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## New concepts in anaerobic digestion processes: recent advances and biological aspects

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1 **Title:** New concepts in anaerobic digestion processes: recent advances and biological aspects

2

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18

19 **Abstract**

20 Waste treatment and the simultaneous production of energy have gained great interest in the world. In the last  
21 decades, scientific efforts have focused largely on improving and developing sustainable bioprocess solutions for  
22 energy recovery from challenging waste. Anaerobic digestion (AD) has been developed as a low-cost organic  
23 waste treatment technology with a simple set-up and relatively limited investment and operating costs. Different  
24 technologies such as, one-stage and two-stage AD have been developed. The viability and performance of these  
25 technologies have been extensively reported, showing the supremacy of two-stage AD in terms of overall energy  
26 recovery from biomass under different substrates, temperatures and pH conditions. However, a comprehensive  
27 review of the advantages and disadvantages of these technologies is still lacking. Since microbial ecology is  
28 critical to developing successful AD, many studies have shown the structure and dynamics of archaeal and  
29 bacterial communities in this type of system. However, the role of Eukarya groups remains largely unknown to  
30 date. In this review, we provide a comprehensive review of the role, abundance, dynamics and structure of  
31 archaeal, bacterial and eukaryal communities during the AD process. The information provided could help  
32 researchers to select the adequate operational parameters to obtain the best performance and biogas production  
33 results.

34

35 **Keywords:** anaerobic digestion; one stage vs two stage; microbiome; Archaea, Bacteria and Eukarya  
36 communities

37

38

## 39 **Introduction**

40 Energy production from renewable sources and efficient waste treatment are two of the more relevant scientific  
41 and social challenges nowadays (De Vrieze et al. 2017). In the last two decades, anaerobic digestion (AD) has  
42 been proven to be a valuable method able to solve both of these issues, combining recycling of different waste  
43 materials with the production of biogas (Oslaj et al. 2010; Tyagi and Lo 2013). Current systems based on AD aim  
44 to convert organic matter into biogas. During this process, hydrolyzing microorganisms hydrolyze organic  
45 polymers (i.e. fats and proteins) producing simple molecules (i.e. sugars, amino acids and fatty acids);  
46 acidogenic microorganisms consume free monomers generating volatile fatty acids (VFAs) and alcohols;  
47 acetogenic microorganisms transform VFA and alcohols into acetic acid, CO<sub>2</sub>, and H<sub>2</sub>; methanogenic archaea  
48 consume acetic acid or hydrogen to generate CH<sub>4</sub> (Gonzalez-Martinez et al. 2016a; Zhang et al. 2016b).

49 AD is a process that can be applied to almost any organic waste. Many different substrates have been  
50 discussed in the literature: agricultural waste, food waste, animal manure, feed waste, energy crops and plant  
51 residues, such as brewery wastewater (Pozo et al. 2002; Chen et al. 2008; Meulepas et al. 2010). In addition to  
52 the digestion of individual substrates, AD reactors can be loaded with mixtures of different residues. This  
53 approach, which is usually termed 'co-digestion' or 'co-fermentation', offers various technical and commercial  
54 advantages. One example is the biostimulating effect coming from the overproduction of nutrients, which can  
55 accelerate the degradation of solid waste (Beyene et al. 2018). Moreover, the application of mono or co-digestion  
56 is an efficient alternative to obtain a stabilized solid waste that can be applied as soil conditioner (Rolando et al.  
57 2011; Gómez et al. 2006).

58 The aim of this review is threefold. First, we will discuss relevant features of AD: the structure of the plants  
59 (one-stage vs two-stage AD), the operational temperature (mesophilic vs thermophilic) and other technologies in  
60 biogas production. A second section will be devoted to describe the role of the microbiome (Archaea, Bacteria  
61 and Eukarya communities) involved in AD and its link to operational and performance parameters and biogas  
62 production. Finally, we will discuss future implications and prospective biotechnologies in AD.

