina* which are more resistant to inhibitors (*i.e.* FAN or VFAs), become predominant (De Vrieze et al., 2012; Venkiteshwaran et al., 2016). In fact, while *Methanosaeta* cannot grow at TAN concentrations greater than $3 \text{ g}\cdot\text{L}^{-1}$, *Methanosarcina* have been found at much higher TAN concentrations (De Vrieze et al., 2012; Poirier et al., 2016). As an illustration, Capson-Tojo et al. (2017a) found *Methanosarcina* to be the dominant methanogens at TAN concentrations up to $3.7 \text{ g}\cdot\text{L}^{-1}$ ($795 \text{ mg FAN}\cdot\text{L}^{-1}$) using FW as substrate in AD batch tests.

Over the past years, the importance of the microbial communities for efficient AD processes has gained attention and many studies have been carried out to further understand the structures of the communities of both bacteria and archaea in AD reactors. In a recent study carried out by Zhang et al. (2016) with sewage sludge and FW as substrates (with final NH_4^+ concentrations up to $2.01 \text{ g}\cdot\text{L}^{-1}$), it was observed that *Methanosaeta* were the main archaea at the beginning of the batch experiment (71 % of the operational taxonomical units; OTUs).

Afterwards, *Methanosarcina* grew during acid production (with transient VFA concentrations up to $24 \text{ g}\cdot\text{L}^{-1}$) and overpassed in abundance *Methanosaeta* because of their greater resistance to VFA and TAN inhibition. Finally, other hydrogenotrophic methanogens (*i.e.* *Methanoculleus*) grew once acetate was totally consumed. Using a high solid-state AD box-type container fed with FW at high TS contents (from 34.4 to 44.5 %) and TAN concentrations ($2.5 \text{ g}\cdot\text{L}^{-1}$), Walter et al. (2016) observed that *Methanosarcina* were the

dominant species accompanied by different hydrogenotrophs (*i.e. Methanobacterium*,
Methanoculleus and *Methanocorpusculum*). Consistently, Zamanzadeh et al. (2016) found
Methanosaeta as the main archaea in mesophilic continuous AD of FW at low concentrations
of FAN ($\leq 200 \text{ mg}\cdot\text{L}^{-1}$). This further supports that the concentration of TAN-FAN is a key
factor that can result in shifts of the archaeal populations. In a recent batch study, Poirier et al.
(2016) identified the key microbial phylotypes resisting to extreme ammonia concentrations
(up to $50 \text{ g TAN}\cdot\text{L}^{-1}$). They achieved high methane yields at TAN concentrations as high as
 $25 \text{ g TAN}\cdot\text{L}^{-1}$, with *Methanosarcina* and *Methanoculleus* as main methanogens and with
relative abundances of *Methanosaeta* lower than 5 % in all AD reactors.

The objective of this study was to evaluate, for the first time, the AD performance of three
microbial inocula from different origins and with different initial archaeal compositions using
FW and cardboard (CB) as substrates. These wastes are the main components of OFMSW
(Kim and Oh, 2011; Y. Zhang et al., 2012) and are generally collected at the same facilities,
and thus their co-digestion is facilitated. Also, they constitute a good waste model substrate,
since the initial proportions of carbon and nitrogen could be easily adjusted. Batch tests were
performed at different substrate loads, TS contents ($\geq 20 \%$) and co-digestion proportions.
Special attention was paid to the archaeal communities and to the FAN and VFA levels.

2. Materials and methods

2.1. Substrate and microbial inoculum

A synthetic FW was prepared according to the VALORGAS report (VALORGAS, 2010). It
was composed of fruits and vegetables (80.7 %), meat (8.2 %), pasta (4.8 %), bread (6.2 %),
dairy products (1.9 %) and biscuits (1.9 %). Its precise composition has been detailed
elsewhere (Capson-Tojo et al., 2017a). Being FW and CB the most common components of
OFMSW, CB (branded “Cartonnages Michel” and shredded to less than 1 mm) was added as

co-substrate to simulate this waste (Hogg et al., 2002), increasing at the same time the C/N ratio of the substrate and thus diluting the TAN concentrations in the reactors and favoring the AD process (Capson-Tojo et al., 2017a). Three different inocula from industrial plants were used: mixture of a centrifuged granular sludge issued from a mesophilic industrial UASB reactor treating sugar factory effluents with a dried digestate. This digestate was used to increase the TS content of the inoculum and was sampled in a thermophilic industrial plant treating OFMSW (Inoc-UASB1); a mixture of sludge and dried digestate issued from the same sources than Inoc-UASB1 but sampled at a different moment (Inoc-UASB2); a sludge issued from an AD industrial plant treating a mixture of different organic waste streams at 35 °C mixed with dried compost (99 % TS; 81 % VS) to increase the TS content of the inoculum (Inoc-OW). The amounts of dried digestate and compost added were 0.5 g per g of inoculum (w/w) (Inoc-UASB1 and Inoc-UASB2) and 0.17-0.34 g per g of inoculum (w/w) (Inoc-OW) respectively, depending on the desired TS content and the initial water content of the sludge.

2.2. Dry batch anaerobic co-digestion tests

Different co-digestion ratios ($4-1 \text{ g TS FW} \cdot \text{g TS CB}^{-1}$), initial TS contents (20-35 %) and substrate to inoculum (S/X) ratios ($0.25-1.00 \text{ g VS} \cdot \text{g VS}^{-1}$) were tested. These values were selected according to previous results and to data gathered from the literature (Capson-Tojo et al., 2016, 2017a). Table 1 summarizes the 10 different experimental conditions that were considered in this study. Each tested condition was run in triplicate. This experimental set-up allowed to produce results which primarily depended on the inoculum source, while evaluating at the same time different initial conditions (*i.e.* S/X and co-digestion ratios and initial TS contents). Therefore, the obtained results were not dependent on the particular operational conditions applied, but only on the type of inoculum used. With this set-up the performance of each reactor was also totally independent between them.

