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

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2   **cardboard**

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9

10   **Abstract**

11   Anaerobic digestion of food waste is a complex process often hindered by high concentrations

12   of volatile fatty acids and ammonia. Methanogenic archaea are more sensitive to these

13   inhibitors than bacteria and thus the structure of their community is critical to avoid reactor

14   acidification. In this study, the performances of three different inocula were compared using

15   batch digestion tests of food waste and cardboard mixtures. Particular attention was paid to

16   the archaeal communities in the inocula and after digestion. While the tests started with

17   inocula rich in *Methanosarcina* led to efficient methane production, VFAs accumulated in the

18   reactors where inocula initially were poor in this archaea and no methane was produced. In

19   addition, higher substrate loads were tolerated when greater proportions of *Methanosarcina*

20   were initially present in the inoculum. Independently of the inoculum origin, *Methanosarcina*

21   were the dominant methanogens in the digestates from the experiments that efficiently

22   produced methane. These results suggest that the initial archaeal composition of the inoculum

23   is crucial during reactor start-up to achieve stable anaerobic digestion at high concentrations

24   of ammonia and organic acids.

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29   **1. Introduction**

30   Novel technologies for treatment and valorization of the organic fraction of municipal solid

31   waste (OFMSW) must be developed to deal with an increasing production and new

32   international regulations. Anaerobic digestion (AD) is a well-known process used for efficient

33   treatment of organic waste with high total solids (TS) contents ($\geq$ 20 %), converting them into

34   biogas and digestate, both added-value end-products. However, AD of highly biodegradable

35   substrates such as food waste (FW), which is a major component of OFMSW, is often

36   associated with accumulation of volatile fatty acids (VFAs), which are detrimental to the AD

37   process. In addition, FW is rich in organic nitrogen, which is reduced to ammonia during AD,

38   leading to high concentrations of total ammonia nitrogen (sum of NH_3 and NH_4^+; TAN) in the

39   digesters (L. Zhang et al., 2012). Accumulation of both VFA and/or TAN might lower the

40   methane yields and can even lead to failure of the AD process (Banks et al., 2008). The

41   reactors are particularly vulnerable to these inhibitions during the start-up period (Fernández

42   et al., 2001). This occurs because the microbial communities are not adapted to the stressful

43   conditions imposed by the substrates and the operational parameters (*i.e.* high organic loading

44   rates). Therefore, to achieve efficient methane yields and productivities with FW as substrate,

45   it is crucial to have well-adapted microbial communities in the digesters, which are resistant

46   to high VFA and free ammonia nitrogen (NH_3; FAN) concentrations.

47   Methanogenic archaea are generally more sensitive to inhibitors than bacteria and thus

48   methanogenesis is usually the first process affected by common inhibitors, such as FAN or

49   VFAs (De Vrieze et al., 2012). Nonetheless, not all methanogenic archaea have the same

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50 resistance to these inhibitors and thus the composition of the archaeal microbial community

51 varies according to the operating conditions (Abbassi-Guendouz et al., 2013). Due to their

52 high substrate affinity, acetotrophs such as *Methanosaeta* are generally predominant under

53 unstressed conditions and thus acetotrophic methanogenesis is the predominant pathway for

54 methane production. On the other hand, under stressful AD conditions, these methanogens are

55 preferentially inhibited and mixotrophic microorganisms (*i.e.* able to consume acetate and

56 hydrogen to produce methane), such as *Methanosarcina* which are more resistant to inhibitors

57 (*i.e.* FAN or VFAs), become predominant (De Vrieze et al., 2012; Venkiteshwaran et al.,

58 2016). In fact, while *Methanosaeta* cannot grow at TAN concentrations greater than 3 $g \cdot L^{-1}$,

59 *Methanosarcina* have been found at much higher TAN concentrations (De Vrieze et al., 2012;

60 Poirier et al., 2016). As an illustration, Capson-Tojo et al. (2017a) found *Methanosarcina* to

61 be the dominant methanogens at TAN concentrations up to 3.7 $g \cdot L^{-1}$ (795 mg $FAN \cdot L^{-1}$) using

62 FW as substrate in AD batch tests.

63 Over the past years, the importance of the microbial communities for efficient AD processes

64 has gained attention and many studies have been carried out to further understand the

65 structures of the communities of both bacteria and archaea in AD reactors. In a recent study

66 carried out by Zhang et al. (2016) with sewage sludge and FW as substrates (with final NH_4^+

67 concentrations up to 2.01 $g \cdot L^{-1}$), it was observed that *Methanosaeta* were the main archaea at

68 the beginning of the batch experiment (71 % of the operational taxonomical units; OTUs).

69 Afterwards, *Methanosarcina* grew during acid production (with transient VFA concentrations

70 up to 24 $g \cdot L^{-1}$) and overpassed in abundance *Methanosaeta* because of their greater resistance

71 to VFA and TAN inhibition. Finally, other hydrogenotrophic methanogens (*i.e.*

72 *Methanoculleus*) grew once acetate was totally consumed. Using a high solid-state AD box-

73 type container fed with FW at high TS contents (from 34.4 to 44.5 %) and TAN

74 concentrations (2.5 $g \cdot L^{-1}$), Walter et al. (2016) observed that *Methanosarcina* were the

3

75 dominant species accompanied by different hydrogenotrophs (*i.e. Methanobacterium*,

76 *Methanoculleus* and *Methanocorpusculum*). Consistently, Zamanzadeh et al. (2016) found

77 *Methanosaeta* as the main archaea in mesophilic continuous AD of FW at low concentrations

78 of FAN (≤ 200 mg·L^{-1}). This further supports that the concentration of TAN-FAN is a key

79 factor that can result in shifts of the archaeal populations. In a recent batch study, Poirier et al.