63

## 64 **Digester configurations: advantages and disadvantages**

65 Since the appearance of AD, a wide variety of digester configurations has been tested such as  
66 thermophilic/mesophilic digestion, dry/wet digestion, one-phase/two-phase digestion or one-stage/two-stage  
67 digestion (Møller et al. 2009; Nizami et al. 2009; Khalid et al. 2011; Mao et al. 2015; Sun et al. 2015; Chen et al.  
68 2016). Among these, the most relevant comparison, as well as the one most debated in the literature, is that based  
69 on the number of stages. However, independently of the digester configuration to obtain a high digestion  
70 efficiency, anaerobic bioreactors should allow a continuously high and sustainable organic load rate operating  
71 with short (Khalid et al. 2011) or long (Bergland et al. 2015) hydraulic retention time (HRT) depending on the  
72 substrate.

73 The simplest possible configuration is the one-stage AD batch reactor, in which the tank is filled with the  
74 feedstock and let stand for a period after which it is emptied (Khalid et al. 2011). Although this kind of system  
75 has very low operational cost, it exhibits some limitations such as high fluctuations in gas production, biogas  
76 losses during emptying the bioreactors and restricted bioreactor heights (Khalid et al. 2011; Zhang et al. 2015;  
77 Sunyoto et al. 2016). A more widely used type of one-stage AD bioreactor is commonly defined 'one-stage  
78 continuously fed systems' (Khalid et al. 2011). In one-stage AD system, hydrolysis, acidogenesis, acetogenesis

79 and methanogenesis take place in the same tank. This implies that acidogenic and methanogenic microbiota have  
80 to cohabit despite the existence of marked differences regarding growth factors and kinetics, nutritional needs  
81 and environmental conditions such as pH and temperature (Gonzalez-Martinez et al. 2016b; De Gioannis et al.  
82 2017). In this context, although the ideal pH range for AD has been reported to be between 6.8–7.4, it is known  
83 that in one-stage AD bioreactor the operational pH sometimes can affect the digestive progress and products  
84 directly. However, two-stage AD process separating the hydrolysis/acidification and  
85 acetogenesis/methanogenesis processes, provides optimal conditions for each of the microbiota, since the  
86 optimal pH levels for acidogenic (5.5–6.5) and methanogenic (7.0) microorganisms can be controlled to increase  
87 the efficiency of the process (Mao et al. 2015). Consequently, in these kinds of systems, the different sub-  
88 processes of AD take place in separate sequential reactors. The most common configuration is the two-stage  
89 continuously fed system, although three-stage systems have been proposed (Angelidaki et al. 2003). Two-stage  
90 AD were originally conceived by Pohland and Ghosh (1971), and soon gained popularity, particularly for  
91 laboratory applications (Nizami et al. 2009). Although overall performance supremacy of two-stage AD has been  
92 variously reported in the literature, one-stage AD are far from being replaced (Møller et al. 2009). According to  
93 Rapport et al. (2012), 90% of the total capacity of the full-scale AD plants installed in Europe at that time was  
94 covered by one-stage systems. The main reasons behind this are probably the simpler structural features and  
95 lower operating costs. On the other hand, two-stage AD provides higher substrate conversion and better energy  
96 recovery, as well as better process stability, resilience and reliability (Salvador et al. 2013; De Gioannis et al.  
97 2017; Shen et al. 2017).

98 Multiple-stage reactors have been developed to improve process stability and efficiency (Achinis et al.  
99 2017). In this sense, Kim et al. (2011) demonstrated significantly higher digestion efficiency of a four-stage AD  
100 system using activated sludge than a single-stage system. Likewise, a novel alternative technique based on a high  
101 working pressure (up to 100 bar), permits the production of biogas with more than 95% methane content. This  
102 technique integrate in a single process both biogas production and *in situ* increased-pressure purification,  
103 generating a clean biogas (99% methane) that can be fed directly into the natural gas networks. However, the  
104 effect of the working pressure on microbiome structure is still unknown (Lindeboom et al. 2011). The  
105 complexity and high cost of this novel technologies are barriers to commercial use and until date, few multiple-  
106 stage AD units operate on a commercial scale.