After adding the required volumes of sludge into the flasks, the corresponding amounts of

substrates (according to Table 1) were supplemented. Finally, the TS contents were adjusted adding water and the flasks were flushed with nitrogen and sealed. As aforementioned, to allow working at the high TS contents desired, the inocula used were mixed with dried digestates (Inoc-UASB1 and Inoc-UASB2) and compost (Inoc-OW). Different blank reactors were carried out to account for the biogas production that could have been produced by the degradation of these materials (Capson-Tojo et al., 2017a, 2017b, 2017c). In addition, both materials were dried at 100 °C for over 24 h to ensure that the impact of the microorganisms present in these media on the methane production was negligible. The working volumes were different according to the operational conditions and the reactor size, varying from 0.4 L to 0.7 L. The duration of the batch experiments was variable (56-98 days). In the systems producing methane, the batch experiments were stopped when a plateau in the biomethane production was observed. On the other hand, longer batch periods were applied when acidification occurred (to ensure that the acid accumulation was irreversible). All the reactors were incubated at 37 °C.

2.3. Analytical methods

2.3.1. Physicochemical characterization of the substrates

The TS and Volatile Solids (VS) contents were determined according to the Standard Methods (APHA, 2005). The protein and carbohydrate concentrations were measured by the modified Lowry method (Frølund et al., 1996) and the Dubois method (Dubois et al., 1956), respectively. The lipid content was determined using a gravimetric method (APHA, 2005), the pH was measured with a WTW pHmeter series inoLab pH720, total Kjeldahl nitrogen (TKN) and TAN contents were determined with an AutoKjeldahl Unit K-370, BUCHI and the total organic carbon (TOC) with a Shimadzu TOC-V_{CSN} Total Organic Carbon Analyzer. A more precise description of the analytical methods can be found in Capson-Tojo et al., (2017a). The biochemical methane potentials (BMPs) of the substrates were determined according to Motte

et al. (2014). The C/N ratio was calculated as TOC divided by TKN. The FAN concentrations were calculated according to Chen et al. (2014) as a function of temperature, pH, and concentration of TAN.

2.3.2. *Gas quantification and analysis*

The total biogas volume was periodically determined by measuring the pressure in the reactor headspace and the gas composition was analyzed by gas chromatography coupled to a catharometer detector, as detailed in Cazier et al. (2015). The methane yields were calculated by dividing the total volume of methane by the amount of VS initially added as substrate.

2.3.3. *Analysis of metabolites and final products of the digestion*

The concentrations of VFAs and ionic species after digestion were measured by gas chromatography and high-performance liquid chromatography, according to Motte et al. (2013). The reactors used in the experiments carried out using the Inoc-OW and Inoc-UASB1 allowed sampling of the digestate during the digestion and therefore, the kinetics of production-consumption of metabolites were also analyzed. The sampling device is described in Capson-Tojo et al. (2017b).

2.4. *Microbial community analysis*

The microbial communities of the inocula and the digestates were characterized by 16S rRNA sequencing. One mL of each sample was first taken and stored at -20 °C until analysis. The DNA from the sample (around 1 g) was extracted using a Fast DNA SPIN kit for soil in accordance with the instructions of the manufacturer (MP Biomedicals). The quality and quantity of the extracted DNA were verified by spectrophotometry using an Infinite 200 PRO NanoQuant (Tecan Group Ltd., Männedorf, Switzerland). The primer pairs 515-532U and 909-928U and their respective linkers were used to amplify the V4-V5 regions of the 16S rRNA genes (over 30 amplification cycles were applied at an annealing temperature of 65 °C). These primer pairs target both bacterial and archaeal 16S rRNA genes, capturing most of their

diversity (Wang and Qian, 2009). The PCR mixtures had a total volume of 50 μ L, containing: 0.5 units of Pfu Turbo DNA polymerase (Stratagene), the corresponding buffer, each deoxynucleotide at 200 mM, each primer at 0.5 mM and 10 ng of genomic DNA. The following PCR sequence was carried out (using a Mastercycler thermal cycler; Eppendorf): after 94 °C for two min, 35 cycles of 94 °C for one min, 65 °C for one min, and 72 °C for one min were applied, with a final extension at 72 °C for 10 min. The obtained products were purified and analyzed using the Illumina MiSeq cartridge (v3 chemistry) for sequencing of paired 300 bp reads at the GenoToul platform (<http://www.genotoul.fr>). Mothur (version 1.35.0) was used for sequence assembling, cleaning and alignment and for assignation of the taxonomic affiliation, as described in Venkiteshwaran et al. (2016).

2.5. Statistical analysis

To analyze potential relationships between variables (*i.e.* methane yields, *Methanosarcina* proportions in the inocula and the digestates, final VFA concentrations, final pH values, initial TS contents and initial S/X and C/N ratios), a principal component analysis (PCA) was performed. The PCA was carried out using the package mixOmics in the software R (version 3.2.5; The R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

3.1. Physicochemical characterization of substrates and inocula

The physico-chemical characteristics of the substrates and the inocula are presented in Table 2. The observed composition of the FW was similar to those found in the literature (Capson-Tojo et al., 2016), with TS contents of 21.6 % and VS/TS of 96.2 %. In agreement with previously reported results, the FW was mainly composed of carbohydrates and had a relatively low C/N ratio, far away from the optimum values of 25 reported in the literature (Mao et al., 2015). The high BMP value of the FW (498 mL CH₄·g VS⁻¹) highlights its great

potential for valorization by AD. In contrast, CB had a high C/N ratio, suggesting that CB can be effectively used as co-substrate for diluting the TAN from FW organic nitrogen. A more extensive characterization of both substrates can be found in Capson-Tojo et al., (2017a). Inoc-UASB1 and Inoc-UASB2 had very similar physico-chemical characteristics, with high TS (70.8 and 74.2 %) and low TAN concentrations ($1.49\text{--}1.50\text{ g}\cdot\text{L}^{-1}$). In contrast, Inoc-OW had much lower TS (5.8 %) and much higher TAN contents ($5.04\text{ g}\cdot\text{L}^{-1}$). Due to this high TAN concentrations (higher than in the two other inocula), it was expected that the microbial community in Inoc-OW was more adapted to typical FW AD conditions, *i.e.* high TAN and high transient VFA concentrations.