80 (2016) identified the key microbial phylotypes resisting to extreme ammonia concentrations

81 (up to 50 g TAN·L^{-1}). They achieved high methane yields at TAN concentrations as high as

82 25 g TAN·L^{-1}, with *Methanosarcina* and *Methanoculleus* as main methanogens and with

83 relative abundances of *Methanosaeta* lower than 5 % in all AD reactors.

84 The objective of this study was to evaluate, for the first time, the AD performance of three

85 microbial inocula from different origins and with different initial archaeal compositions using

86 FW and cardboard (CB) as substrates. These wastes are the main components of OFMSW

87 (Kim and Oh, 2011; Y. Zhang et al., 2012) and are generally collected at the same facilities,

88 and thus their co-digestion is facilitated. Also, they constitute a good waste model substrate,

89 since the initial proportions of carbon and nitrogen could be easily adjusted. Batch tests were

90 performed at different substrate loads, TS contents (≥ 20 %) and co-digestion proportions.

91 Special attention was paid to the archaeal communities and to the FAN and VFA levels.

92

93 **2. Materials and methods**

94 *2.1. Substrate and microbial inoculum*

95 A synthetic FW was prepared according to the VALORGAS report (VALORGAS, 2010). It

96 was composed of fruits and vegetables (80.7 %), meat (8.2 %), pasta (4.8 %), bread (6.2 %),

97 dairy products (1.9 %) and biscuits (1.9 %). Its precise composition has been detailed

98 elsewhere (Capson-Tojo et al., 2017a). Being FW and CB the most common components of

99 OFMSW, CB (branded ''Cartonnages Michel'' and shredded to less than 1 mm) was added as

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100   co-substrate to simulate this waste (Hogg et al., 2002), increasing at the same time the C/N

101   ratio of the substrate and thus diluting the TAN concentrations in the reactors and favoring the

102   AD process (Capson-Tojo et al., 2017a). Three different inocula from industrial plants were

103   used: mixture of a centrifuged granular sludge issued from a mesophilic industrial UASB

104   reactor treating sugar factory effluents with a dried digestate. This digestate was used to

105   increase the TS content of the inoculum and was sampled in a thermophilic industrial plant

106   treating OFMSW (Inoc-UASB1); a mixture of sludge and dried digestate issued from the

107   same sources than Inoc-UASB1 but sampled at a different moment (Inoc-UASB2); a sludge

108   issued from an AD industrial plant treating a mixture of different organic waste streams at 35

109   °C mixed with dried compost (99 % TS; 81 % VS) to increase the TS content of the inoculum

110   (Inoc-OW). The amounts of dried digestate and compost added were 0.5 g per g of inoculum

111   (w/w) (Inoc-UASB1 and Inoc-UASB2) and 0.17-0.34 g per g of inoculum (w/w) (Inoc-OW)

112   respectively, depending on the desired TS content and the initial water content of the sludge.

113   *2.2. Dry batch anaerobic co-digestion tests*

114   Different co-digestion ratios (4-1 g TS FW·g TS CB^{-1}), initial TS contents (20-35 %) and

115   substrate to inoculum (S/X) ratios (0.25-1.00 g VS·g VS^{-1}) were tested. These values were

116   selected according to previous results and to data gathered from the literature (Capson-Tojo et

117   al., 2016, 2017a). Table 1 summarizes the 10 different experimental conditions that were

118   considered in this study. Each tested condition was run in triplicate. This experimental set-up

119   allowed to produce results which primarily depended on the inoculum source, while

120   evaluating at the same time different initial conditions (*i.e.* S/X and co-digestion ratios and

121   initial TS contents). Therefore, the obtained results were not dependent on the particular

122   operational conditions applied, but only on the type of inoculum used. With this set-up the

123   performance of each reactor was also totally independent between them.

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

125   substrates (according to Table 1) were supplemented. Finally, the TS contents were adjusted

126   adding water and the flasks were flushed with nitrogen and sealed.

127   As aforementioned, to allow working at the high TS contents desired, the inocula used were

128   mixed with dried digestates (Inoc-UASB1 and Inoc-UASB2) and compost (Inoc-OW).

129   Different blank reactors were carried out to account for the biogas production that could have

130   been produced by the degradation of these materials (Capson-Tojo et al., 2017a, 2017b,

131   2017c). In addition, both materials were dried at 100 °C for over 24 h to ensure that the impact

132   of the microorganisms present in these media on the methane production was negligible. The

133   working volumes were different according to the operational conditions and the reactor size,

134   varying from 0.4 L to 0.7 L. The duration of the batch experiments was variable (56-98 days).

135   In the systems producing methane, the batch experiments were stopped when a plateau in the

136   biomethane production was observed. On the other hand, longer batch periods were applied

137   when acidification occurred (to ensure that the acid accumulation was irreversible). All the

138   reactors were incubated at 37 °C.