107

### 108 **Thermophilic and mesophilic conditions**

109 A further relevant way to classify AD systems is to consider their operating temperature. Although the biogas  
110 process can proceed at different temperatures, mesophilic (30–40°C) and thermophilic (50–60 °C) conditions are  
111 commonly used (Møller et al. 2009; Wang et al. 2018). Temperature is, indeed, one of the main environmental  
112 factors affecting physical parameters such as viscosity, surface tension and mass transfer properties. Moreover,  
113 small changes in the temperature can result in a reduction in process efficiency, so its stability is also important  
114 (Angelidaki et al. 2003). Above all, temperature must be considered in relation to microbial growth and reactions  
115 (Amani et al. 2010; Gonzalez-Martinez et al. 2017) and changes in the structure and dynamics of prokaryotic and  
116 eukaryotic groups (see Section 2). The groups of microbes that have been identified for AD are mesophilic and  
117 thermophilic strains. While great diversity exists between mesophilic and thermophilic bacteria, with the latter

118 showing both higher specific growth and decay rates, methanogen growth is mostly favoured by both mesophilic  
119 and thermophilic temperatures (Li et al. 2015; Kundu et al. 2017).

120 Neither of the two conditions (i.e. mesophilic or thermophilic) is absolutely preferable. Although mesophilic  
121 digestion has some disadvantages (i.e. lower metabolic rate, lower rate and efficiency of particulate matter  
122 hydrolysis, smaller degree of pathogen deactivation and lower biogas production yields) (Liu et al. 2017), it has  
123 important advantages, such as a lower VFA concentration in the final effluents, maintenance of a higher organic  
124 loading rate (OLR) (Bayr et al. 2012) and a more stable performance (Guo et al. 2014), compared to  
125 thermophilic digestion (Appels et al. 2008; Wang et al. 2017). On the other hand, thermophilic temperatures can  
126 produce large quantities of dissolved solids in the digester supernatant and more odours, and have acidification  
127 potential and higher energy requirements. For these reasons, two-stage AD offers the opportunity to operate  
128 thermophilic hydrolysis/acidogenesis and mesophilic methanogenesis, as a good compromise. Of note, a  
129 different approach not requiring an extra heat supply, named '*ambient/seasonal temperature AD*', has also been  
130 used for organic waste. However, the changes in temperature induce less stability and lower methane production  
131 compared with the mesophilic process (Mao et al. 2015).

132

### 133 **Biogas production**

134 Currently, AD is implemented in various ways worldwide. In the Western world there are, to date, about ten  
135 thousands of operational AD plants (Yousuf et al. 2016; Vasco-Correa et al. 2018). A comparable amount can be  
136 found in Asia, where rural communities use small-scale household digesters for domestic necessities (Surendra  
137 et al. 2014). Similar small-scale digesters have also been installed in rural regions of Latin America and Africa  
138 during the last few years (REN21, 2016). Laws on the subject of environmental protection and waste treatment,  
139 as well as new emerging candidate substrates and innovative technologies, will surely guide the evolution of AD.

140 Different compositions of mixed substrates have been reported to increase the production of biogas, such as  
141 mixing municipal solid waste with industrial sludge (Ağdağ and Sponza 2007) or olive mill wastewater with  
142 olive mill solid waste (Fezzani and Cheikh 2010). In addition, co-digestion has been proved to stabilize reactor  
143 performance (Lo et al. 2010; Beyene et al. 2018). Interestingly, the use of this approach with substrates rich in  
144 carbon has been proposed as a solution to reduce ammonia and other toxic substances (Rajagopal et al. 2013;  
145 Fitamo et al. 2017). Moreover, co-digestion is an efficient strategy to degrade those kinds of waste that are  
146 difficult to process as a unique substrate. Recently, Park et al. (2016) tested different mixtures in order to  
147 optimize the processing of sewage sludge, obtaining optimal results in combination with food waste. As a further  
148 solution, Shen et al. (2017) proved that the combination of sewage sludge and pyro-biochar can improve  
149 biomethane production, compared with the digestion of sewage sludge alone.