3.2. Anaerobic digestion performances

As shown in Table 1, while some batch tests produced methane efficiently, others did not produce methane significantly (methane yields marked with *). This occurred because VFA rapidly accumulated at the beginning of the batch AD process, decreasing the pH initially and overloading the methanogens in the acidified reactors. Basically, if the present archaea were not able to rapidly consume the accumulated VFAs, the pH decreased to values where methanogenesis was inhibited.

To illustrate this initial VFA accumulation-consumption, Figure 1 presents the evolution of the total VFA concentrations in the experiments carried out using Inoc-OW as inoculum (efficient methane production; Figure 1.A) and Inoc-UASB1 as inoculum (no methane production; Figure 1.B). The acidified systems corresponded to all the tests inoculated with Inoc-UASB1 (regardless the experimental conditions applied) and the experiment started at an S/X ratio of $1.00\text{ g VS}\cdot\text{g VS}^{-1}$ with Inoc-UASB2. In the tests started with Inoc-UASB1, VFA concentrations up to $33.7\text{ g COD}\cdot\text{L}^{-1}$ (calculated in COD units, 45 % acetic acid, 39 % butyric acid, 8 % caproic acid, 4 % propionic acid and 3 % valeric acid) were detected, causing a pH drop to values down to 5.6 (Table 3). In contrast, all the batch tests inoculated with Inoc-OW

produced methane efficiently, as well as the experiments carried out at low loads (S/X ratio of 0.25 g VS·g VS⁻¹) with Inoc-UASB2. Although high transient VFA concentrations, up to 22.6 g COD·L⁻¹, were observed in these reactors (Figure 1.A), the methanogens efficiently consumed the accumulated VFAs, producing methane and avoiding a pH drop. A possible explanation for the different results obtained using the Inoc-OW is that the high initial TAN concentrations in this inoculum buffered the initial peak of VFAs (up to 22.6 g COD·kg⁻¹; 64 % COD acetic acid, 23 % butyric acid, 10 % propionic acid and 3 % valeric acid), alleviating the pH drop. However, such TAN-buffering effect cannot explain the different performances observed between the methane-producing reactors started with Inoc-UASB2 and those inoculated with Inoc-UASB1, with very similar initial TAN concentrations and working conditions.

In order to elucidate the reasons behind these observations, analyses of the microbial communities were performed. At this point, it must be mentioned that the methanogenic activity of all the used inocula was previously verified using ethanol as substrate. In fact, all the blank tests defined to determine the endogenous respiration produced significant amounts of methane. These reactors served, not only to account for the endogenous respiration of the inocula, but also to verify their activity and to corroborate that the observed acidification was related to the addition of the substrates.

3.3. Microbial composition of the inocula and the digestates

In an attempt to explain the different behaviors observed, the structures of the microbial communities of the initial inocula and the digestates sampled at the end of each batch tests were analyzed. Due to their relevance for methane production, the composition of the archaeal communities was specifically investigated. Figure 2 and 3 show the relative abundances of archaea found in the three inocula (Figure 2) and in the digestates (Figure 3). It must be mentioned that the OTUs were defined from the 16S rRNA copies.

As shown in Figure 2, the initial archaeal communities varied widely according to the inoculum origin. Inoc-UASB1 and Inoc-UASB2 (non-acclimated to high TAN or VFA concentrations) were rich in the hydrogenotroph *Methanobacterium* and the acetotroph *Methanosaeta*, both relatively vulnerable to TAN inhibition, *i.e.* not surviving over 3 g TAN·L⁻¹ (De Vrieze et al., 2012). In contrast, *Methanosarcina* was already the dominant species in the Inoc-OW, with the highest initial TAN concentrations, followed by the hydrogenotrophs *Methanothermobacter* and *Methanobrevibacter*. Therefore, for practical reasons these inocula were classified according to their initial relative abundance of *Methanosarcina*: negligible proportions in Inoc-UASB1 (0.47 %; MS-Rare), 6.36 % of the total archaeal OTUs in Inoc-UASB2 (MS-Poor) and up to 52.6 % in Inoc-OW (MS-Rich). As the experiments performed with Inoc-UASB1 (MS-Rare) did not produce any methane and those inoculated with Inoc-OW (MS-Rich) generated methane at higher substrate loads than those inoculated with Inoc-UASB2 (MS-Poor), these results suggest that *Methanosarcina*-scarce inocula were sensitive to inhibition when compared to inocula with higher initial proportions of *Methanosarcina*.

The archaeal populations in the digestates (shown in Figure 3) support this assumption. Regardless of the initial inoculum, the predominant species in all the batch tests that produced methane was *Methanosarcina*, with relative abundances ranging from 48.8 % to 61.8 %. This suggests that, at the high TAN levels (up to 5.05 g·L⁻¹) and transient VFA concentrations (up to 22.6 g·L⁻¹) that are associated with batch high-solids AD environments (Table 3) the growth of members of this genus was favored, which is in agreement with different results presented in the literature (Hao et al., 2015).