139   *2.3. Analytical methods*

140   *2.3.1. Physicochemical characterization of the substrates*

141   The TS and Volatile Solids (VS) contents were determined according to the Standard

142   Methods (APHA, 2005). The protein and carbohydrate concentrations were measured by the

143   modified Lowry method (Frølund et al., 1996) and the Dubois method (Dubois et al., 1956),

144   respectively. The lipid content was determined using a gravimetric method (APHA, 2005), the

145   pH was measured with a WTW pHmeter series inoLab pH720, total Kjeldahl nitrogen (TKN)

146   and TAN contents were determined with an AutoKjehdahl Unit K-370, BUCHI and the total

147   organic carbon (TOC) with a Shimadzu TOC-V_{CSN} Total Organic Carbon Analyzer. A more

148   precise description of the analytical methods can be found in Capson-Tojo et al., (2017a). The

149   biochemical methane potentials (BMPs) of the substrates were determined according to Motte

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150 et al. (2014). The C/N ratio was calculated as TOC divided by TKN. The FAN concentrations

151 were calculated according to Chen et al. (2014) as a function of temperature, pH, and

152 concentration of TAN.

2.3.2. Gas quantification and analysis

154 The total biogas volume was periodically determined by measuring the pressure in the reactor

155 headspace and the gas composition was analyzed by gas chromatography coupled to a

156 catharometer detector, as detailed in Cazier et al. (2015). The methane yields were calculated

157 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

159 The concentrations of VFAs and ionic species after digestion were measured by gas

160 chromatography and high-performance liquid chromatography, according to Motte et al.

161 (2013). The reactors used in the experiments carried out using the Inoc-OW and Inoc-UASB1

162 allowed sampling of the digestate during the digestion and therefore, the kinetics of

163 production-consumption of metabolites were also analyzed. The sampling device is described

164 in Capson-Tojo et al. (2017b).

2.4. Microbial community analysis

166 The microbial communities of the inocula and the digestates were characterized by 16S rRNA

167 sequencing. One mL of each sample was first taken and stored at -20 °C until analysis. The

168 DNA from the sample (around 1 g) was extracted using a Fast DNA SPIN kit for soil in

169 accordance with the instructions of the manufacturer (MP Biomedicals). The quality and

170 quantity of the extracted DNA were verified by spectrophotometry using an Infinite 200 PRO

171 NanoQuant (Tecan Group Ltd., Männedorf, Switzerland). The primer pairs 515-532U and

172 909-928U and their respective linkers were used to amplify the V4-V5 regions of the 16S

173 rRNA genes (over 30 amplification cycles were applied at an annealing temperature of 65 ℃).

174 These primer pairs target both bacterial and archaeal 16S rRNA genes, capturing most of their

175 diversity (Wang and Qian, 2009). The PCR mixtures had a total volume of 50 μL, containing:

176 0.5 units of Pfu Turbo DNA polymerase (Stratagene), the corresponding buffer, each

177 deoxynucleotide at 200 mM, each primer at 0.5 mM and 10 ng of genomic DNA. The

178 following PCR sequence was carried out (using a Mastercycler thermal cycler; Eppendorf):

179 after 94 ℃ for two min, 35 cycles of 94 ℃ for one min, 65 ℃ for one min, and 72 ℃ for one

180 min were applied, with a final extension at 72 ℃ for 10 min. The obtained products were

181 purified and analyzed using the Illumina MiSeq cartridge (v3 chemistry) for sequencing of

182 paired 300 bp reads at the GenoToul platform (http://www.genotoul.fr). Mothur (version

183 1.35.0) was used for sequence assembling, cleaning and alignment and for assignation of the

184 taxonomic affiliation, as described in Venkiteshwaran et al. (2016).

185 *2.5. Statistical analysis*

186 To analyze potential relationships between variables (*i.e.* methane yields, *Methanosarcina*

187 proportions in the inocula and the digestates, final VFA concentrations, final pH values, initial

188 TS contents and initial S/X and C/N ratios), a principal component analysis (PCA) was

189 performed. The PCA was carried out using the package mixOmics in the software R (version

190 3.2.5; The R Foundation for Statistical Computing, Vienna, Austria).

191

192 **3. Results and discussion**

193 *3.1. Physicochemical characterization of substrates and inocula*

194 The physico-chemical characteristics of the substrates and the inocula are presented in Table

195 2. The observed composition of the FW was similar to those found in the literature (Capson-

196 Tojo et al., 2016), with TS contents of 21.6 % and VS/TS of 96.2 %. In agreement with

197 previously reported results, the FW was mainly composed of carbohydrates and had a

198 relatively low C/N ratio, far away from the optimum values of 25 reported in the literature

199 (Mao et al., 2015). The high BMP value of the FW (498 mL $CH_4 \cdot$ g VS^{-1}) highlights its great

200   potential for valorization by AD. In contrast, CB had a high C/N ratio, suggesting that CB can

201   be effectively used as co-substrate for diluting the TAN from FW organic nitrogen. A more

202   extensive characterization of both substrates can be found in Capson-Tojo et al., (2017a).