150 As an example, the Korean government recently solicited the use as an AD substrate of organic waste from  
151 ocean dumping or landfill, with the aim to produce renewable energy; this raises the issue of efficiently  
152 degrading septage and sewage sludge, and the consequent investigation of different mixtures for co-digestion  
153 approaches (Park et al. 2016). Otherwise, good availability of a specific kind of waste can turn it into a candidate  
154 substrate. In Colombia, for example, the massive production of coffee generates a large amount of coffee  
155 mucilage, a crop residue rich in carbohydrates. This organic matter has been successfully used in co-digestion  
156 with pig manure to produce biohydrogen, taking advantage of two types of organic waste readily available in the

157 same geographical region (Hernández et al. 2014). Finally, technical innovations will help the scale-up of  
158 currently experimental systems.

159 Biohythane is a promising sustainable alternative to hythane. It is more environmentally friendly, requires a  
160 shorter fermentation time and offers better energy recovery than traditional biogas. Despite research interest in  
161 the production of this gas, numerous challenges have still to be addressed in order to allow large-scale  
162 production of biohythane by means of AD (Liu et al. 2018). Similarly, technical improvements are needed for  
163 the realization of full-scale three-stage AD plants. Hitherto, an in-lab preliminary study has proved that this  
164 approach could considerably improve the production of methane (Zhang et al. 2017). A further promising  
165 strategy to increase biogas yield and system performance is the application of selected microbial consortia, often  
166 taken from another operating plant. However, more accurate knowledge concerning adaptation of the inoculum  
167 is required in order to maximize the potential advantages of this approach (Wojcieszak et al. 2017).

168

### 169 **Archaea, Bacteria and Eukarya communities in anaerobic digestion processes**

170 Integration of microbial aspects within the framework of AD is critical to achieve the desired performance and  
171 biogas production. The microbiome as an entity does not work as a randomized mix, and scientific efforts focus  
172 largely on linking operational and performance parameters with the structure of microbial communities. Here,  
173 we highlight engineering of the microbiome, focusing on the most crucial Archaea, Bacteria and Eukarya  
174 groups.

175

### 176 **Abundance, structure and dynamics of the microbiome in anaerobic digestion processes**

177 Microbial ecologists and engineers have shown increasing interest concerning insight into the microbiome in  
178 anaerobic digesters. So far, the most crucial microorganisms have been identified although few authors have  
179 linked operational and performance parameters and microbiome response at laboratory or full-scale conditions  
180 (Carballa et al. 2011; Werner et al. 2011; Carballa et al. 2015; Gonzalez-Martinez et al. 2016b; De Vrieze et al.  
181 2017; Kundu et al. 2017; Wang et al. 2018). Since a strong syntrophic relationship exists between acetogenic and  
182 methanogenic organisms involved in AD, biomonitoring of the system could be an important feature for  
183 engineers to obtain a highly efficient microbiome and to predict and prevent system failure (Amani et al. 2010).  
184 For example, Kundu et al. (2013) showed that a high degree of microbial diversity could be indicative of stable  
185 AD performance. Recently, a methodological approach to link microbial and operational data has also been  
186 described (de Los Reyes III et al. 2015).

187 The development of next-generation sequencing technologies has offered an opportunity to describe the  
188 microorganisms present (DNA) or active (RNA) in engineered ecosystems as well as their abundance (Muñoz-  
189 Palazon et al. 2018). Nevertheless, a combined DNA–RNA approach would result in a more accurate  
190 methodology to link the microbial community's structure and its metabolic ability requirements (Kaeffer et al.  
191 2014; Maus et al. 2016). Identification of the critical representative species by means of these techniques can  
192 help to increase the efficiency and stability of AD (Venkiteshwaran et al. 2015; Dang et al. 2017). In this sense,  
193 the presence of sulphate-reducing bacteria in AD can decrease methane production because of substrate  
194 competition and sulphide inhibition of the methanogenic community (Chen et al. 2008; Sasaki et al. 2011). Thus,  
195 biomonitoring tools can help to prevent inefficiencies in AD.