This can be explained by the high resistance of *Methanosarcina* to inhibition by these compounds (De Vrieze et al., 2012). As aforementioned, acetotrophs such as *Methanosaeta* are generally predominant under unstressed conditions due to their higher substrate affinity

and favored thermodynamics when compared to hydrogenotrophs. However, under stressful AD conditions (*i.e.* high FAN or VFAs concentrations) these methanogens are inhibited and the growth of mixotrophic and hydrogenotrophic microorganisms, which are more resistant to inhibitors, is favored (De Vrieze et al., 2012; Venkiteshwaran et al., 2016). Therefore, hydrogenotrophic methanogenesis, which otherwise would have been a secondary methane-producing pathway, becomes predominant. This finding is in agreement with a recent study using ^{14}C radiolabeling, which showed that at TAN concentrations over $2\text{ g}\cdot\text{L}^{-1}$, hydrogenotrophic methanogenesis was predominant (68-75 % of the methane produced) over the acetoclastic pathway (Jiang et al., 2017). Therefore, the growth of hydrogenotrophic/mixotrophic microorganisms (such as those belonging to the genus *Methanosarcina*) was favored under these conditions.

In addition, when comparing the tests that produced methane with the others, the importance of *Methanosarcina* to achieve efficient methanogenesis is also highlighted. Using Inoc-UASB1 (MS-Rare), where the proportion of this group of archaea was initially negligible, no efficient methane production was achieved under any condition, even at relatively low organic loads ($0.25\text{ g VS}\cdot\text{g VS}^{-1}$). On the other hand, the experiments operated under equivalent conditions but inoculated with Inoc-UASB2 (MS-Poor; with 6.36 % of *Methanosarcina* initially) showed efficient methane production, likely due to the presence and the emergence of this group of methanogens. This is supported by the fact that a minimum of 48.8 % of *Methanosarcina* was observed in these tests after AD, indicating that the growth of this group of archaea prevailed. Moreover, when looking at the results obtained using Inoc-OW (MS-Rich; 52.5 % *Methanosarcina* initially), high methane yields were achieved at substrate loads up to $1\text{ g VS}\cdot\text{g VS}^{-1}$, values where Inoc-UASB2 (MS-Poor; with lower initial proportions of *Methanosarcina*; 6.36 %) led to acidification and no methane was produced.

Regarding the archaeal composition of the acidified (non-methane producing) reactors,

300 *Methanobacterium* was predominant in all of them, with negligible proportions of other
301 archaeal genus. As these archaea were already predominant in both UASB1 and UASB2
302 inocula, this simply indicates that, as no methane was significantly produced, no growth of
303 other methanogenic microorganisms occurred in these conditions.

304 These results are in accordance with a recent review article focused on the microbial
305 communities of FW AD. In their bibliographic study, Wang et al. (2017) pointed out that
306 *Methanosarcina* was a predominant methanogen during dry FW AD and that the presence of
307 this archaea could potentially act as an indicator of a stable and efficient dry AD process. It
308 must also be mentioned that, together with this particular archaea, the development of other
309 microorganisms growing in syntrophy with *Methanosarcina* might have also been of critical
310 importance. The growth of other hydrogenotrophic methanogens (such as
311 *Methanothermobacter* or *Methanoculleus*) might have contributed greatly to the metabolic
312 shift towards hydrogenotrophic methanogenesis as main methane-producing pathway.

313 Moreover, if it is assumed that this was the main route for methane production using FW and
314 CB as substrates (Capson-Tojo et al., 2017d), the growth of syntrophic acetate oxidizers has
315 also been essential to degrade acetate to hydrogen, facilitating the production of methane by
316 the hydrogenotrophs. Finally, the fact that all the reactors producing methane (regardless of
317 the inoculum used) had *Methanosarcina* as predominant methanogen suggest that the
318 relevance of its growth was independent of the particular characteristics of each inoculum (*i.e.*
319 initial VS concentration or sludge mixture). At this point, it must be mentioned that, before
320 concluding the suitability of adapted inocula (such as Inoc-OW) for dry AD of FW
321 (with/without CB), experiments at higher TS contents (*i.e.* 27-30 %) must be carried out.

322 The results from the PCA carried out using the methane yields, the *Methanosarcina*
323 proportions in the inocula and the digestates, the final VFA concentrations and pH values and
324 the initial TS contents and S/X and C/N ratios as entries further support the critical

importance of *Methanosarcina* to achieve an efficient AD process. Figure 4 shows the obtained results.

As it can be observed in the correlation circle, the first component accounted for 54.3 % of the variance, which was mainly attributed to the final pH, the methane yields, the *Methanosarcina* proportions in both the inocula and the digestates and the final VFA concentrations. The pH, methane yields and *Methanosarcina* proportions in the digestates were all strongly positively correlated between them and negatively correlated to the VFA concentrations. Also in agreement with the results presented above, the initial *Methanosarcina* proportions were also significantly correlated with the final pH, the methane yields and the final *Methanosarcina* proportions. Interestingly, the initial working conditions (*i.e.* TS content and initial S/X and C/N ratios) were not correlated to either of the aforementioned variables (*i.e.* pH, methane yields, VFA concentrations and *Methanosarcina* proportions in the digestates), suggesting that similar results were obtained regardless the initial conditions defined in the batch reactors. To summarize, regardless of the initial conditions, methane was efficiently produced in the reactors where *Methanosarcina* was predominant after the digestion, consuming the accumulated VFAs and avoiding a pH drop and reactor acidification.

The obtained results highlight the critical relevance of the initial composition of the archaeal populations in the inoculum to achieve efficient AD, especially during reactor start-up. In particular, the results suggest the great importance of *Methanosarcina* and other hydrogenotrophs within the archaeal populations to achieve efficient dry AD of FW. The structure of the archaeal community used to start up batch FW AD may also explain the high variability of the substrate loading limits reported in the literature, ranging from below 0.5 g VS·g VS⁻¹ to over 2 g VS·g VS⁻¹ (Capson-Tojo et al., 2016). These results could have great implications in industrial scale AD installations, in particular for the start-up of continuous

AD systems and for the initial conditions applicable for batch AD systems.