203   Inoc-UASB1 and Inoc-UASB2 had very similar physico-chemical characteristics, with high

204   TS (70.8 and 74.2 %) and low TAN concentrations (1.49-1.50 g·L^{-1}). In contrast, Inoc-OW

205   had much lower TS (5.8 %) and much higher TAN contents (5.04 g·L^{-1}). Due to this high

206   TAN concentrations (higher than in the two other inocula), it was expected that the microbial

207   community in Inoc-OW was more adapted to typical FW AD conditions, *i.e.* high TAN and

208   high transient VFA concentrations.

209   *3.2. Anaerobic digestion performances*

210   As shown in Table 1, while some batch tests produced methane efficiently, others did not

211   produce methane significantly (methane yields marked with *). This occurred because VFA

212   rapidly accumulated at the beginning of the batch AD process, decreasing the pH initially and

213   overloading the methanogens in the acidified reactors. Basically, if the present archaea were

214   not able to rapidly consume the accumulated VFAs, the pH decreased to values were

215   methanogenesis was inhibited.

216   To illustrate this initial VFA accumulation-consumption, Figure 1 presents the evolution of

217   the total VFA concentrations in the experiments carried out using Inoc-OW as inoculum

218   (efficient methane production; Figure 1.A) and Inoc-UASB1 as inoculum (no methane

219   production; Figure 1.B). The acidified systems corresponded to all the tests inoculated with

220   Inoc-UASB1 (regardless the experimental conditions applied) and the experiment started at an

221   S/X ratio of 1.00 g VS·g VS^{-1} with Inoc-UASB2. In the tests started with Inoc-UASB1, VFA

222   concentrations up to 33.7 g COD·L^{-1} (calculated in COD units, 45 % acetic acid, 39 % butyric

223   acid, 8 % caproic acid, 4 % propionic acid and 3 % valeric acid) were detected, causing a pH

224   drop to values down to 5.6 (Table 3). In contrast, all the batch tests inoculated with Inoc-OW

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225 produced methane efficiently, as well as the experiments carried out at low loads (S/X ratio of

226 0.25 g VS·g VS^{-1}) with Inoc-UASB2. Although high transient VFA concentrations, up to 22.6

227 g COD·L^{-1}, were observed in these reactors (Figure 1.A), the methanogens efficiently

228 consumed the accumulated VFAs, producing methane and avoiding a pH drop. A possible

229 explanation for the different results obtained using the Inoc-OW is that the high initial TAN

230 concentrations in this inoculum buffered the initial peak of VFAs (up to 22.6 g COD·kg^{-1}; 64

231 % COD acetic acid, 23 % butyric acid, 10 % propionic acid and 3 % valeric acid), alleviating

232 the pH drop. However, such TAN-buffering effect cannot explain the different performances

233 observed between the methane-producing reactors started with Inoc-UASB2 and those

234 inoculated with Inoc-UASB1, with very similar initial TAN concentrations and working

235 conditions.

236 In order to elucidate the reasons behind these observations, analyses of the microbial

237 communities were performed. At this point, it must be mentioned that the methanogenic

238 activity of all the used inocula was previously verified using ethanol as substrate. In fact, all

239 the blank tests defined to determine the endogenous respiration produced significant amounts

240 of methane. These reactors served, not only to account for the endogenous respiration of the

241 inocula, but also to verify their activity and to corroborate that the observed acidification was

242 related to the addition of the substrates.

243 *3.3. Microbial composition of the inocula and the digestates*

244 In an attempt to explain the different behaviors observed, the structures of the microbial

245 communities of the initial inocula and the digestates sampled at the end of each batch tests

246 were analyzed. Due to their relevance for methane production, the composition of the archaeal

247 communities was specifically investigated. Figure 2 and 3 show the relative abundances of

248 archaea found in the three inocula (Figure 2) and in the digestates (Figure 3). It must be

249 mentioned that the OTUs were defined from the 16S rRNA copies.

250 As shown in Figure 2, the initial archaeal communities varied widely according to the

251 inoculum origin. Inoc-UASB1 and Inoc-UASB2 (non-acclimated to high TAN or VFA

252 concentrations) were rich in the hydrogenotroph *Methanobacterium* and the acetotroph

253 *Methanosaeta*, both relatively vulnerable to TAN inhibition, *i.e.* not surviving over 3 g

254 TAN·L^{-1} (De Vrieze et al., 2012). In contrast, *Methanosarcina* was already the dominant

255 species in the Inoc-OW, with the highest initial TAN concentrations, followed by the

256 hydrogenotrophs *Methanothermobacter* and *Methanobrevibacter*. Therefore, for practical

257 reasons these inocula were classified according to their initial relative abundance of

258 *Methanosarcina*: negligible proportions in Inoc-UASB1 (0.47 %; MS-Rare), 6.36 % of the

259 total archaeal OTUs in Inoc-UASB2 (MS-Poor) and up to 52.6 % in Inoc-OW (MS-Rich). As

260 the experiments performed with Inoc-UASB1 (MS-Rare) did not produce any methane and

261 those inoculated with Inoc-OW (MS-Rich) generated methane at higher substrate loads that

262 those inoculated with Inoc-UASB2 (MS-Poor), these results suggest that *Methanosarcina*-

263 scarce inocula were sensitive to inhibition when compared to inocula with higher initial

264 proportions of *Methanosarcina*.