196 The AD process comprises four interdependent steps in which microorganisms responsible for a specific  
197 stage provide the intermediates for the next. Microbial community structure and dynamics are important to  
198 sustain functional redundancy and to maintain a well-balanced process (Allison and Martiny 2008; Ziganshin et  
199 al. 2013). Archaea, Bacteria and Eukarya communities form the microbiome of the anaerobic digester and  
200 change during the stages of the AD process (Matsubayashi et al. 2017).

201 Archaea play a central role during methanogenic processes of AD, and it has been reported that these  
202 microorganisms can be related to different operational parameters (Zhang et al. 2012; Smith et al. 2014; Hao et  
203 al. 2016). Synthesis of CH<sub>4</sub> is carried out both by acetoclastic (e.g. *Methanosaeta*, *Methanosarcina* and  
204 *Methanotherix*) and hydrogenotrophic methanogens (e.g. *Methanobacterium*, *Methanomicrobium*,  
205 *Methanococcus*, *Methanobrevibacter*, *Methanomassilii* and *Methanospirillum*) using acetic acid, or by using H<sub>2</sub>  
206 and CO<sub>2</sub> or methyl compounds to synthesize CH<sub>4</sub> (Calderón et al. 2013; Gonzalez-Martinez et al. 2016b). The  
207 characteristics and properties of the main methanogens involved in an AD as well as their substrates and  
208 products have been reported (McHugh et al. 2003; Amani et al. 2010; Goswani et al. 2016; Kundu et al. 2017).  
209 In most of the studies in the literature, Archaea diversity decreases with temperature elevation (Kundu et al.  
210 2012; Guo et al. 2014), an effect more remarkable than changes in OLR which abrupt increase (from 1 to 8 g VS  
211 L<sup>-1</sup> d<sup>-1</sup>) seemed to have little influence on the microbial community (Gou et al. 2014). Hao et al. (2016)  
212 compared the effect of total solid (TS) concentrations on archaeal diversity in sludge-fed digesters. Under high  
213 TS conditions (TS > 44 g/L), the relative abundance of *Methanosarcinaceae* and *Methanobacteriaceae* families  
214 increased whereas when digesters operated at lower-TS (TS ≤ 44 g/L) only *Methanosaetaceae* family was  
215 favoured. Under the use of continuous lab and full-scale reactors and food waste substrate the genus  
216 *Methanosarcina* is dominant under thermophilic conditions, with abundance higher than 80%, although  
217 *Methanothermobacter* and *Methanoculleus* are also favoured (Cho et al. 2013; Wang et al. 2018), whereas  
218 *Methanosaeta* is dominant under mesophilic conditions (accounting for >25% of relative abundance) (Gonzalez-  
219 Martinez et al. 2016b). On the other hand, *Methanosaeta* instead of *Methanosarcina* is favoured under low acid  
220 concentrations. Since VFA accumulation results in lower values for pH, Guo et al. (2014) showed a decrease in  
221 archaeal diversity when VFAs produced in the hydrolytic step are not consumed by methanogens. In fact,  
222 acetoclastic methanoarchaea have a positive correlation with VFAs and NH<sub>4</sub><sup>+</sup> (Lin et al. 2012). Methanogen  
223 diversity is also sensitive to a pH value lower than 6.5, particularly during acid and acetate accumulation (Bräuer  
224 et al. 2006). In general, lower hydraulic retention time values decrease archaeal diversity by selecting organisms  
225 with a high growth rate and poor substrate affinity. In this sense, *Methanosaetaceae* (slower growth rate)  
226 predominate when HRT > 5 days, while *Methanosarcinaceae*, *Methanobacteriales* and *Methanomicrobiales*  
227 (faster growth rate) become dominant at HRT < 2 days (Padmasiri et al. 2007; Chelliapan et al. 2011). Regueiro  
228 et al. (2014) reported that *Methanosaeta* is crucial for reaching stable reactor performance although the archaeal  
229 community structure is affected by substrate type. Moreover, taking into account operational performance  
230 parameters, Kundu et al. (2017) indicated *Methanosaetaceae* as the best candidate for biomonitoring based on its  
231 sensitivity to fluctuations in the AD process.