4. Conclusions

AD performances of three different inocula were compared using FW and CB as substrates. Particular attention was paid to the compositions of the archaeal communities in the inocula and in the digestates. Regardless of the inoculum used, *Methanosarcina* was the dominant methanogen in all the experiments where methane was produced, suggesting that these archaea played a critical role in methane production at high TAN and VFA concentrations. Higher proportions of *Methanosarcina* in the inocula also allowed greater substrate loads. The initial composition of the archaeal communities in the inoculum was found to be crucial, mainly in batch systems and during reactor start-up. This may have huge implications for industrial-scale installations treating FW and CB.

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454 **Figure and table captions**

455 **Figure 1.** Evolution of the total VFA concentrations in the experiments carried out using
456 Inoc-OW (A) and Inoc-UASB1 (B) as inocula. The legends indicate the substrate used, the
457 S/X ratio and the initial TS content. FW stands for food waste, CB for cardboard, TS for total
458 solids, VS for volatile solids and VFA for volatile fatty acids

459 **Figure 2.** Relative abundances of archaeal OTUs in the inocula. The inocula were named
460 “MS-Rare”, “MS-Poor” and “MS-Rich” according to their low to high initial relative
461 abundances of *Methanosarcina*

462 **Figure 3.** Relative abundances of archaeal OTUs in the digestates from the batch tests that
463 produced methane (up) and the acidified reactors (down). The methane yields are also
464 presented. The inocula were named “MS-Rare”, “MS-Poor” and “MS-Rich” according to
465 their initial relative abundances of *Methanosarcina*

466 **Figure 4.** Correlation circle using as input data the initial total solid contents (TS) and C/N
467 and S/X ratios, the *Methanosarcina* proportions in the inocula ($Sarcina_0$), the *Methanosarcina*
468 proportions in the digestates ($Sarcina_f$) and the final pH, methane yields (CH_4) and volatile
469 fatty acids concentrations (VFA). This circle resulted from the projection in plans formed by
470 the two first principal components, accounting for 85.6 % of the variance

471 **Table 1.** Operational conditions of the batch experiments and obtained methane yields.
472 “UASB1”, “UASB2” and “OW” stand for the inoculum

473 **Table 2.** Physico-chemical characteristics of the substrates and the inocula

474 **Table 3.** Concentrations of VFAs, TAN and FAN at the beginning and the end of the batch
475 tests presented in Table 1. The values of the pH and the incubation times are also presented

Graphical abstract

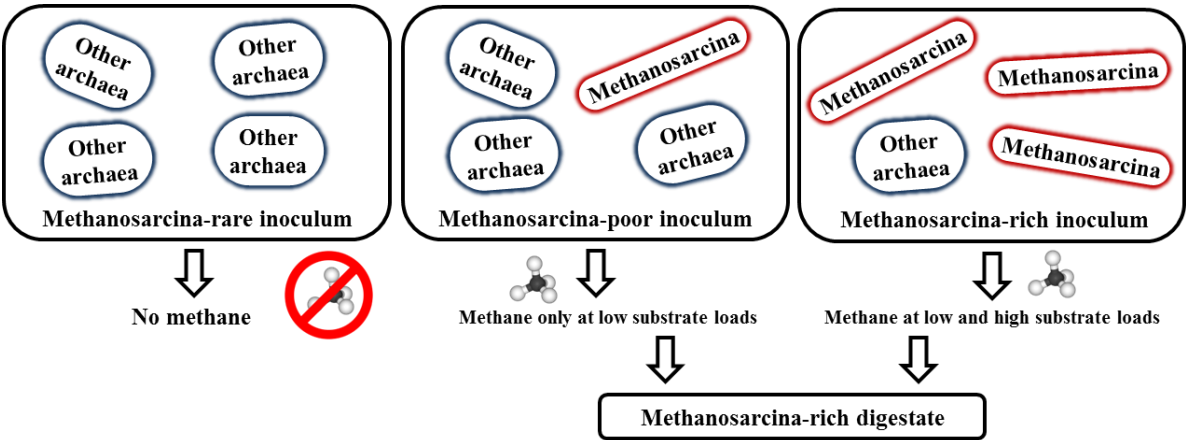


Table 1. Operational conditions of the batch experiments and obtained methane yields.

“UASB1”, “UASB2” and “OW” stand for the inoculum

| Inoculum | Substrate | Substrate C/N ratio | Co-dig. ratio (g TS FW·g TS CB ⁻¹) | S/X (g VS·g VS ⁻¹) | Initial TS (%) | Methane yield (mL CH ₄ ·g VS ⁻¹) |
|----------|-----------|------------------------|---|-----------------------------------|-------------------|--|
| UASB1 | FW+CB | 23.1 | 1.75 | 0.25 | 30.0 | 11±3* |
| UASB1 | FW+CB | 27.8 | 1.00 | 0.25 | 30.0 | 8±2* |
| UASB1 | FW+CB | 27.8 | 1.00 | 0.25 | 35.0 | 17±2* |
| UASB1 | FW | 16.3 | - | 0.25 | 20.0 | 1±1* |
| UASB2 | FW+CB | 22.7 | 1.86 | 0.25 | 27.5 | 409±11 |
| UASB2 | FW+CB | 27.8 | 1.00 | 0.25 | 27.5 | 393±9 |
| UASB2 | FW+CB | 27.8 | 1.00 | 0.25 | 35.0 | 401±16 |
| UASB2 | FW+CB | 19.3 | 4.00 | 1.00 | 27.5 | 0±0* |
| OW | FW | 16.3 | - | 0.25 | 20.0 | 464±14 |
| OW | FW | 16.3 | - | 1.00 | 20.0 | 375±17 |