265 The archaeal populations in the digestates (shown in Figure 3) support this assumption.

266 Regardless of the initial inoculum, the predominant species in all the batch tests that produced

267 methane was *Methanosarcina*, with relative abundances ranging from 48.8 % to 61.8 %. This

268 suggests that, at the high TAN levels (up to 5.05 g·L^{-1}) and transient VFA concentrations (up

269 to 22.6 g·L^{-1}) that are associated with batch high-solids AD environments (Table 3) the

270 growth of members of this genus was favored, which is in agreement with different results

271 presented in the literature (Hao et al., 2015).

272 This can be explained by the high resistance of *Methanosarcina* to inhibition by these

273 compounds (De Vrieze et al., 2012). As aforementioned, acetotrophs such as *Methanosaeta*

274 are generally predominant under unstressed conditions due to their higher substrate affinity

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275   and favored thermodynamics when compared to hydrogenotrophs. However, under stressful

276   AD conditions (*i.e.* high FAN or VFAs concentrations) these methanogens are inhibited and

277   the growth of mixotrophic and hydrogenotrophic microorganisms, which are more resistant to

278   inhibitors, is favored (De Vrieze et al., 2012; Venkiteshwaran et al., 2016). Therefore,

279   hydrogenotrophic methanogenesis, which otherwise would have been a secondary methane-

280   producing pathway, becomes predominant. This finding is in agreement with a recent study

281   using ^{14}C radiolabeling, which showed that at TAN concentrations over 2 $g \cdot L^{-1}$,

282   hydrogenotrophic methanogenesis was predominant (68-75 % of the methane produced) over

283   the acetoclastic pathway (Jiang et al., 2017). Therefore, the growth of

284   hydrogenotrophic/mixotrophic microorganisms (such as those belonging to the genus

285   *Methanosarcina*) was favored under these conditions.

286   In addition, when comparing the tests that produced methane with the others, the importance

287   of *Methanosarcina* to achieve efficient methanogenesis is also highlighted. Using Inoc-

288   UASB1 (MS-Rare), where the proportion of this group of archaea was initially negligible, no

289   efficient methane production was achieved under any condition, even at relatively low organic

290   loads (0.25 g $VS \cdot g \ VS^{-1}$). On the other hand, the experiments operated under equivalent

291   conditions but inoculated with Inoc-UASB2 (MS-Poor; with 6.36 % of *Methanosarcina*

292   initially) showed efficient methane production, likely due to the presence and the emergence

293   of this group of methanogens. This is supported by the fact that a minimum of 48.8 % of

294   *Methanosarcina* was observed in these tests after AD, indicating that the growth of this group

295   of archaea prevailed. Moreover, when looking at the results obtained using Inoc-OW (MS-

296   Rich; 52.5 % *Methanosarcina* initially), high methane yields were achieved at substrate loads

297   up to 1 g $VS \cdot g \ VS^{-1}$, values where Inoc-UASB2 (MS-Poor; with lower initial proportions of

298   *Methanosarcina*; 6.36 %) led to acidification and no methane was produced.

299   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

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

326 obtained results.

327 As it can be observed in the correlation circle, the first component accounted for 54.3 % of the

328 variance, which was mainly attributed to the final pH, the methane yields, the

329 *Methanosarcina* proportions in both the inocula and the digestates and the final VFA

330 concentrations. The pH, methane yields and *Methanosarcina* proportions in the digestates

331 were all strongly positively correlated between them and negatively correlated to the VFA

332 concentrations. Also in agreement with the results presented above, the initial

333 *Methanosarcina* proportions were also significantly correlated with the final pH, the methane

334 yields and the final *Methanosarcina* proportions. Interestingly, the initial working conditions

335 (*i.e.* TS content and initial S/X and C/N ratios) were not correlated to either of the

336 aforementioned variables (*i.e.* pH, methane yields, VFA concentrations and *Methanosarcina*

337 proportions in the digestates), suggesting that similar results were obtained regardless the

338 initial conditions defined in the batch reactors. To summarize, regardless of the initial

339 conditions, methane was efficiently produced in the reactors were *Methanosarcina* was

340 predominant after the digestion, consuming the accumulated VFAs and avoiding a pH drop

341 and reactor acidification.

342 The obtained results highlight the critical relevance of the initial composition of the archaeal

343 populations in the inoculum to achieve efficient AD, especially during reactor start-up. In

344 particular, the results suggest the great importance of *Methanosarcina* and other

345 hydrogenotrophs within the archaeal populations to achieve efficient dry AD of FW. The

346 structure of the archaeal community used to start up batch FW AD may also explain the high

347 variability of the substrate loading limits reported in the literature, ranging from below 0.5 g

348 VS·g VS^{-1} to over 2 g VS·g VS^{-1} (Capson-Tojo et al., 2016). These results could have great

349 implications in industrial scale AD installations, in particular for the start-up of continuous

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

351

352   **4. Conclusions**

353   AD performances of three different inocula were compared using FW and CB as substrates.

354   Particular attention was paid to the compositions of the archaeal communities in the inocula

355   and in the digestates. Regardless of the inoculum used, *Methanosarcina* was the dominant

356   methanogen in all the experiments where methane was produced, suggesting that these

357   archaea played a critical role in methane production at high TAN and VFA concentrations.