232 The presence of bacterial genera such as *Desulfotomaculum*, *Desulfovibrio*, *Syntrophobacter*,  
233 *Syntrophomonas*, *Syntrophospora*, *Clostridium*, *Bacteroides*, *Bifidobacterium*, *Butyrivibrio*, *Pseudomonas*,  
234 *Bacillus*, *Streptococcus* and *Eubacterium* has been related to acid formation and hydrogen release (Yamada et al.  
235 2006; Gonzalez-Martinez et al. 2016a), and synergistic cooperation with methanogenic archaeal groups in



236 methanogenesis bioreactors has also been considered (Demirel and Scherer 2008). González-Martínez et al.  
237 (2016b) studied archaeal and bacterial community dynamics of a bench-scale two-stage anaerobic digester. An  
238 overview of the response of key archaeal and bacterial phylotypes to changes in performance parameters is  
239 presented in Fig. 1a and 1b, respectively.

240 In the acidogenic phase, organic matter is biodegraded to VFAs by bacterial communities. During this phase,  
241 *Bacteroidetes*, *Chloroflexi*, *Cloacimonetes*, *Firmicutes* and *Proteobacteria* are the predominant phyla. Moreover,  
242 *Microthrix* spp. are usually associated with operational dysfunction while *Firmicutes* species in the digesters are  
243 important acetogens utilizing simple and complex carbohydrates (Tracy et al. 2012). *Synergistetes* spp. can  
244 utilize amino acids as an energy source to produce VFAs for methanogens (Vartoukian et al. 2007), whereas  
245 *Proteobacteria* have been recognized as one of the main consumers of VFAs (Ariesyady et al. 2007). Moreover,  
246 *Syntrophomonas* strains are present during this phase and are able to syntrophically degrades straight-chain fatty  
247 acids (4–8 carbon atoms) into propionate, acetate and methane in co-culture with methanogens (Zhang et al.  
248 2005).

249 Changes in operational and performance parameters influence bacterial diversity. Hao et al. (2016) found that  
250 under high TS conditions, the relative abundance of *Thermoanaerobacteraceae*, *Syntrophomonadaceae*,  
251 *Rhodobacteraceae*, *Comamonadaceae* and *Xanthomonadaceae* families were enriched. In contrast, digesters at  
252 lower-TS favoured *Syntrophaceae*, *Syntrophobacteraceae*, *Anaerolineaceae*, *Rikenellaceae* and *WCHB01-69*  
253 and *Candidatus Cloacamonas* families. Under thermophilic and mesophilic conditions, Guo et al. (2014) found  
254 that *Firmicutes* was the common phylum appearing at both temperatures, accounting for 10–20% of relative  
255 abundance. *Thermotogae* (60–80% of relative abundance) and *Bacteroidetes* (5–45% of relative abundance)  
256 were the dominant taxa under both conditions, respectively. *Proteobacteria* were present in limited amounts and  
257 only in thermophilic AD whereas *Synergistetes* appeared in both reactors. Although the relative abundance of  
258 *Chloroflexi*, *Actinobacteria* and *Spirochaetes* was higher than that in thermophilic AD, they were poorly  
259 represented, accounting for <3% of relative abundance. Finally, *Gelria*, uncultured *Lachnospiraceae*,  
260 *Ruminococcaceae Incertae Sedis*, *Sporanaerobacter*, *Tepidanaerobacter*, *Petrobacter* and *Anaerobaculum* were  
261 related to performance variations with OLR elevation.

262 Adaptation of bacterial communities during the start-up stage of thermophilic and mesophilic AD was  
263 explored by Wu et al. (2016) and González-Martínez et al. (2016b), respectively. Under thermophilic conditions,  
264 the relative abundance of *Firmicutes* increased gradually; on the contrary, *Proteobacteria* and *Thermotogae*  
265 decreased. Under mesophilic conditions, the more abundant microorganisms were related to *Clostridiaceae*  
266 (21.49%), *Treponema* (5.10%), *Synergistetes* (4.11%) and *Paenibacillaceae* (3.25%) whereas *Cloacamonas* and  
267 *Comamonas* were present at >3% abundance only at the beginning of AD, decreasing after that. Zhang et al.  
268 (2016a) analysed the microbial community in the anaerobic co-digestion of food waste and sewage sludge.  
269 *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were found as the predominant phyla in the  
270 bacterial community. *Firmicutes* increased significantly on day 5 at acidification phase corresponding to VFAs  
271 accumulation. After that, the abundance of *Firmicutes* and *Bacteroidetes* increased much more from day 12 at  
272 the active methane production phase. *Proteobacteria* and *Actinobacteria* decreased significantly during the  
273 experimental period. The greatest changes in these four dominant phyla all appeared on day 5, which could be an  
274 indicator of the acidification phase corresponding to VFA accumulation. Hydrolytic bacteria are known to have a  
275 lower sensitivity to changes in environmental factors, such as pH and temperature, than methanogens.