* These values were considered as indicators of an inefficient AD process

Table 2. Physico-chemical characteristics of the substrates and the inocula

| Parameter/Element | Model food waste | Cardboard | Inoc-UASB1 | Inoc-UASB2 | Inoc-OW |
|---|------------------|-----------|------------|------------|-----------|
| TS % (wet basis) | 21.6±0.7 | 92.7±3.7 | 70.8±2.2 | 74.2±3.1 | 5.81±0.02 |
| VS (% TS) | 96.2±0.1 | 77.5±0.2 | 70.9±1.4 | 59.1±0.4 | 59.1±0.1 |
| pH | 5.60 | 7.10 | - | - | 8.01 |
| Carbohydrates (g·kg TS ⁻¹) | 687±15 | 958±5 | - | - | - |
| Proteins (g·kg TS ⁻¹) | 169±10 | 0 | - | - | - |
| Lipids (g·kg TS ⁻¹) | 72.3±1.5 | 0 | - | - | - |
| BMP (ml CH ₄ ·g VS ⁻¹) | 498±42 | 250±3 | - | - | - |
| TAN (g·L ⁻¹) | 0 | 0 | 1.50 | 1.49 | 5.04 |
| TKN (g·kg TS ⁻¹) | 27.08±1.64 | 2.00±0.02 | - | - | - |
| TOC (g·kg TS ⁻¹) | 442±7 | 366±6 | - | - | - |
| C/N | 16.3 | 183 | - | - | - |

Table 3. Concentrations of VFAs, TAN and FAN at the beginning and the end of the batch tests presented in Table 1. The values of the pH and the incubation times are also presented

| Inoculum | Substrate C/N ratio | Initial TS (%) | Incubation time (d) | pH | | Total VFAs (g COD·L ⁻¹) | | TAN (mg·L ⁻¹) | | FAN (mg·L ⁻¹) | |
|----------|------------------------|-------------------|------------------------|-----------------|---------|--|-----------------|------------------------------|----------|------------------------------|---------|
| | | | | Initial | Final | Initial | Final | Initial | Final | Initial | Final |
| UASB1 | 23.1 | 30.0 | 83 | na ¹ | 5.9±0.1 | nd ² | 33.3±0.5 | 530 | 1250±30 | na ¹ | 1±0 |
| UASB1 | 27.8 | 30.0 | 83 | na ¹ | 5.9±0.2 | nd ² | 33.7±0.3 | 450 | 1060±30 | na ¹ | 1±0 |
| UASB1 | 27.8 | 35.0 | 83 | na ¹ | 6.3±0.0 | nd ² | 24.7±1.6 | 350 | 1340±10 | na ¹ | 3±0 |
| UASB1 | 16.3 | 20.0 | 83 | na ¹ | 5.7±0.2 | nd ² | 25.6±0.6 | 310 | 1090±140 | na ¹ | 1±0 |
| UASB2 | 22.7 | 27.5 | 98 | na ¹ | 8.3±0.0 | nd ² | nd ² | 470 | 2900±210 | na ¹ | 576±32 |
| UASB2 | 27.8 | 27.5 | 98 | na ¹ | 8.4±0.0 | nd ² | nd ² | 470 | 2600±160 | na ¹ | 567±20 |
| UASB2 | 27.8 | 35.0 | 98 | na ¹ | 8.5±0.0 | nd ² | nd ² | 600 | 3200±180 | na ¹ | 795±55 |
| UASB2 | 19.3 | 27.5 | 98 | na ¹ | 5.4±0.5 | nd ² | 64.4±14.5 | 370 | 1800±90 | na ¹ | 1±1 |
| OW | 16.3 | 20.0 | 56 | 8.1 | 8.3±0.0 | nd ² | nd ² | 4140 | 4800±480 | 570 | 948±119 |
| OW | 16.3 | 20.0 | 56 | 8.1 | 8.4±0.0 | nd ² | nd ² | 3770 | 5050±160 | 519 | 1171±50 |