358   Higher proportions of *Methanosarcina* in the inocula also allowed greater substrate loads. The

359   initial composition of the archaeal communities in the inoculum was found to be crucial,

360   mainly in batch systems and during reactor start-up. This may have huge implications for

361   industrial-scale installations treating FW and CB.

362

363   **Acknowledgement**

367

368   **References**

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453

Figure and table captions

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

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

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

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 (Sarcina$_0$), the *Methanosarcina* proportions in the digestates (Sarcina$_f$) and the final pH, methane yields (CH$_4$) 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

Table 1. Operational conditions of the batch experiments and obtained methane yields. "UASB1", "UASB2" and "OW" stand for the inoculum

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

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
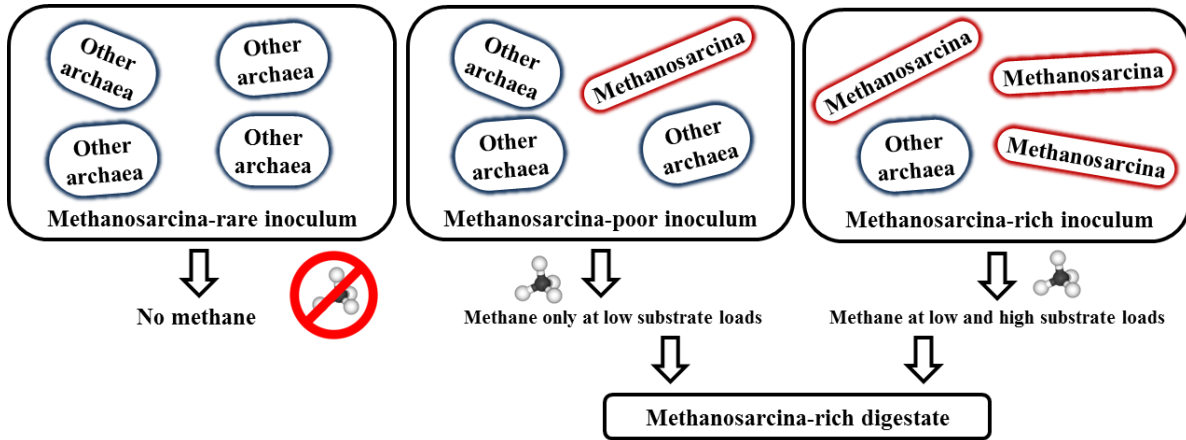
Graphical abstract

Table1

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

Table2

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	-	-	-

Table3

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
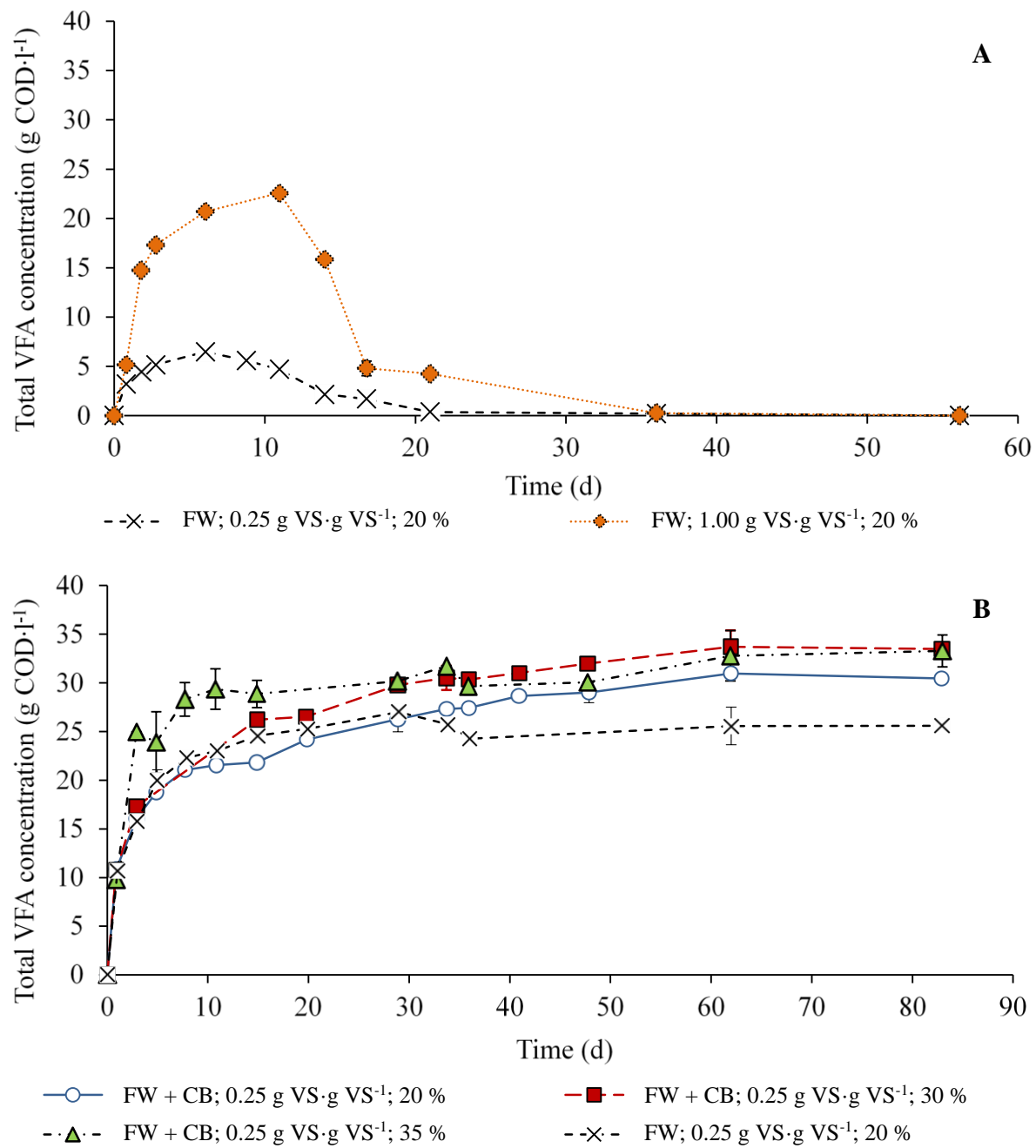2. Not detectable due to too low concentrations