276 Although the role of eukaryotes in performance, predation on bacteria and excess sludge production has been  
277 reported during aerobic treatment processes (Ntougias et al. 2011), it is also important to investigate the  
278 diversity, roles and functions of eukaryotes in AD. Few authors have reported on diversity and roles/functions in  
279 AD (Luo et al. 2005; Matsubayashi et al. 2017). Under mesophilic AD, *Rotifera* and *Phragmoplastophyta* are the  
280 most representative phyla and the majority of eukaryal phylotypes belong to Fungi (42.2%), followed by  
281 Animalia (28.8%), Protista (13.3%) and finally Plantae (8.9%). In addition, Luo et al.(2005) described the  
282 microeukaryotic community in anaerobic sulphide- and sulphur-rich springs, whereas Matsubayashi et al. (2017)  
283 constructed clone libraries by sequencing the 18S rRNA gene in anaerobic sludge digesters (Table 1). The latter  
284 study suggested that prokaryotic and eukaryotic community structures do not work independently, and that the  
285 functional features of both communities are closely related.

286 Very limited information on the physiology of anaerobic or facultative anaerobic eukaryotic organisms is  
287 available to date. Some of the Fungi found in AD contribute to the degradation of some organic matter in  
288 anaerobic environments and they could be implicated in the hydrolysis of organic matter in anaerobic sludge  
289 digestion processes. Previous studies have demonstrated that phylotypes in Plantae, Animalia and Fungi can  
290 produce CH<sub>4</sub> (Liu et al. 2015; Gorrasi et al. 2014).

291 Regarding the dynamics of the microbiome during AD, contrasting results have been obtained, showing large  
292 changes (>25%) from bench-scale mesophilic anaerobic digesters inoculated with sludge from wastewater  
293 treatment plants (Schauer-Gimenez et al. 2010; De Vrieze et al. 2013) or high consistency from reactors with an  
294 upflow configuration with anaerobic granular biomass (Werner et al. 2011). Given the presence of a wide variety  
295 of microorganisms in the influent of AD, dynamic changes in community diversity are likely the result of  
296 proliferation of organisms that are adapted to the selective pressures in each bioreactor. However, a core  
297 microbiome dominates the reactors, showing the strong selective pressures present in this type of environment  
298 (Town et al. 2014; Gonzalez-Martinez et al. 2016b). Maspolim et al. (2015) compared the microbial community  
299 dynamics in single-stage and 2-phase anaerobic AD systems treating municipal sludge and the analysis revealed  
300 that microbial adaptation occurred as the sludge formed a different community in each reactor at 30 d HRT but  
301 no significant microbial changes occurred at lower HRTs. Engineering of the microbiome by adjusting  
302 operational parameters leads to a stable microbial structure (Vanwonterghem et al. 2014; De Vrieze et al. 2016).  
303 Accurate monitoring of the microbial community requires that the metabolic potential and mode of interaction  
304 between the different microorganisms are distinguished from sudden unwanted changes related to unfavourable  
305 operational conditions. While generalist microorganisms are able to occupy a broad range of niches based on  
306 their greater phenotypic plasticity (van Tienderen 1997), specialists occupy only a limited number of niches and  
307 show high levels of specificity. The former can be considered as keepers of process stability (Matias et al. 2013)  
308 whereas the latter may drive evolution towards new traits in the microbial community and could be of direct  
309 interest in the search for new potential.