1. Not available due to the high TS contents of the inoculum
2. Not detectable due to too low concentrations

Figure1

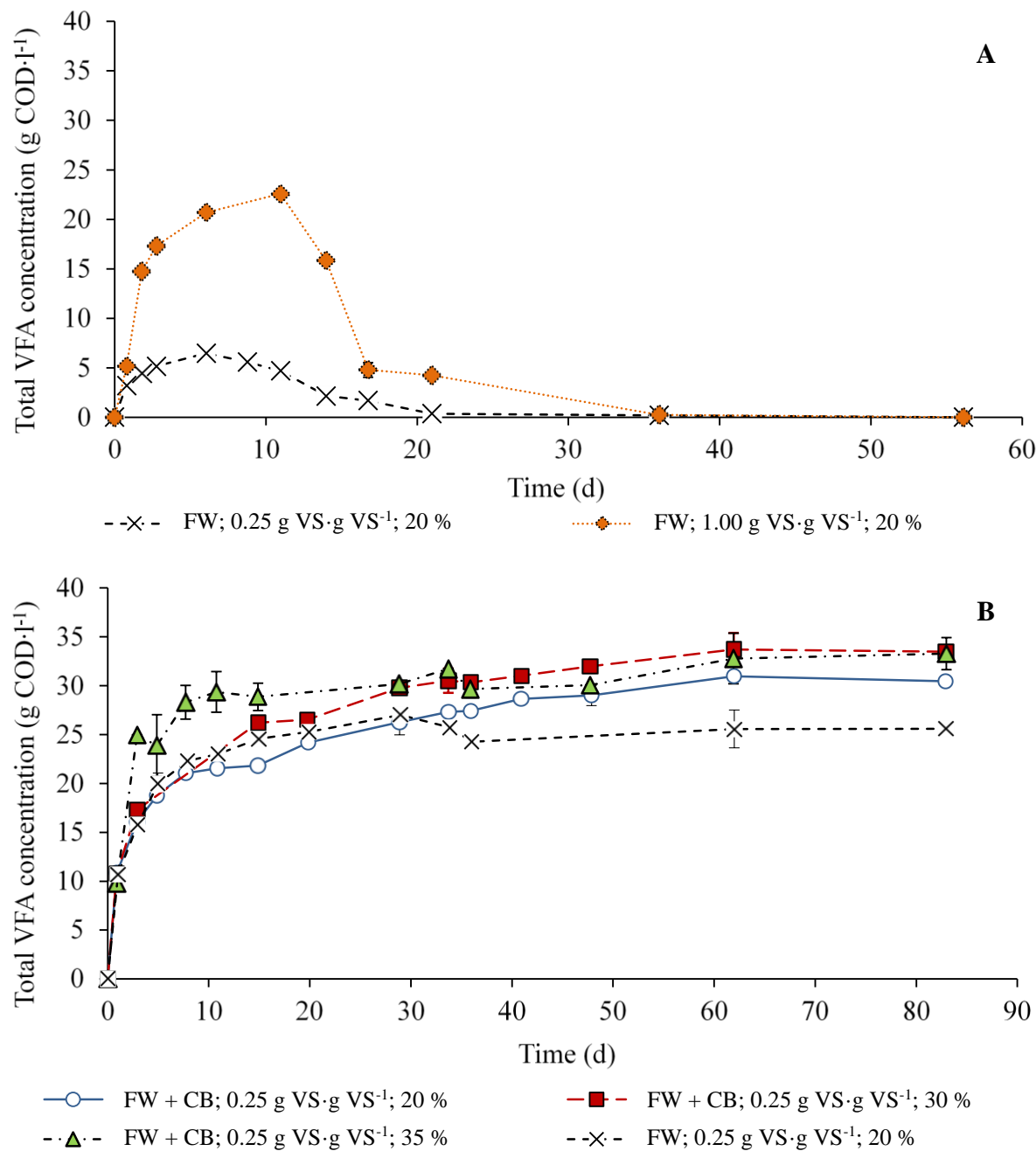


Figure 1. Evolution of the total VFA concentrations in the experiments carried out using Inoc-OW (A) and Inoc-UASB1 (B) as inocula. The legends indicate the substrate used, the S/X ratio and the initial TS content. FW stands for food waste, CB for cardboard, TS for total solids, VS for volatile solids and VFA for volatile fatty acids