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
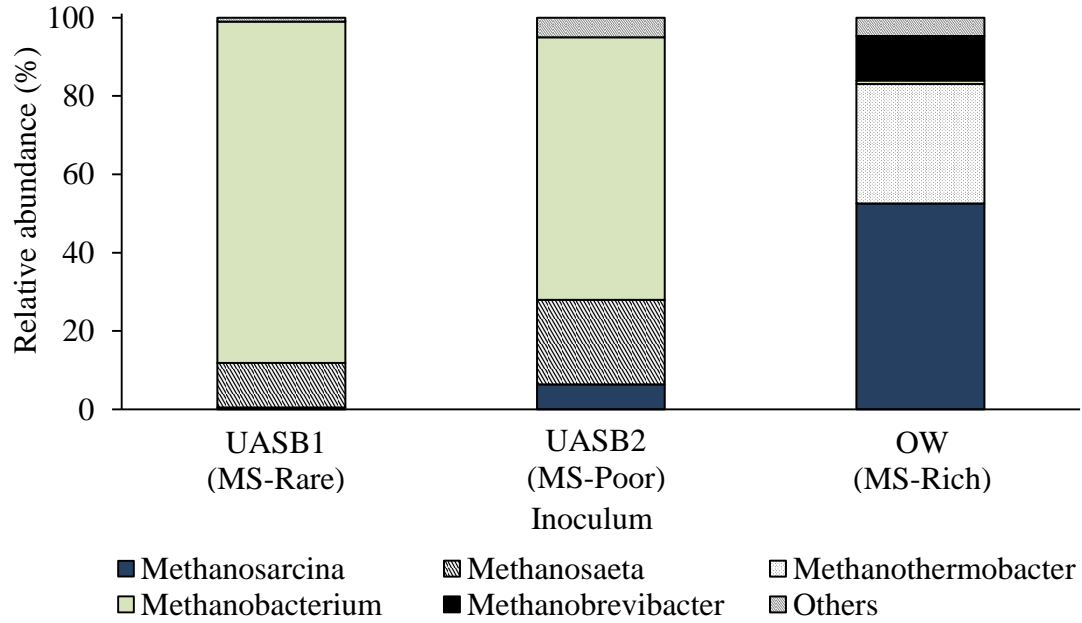
Figure2

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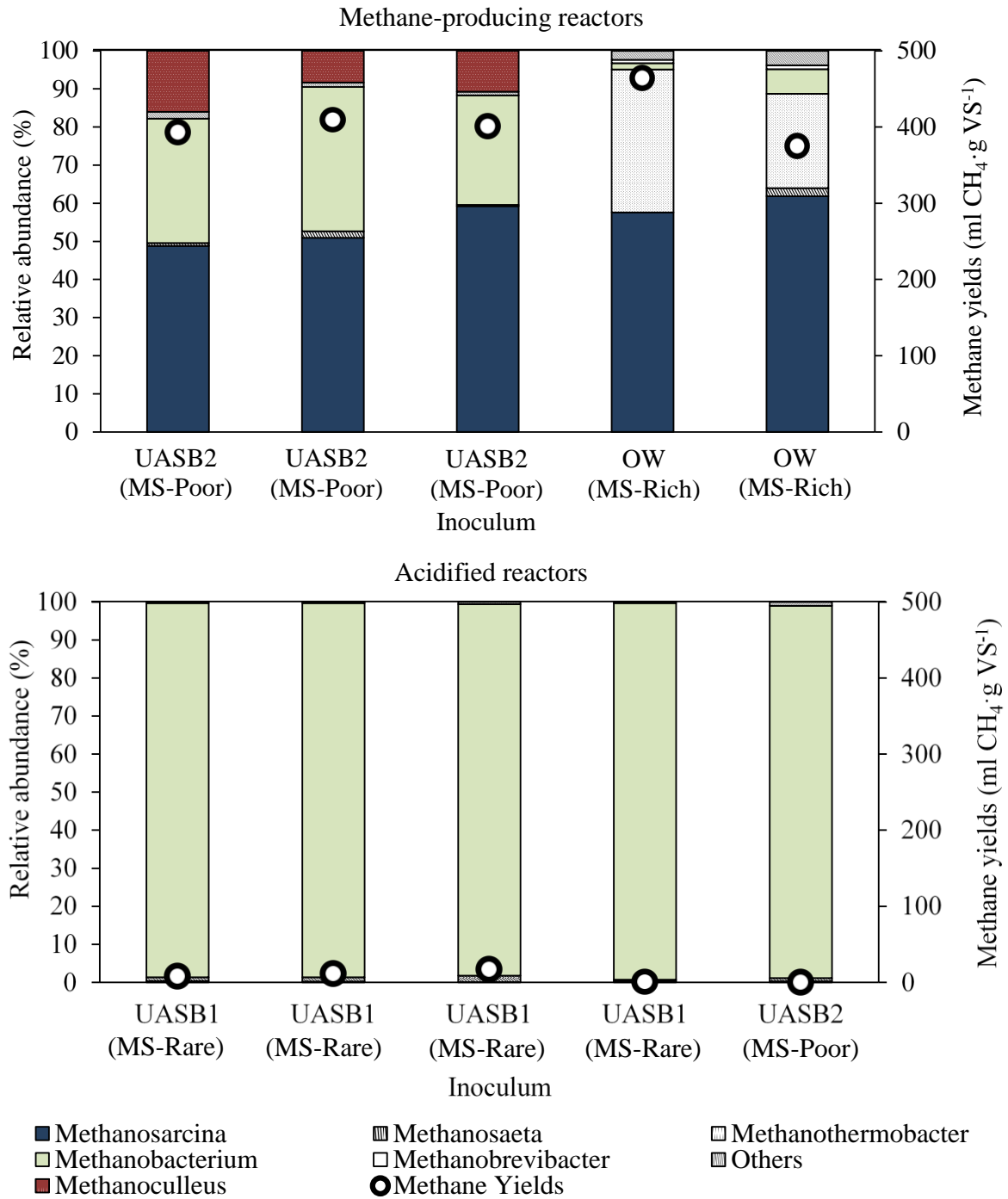
Methanosarcina

Figure3

Figure 3. Relative abundances of archaeal OTUs in the digestates from the batch tests that produced methane (up) and the acidified reactors (down). The methane yields are also presented. The inocula were named "MS-Rare", "MS-Poor" and "MS-Rich" according to their initial relative abundances of *Methanosarcina*
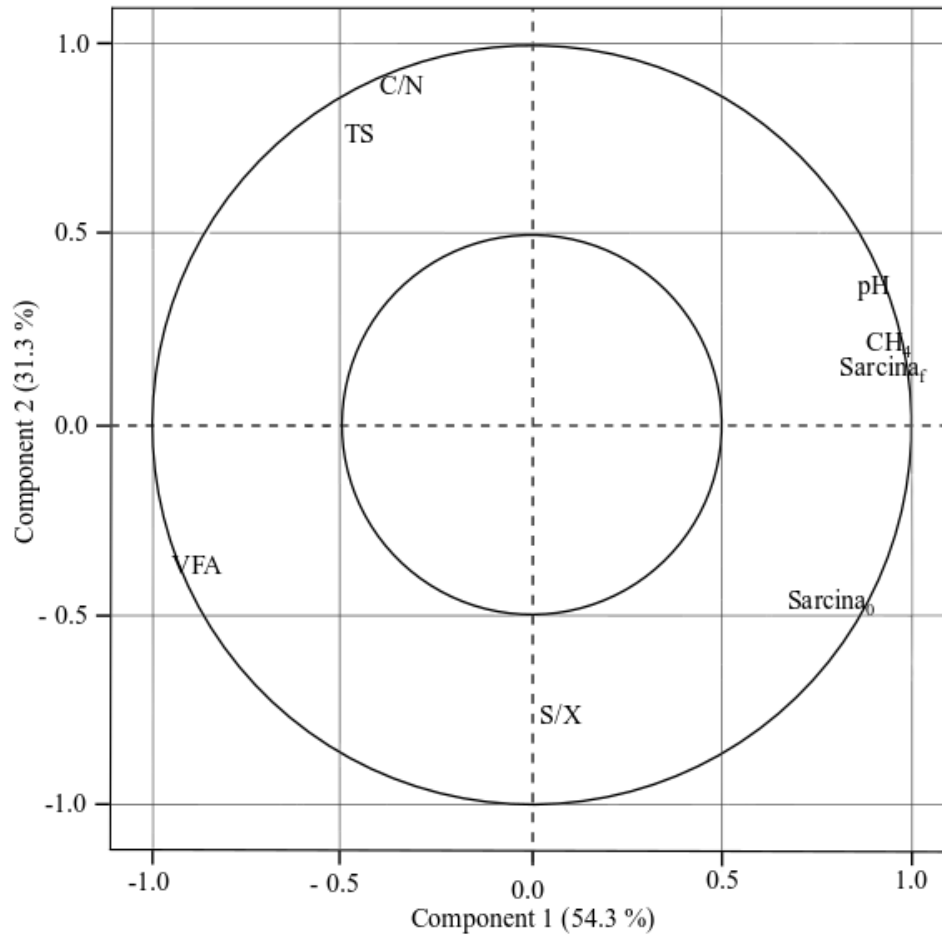
Figure4

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 (Sarcina$_0$), the *Methanosarcina* proportions in the digestates (Sarcina$_f$) and the final pH, methane yields (CH$_4$) 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