Figure2

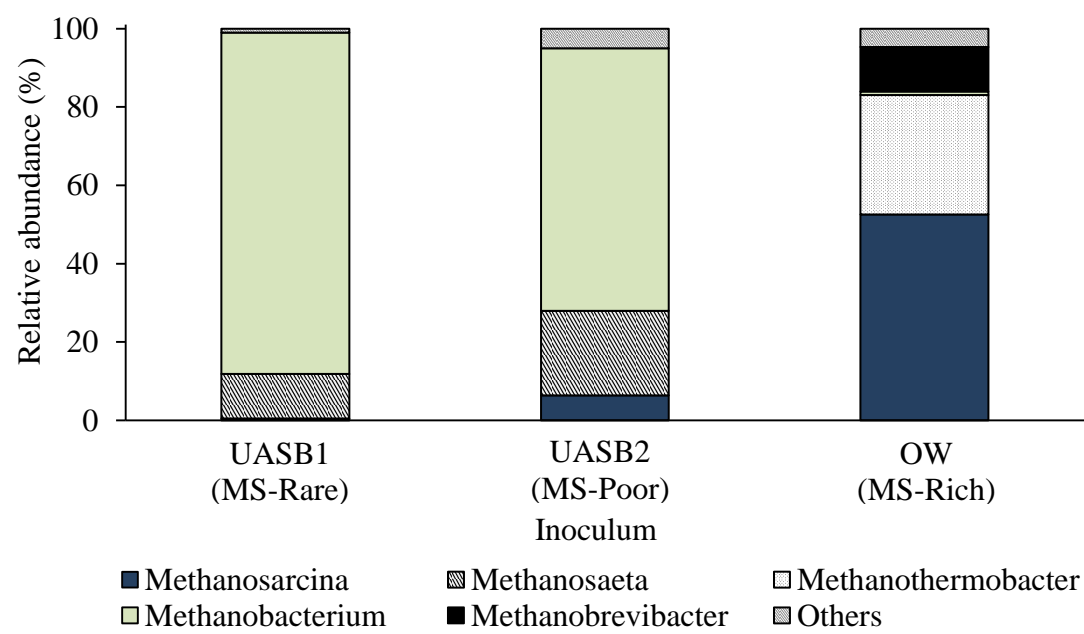


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Figure3

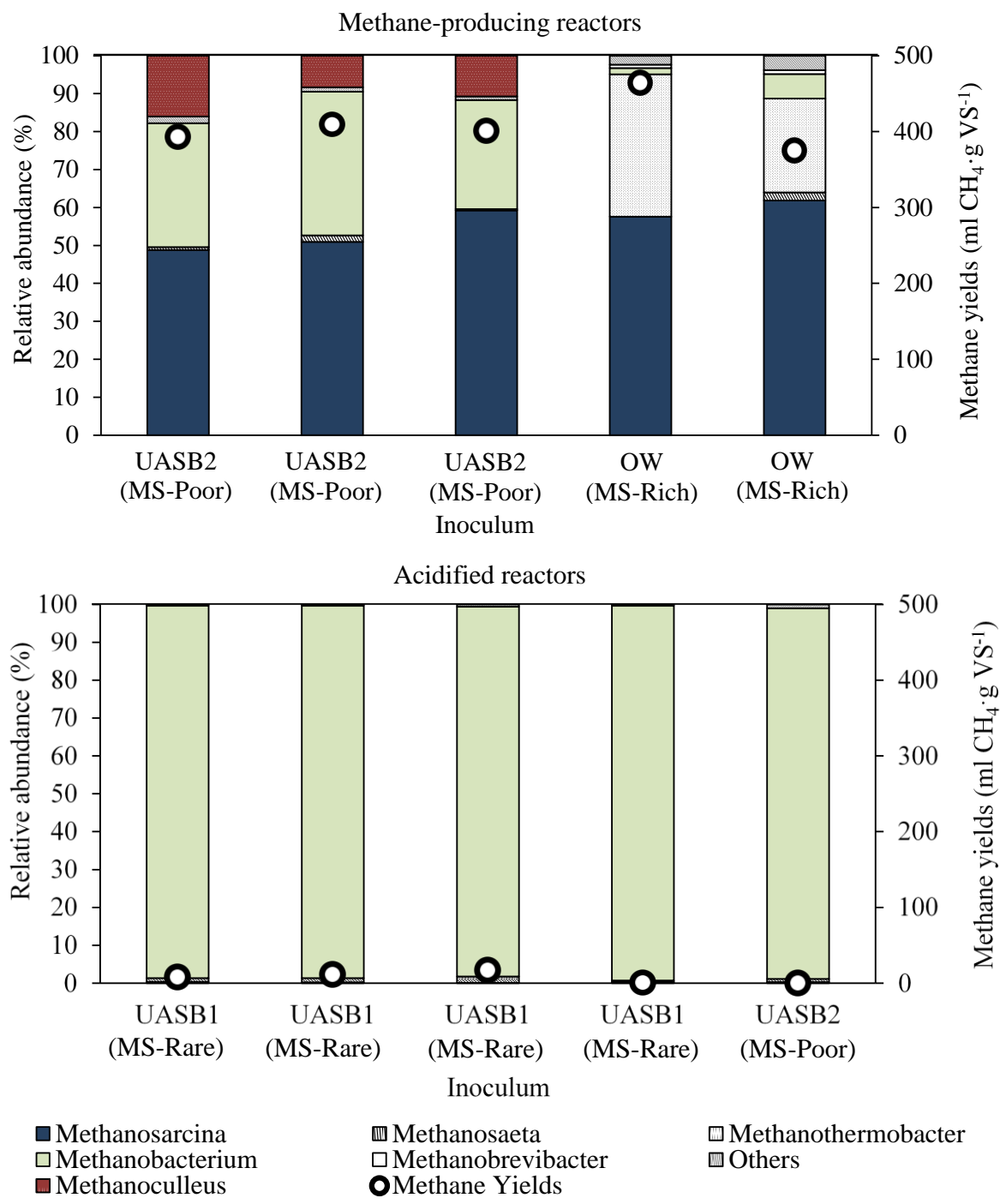


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Figure4

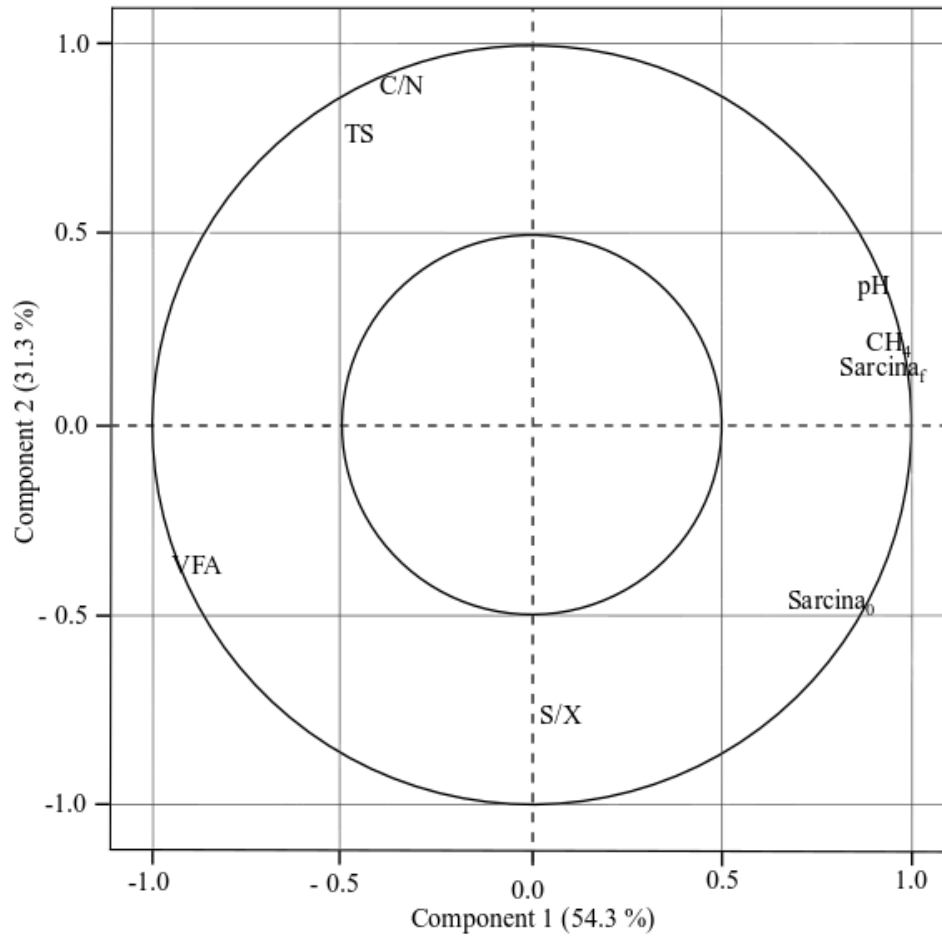


Figure 4. Correlation circle using as input data the initial total solid contents (TS) and C/N and S/X ratios, the *Methanosarcina* proportions in the inocula (Sarcina₀), the *Methanosarcina* proportions in the digestates (Sarcina_f) and the final pH, methane yields (CH₄) and volatile fatty acids concentrations (VFA). This circle resulted from the projection in plans formed by the two first principal components, accounting for 85.6 % of the variance