310 The dynamics of prokaryotic organisms have been described during the start-up stage of AD (Gonzalez-  
311 Martinez et al. 2016b) as showing substantial changes under unstable conditions. Thus, a challenge exists to  
312 develop a useful biomonitoring tool for environmental engineers. Many studies have indicated that  
313 *Methanosaeta* and *Methanosarcina* are competitive genera in the AD process. During the acidification phase,  
314 *Methanosaeta*, an acetoclastic methanogen, is the dominant genus but its abundance decreases significantly  
315 during the methane production phase. In the latter phase, the acetoclastic methanogen *Methanosarcina* increases

316 significantly. *Methanosarcina* is more tolerant to inhibitors than *Methanosaeta* (Cho et al. 2013). At the end of  
317 AD, *Methanoculleus*, a hydrogenotrophic methanogen, becomes dominant because of the exhaustion of acetic  
318 acid. Previous studies have reported that for continuous and fed-batch systems, bacterial community dynamics  
319 show larger changes than those for the archaeal community, but there is similar diversity, and VFA-producers  
320 show greater relative abundance. Generally considered, the hydrolysing bacterial groups *Bacteroides*,  
321 *Cloacamonas*, *Clostridiaceae* and *Rikenellaceae* are dominant at the beginning of AD and finally change to  
322 other bacterial groups such as *Clostridiaceae*, *Fervidobacterium*, *Paenibacillus* and *Spirochaetes* (Ghasimi et al.  
323 2015;Gonzalez-Martinez et al. 2016b).

324

### 325 **Microbial and Eukaryal groups involved in biogas production**

326 AD for methane production has already been widely adopted (Cavinato et al. 2013; Carrere et al. 2016) using  
327 methanogenic microorganisms able to utilize simple organic substrates, such as acetate, CO<sub>2</sub>/H<sub>2</sub>, methanol and  
328 formate (de Bok et al. 2004). A deep insight into the main archaeal and bacterial phylotypes of AD involved in  
329 biogas production under different operational conditions can be seen in Hao et al. (2016). There are three main  
330 types of methanogen, namely acetoclastic, hydrogenotrophic and methylotrophic. Most archaea produce methane  
331 by the hydrogenotrophic route and only those belonging to the order *Methanosarcinales* produce it by the  
332 acetoclastic route. *Methanobacterium*, *Methanothermobacter*, and *Methanospirillum* are the most commonly  
333 identified hydrogenotrophic methanogens in the AD process. Acetoclastic methanogens belong to two genera:  
334 *Methanosaeta* and *Methanosarcina* (Venkiteshwaran et al. 2015; Gonzalez-Martinez et al 2016b). *Methanosaeta*  
335 can be considered a key methanogen in the AD process, given its unique morphology and physiology (De Vrieze  
336 et al. 2012; 2015), catalysing renewable energy production from organic waste streams.

337 Bacteria can support methane production during the process of methanogenesis by hydrolysis of organic  
338 matter. Positive correlation of *Cytophaga*, *Herbaspirillum*, *Symbiobacterium*, *Comamonas* and *Allochromatium*  
339 with biogas production has been found (Gonzalez-Martinez et al. 2016b). The genera *Cytophaga* and  
340 *Symbiobacterium* are important organic matter degraders in AD in the hydrolysis and acidogenesis processes,  
341 respectively (Panichnumsin et al. 2012; Yi et al. 2014).The importance of *Herbaspirillum* sp. remains widely  
342 unclear due to its inability to carry out fermentation (Schmid et al. 2006), but its relationship to biogas  
343 production (Gonzalez-Martinez et al. 2016b) and degradation of complex organic matter has been reported (Guo  
344 et al. 2015).

345 The role of Eukarya in the production of methane remains largely unknown although Plantae, Animalia and  
346 Fungi eukaryal phylotypes have been reported to direct produce CH<sub>4</sub>,even in the presence of oxygen (Liu et al.  
347 2015; Gorrasi et al. 2014). However, the mechanisms involved in this pathway remain largely unclear and it has  
348 been proposed that CH<sub>4</sub> originates from organic methyl-type compounds in response to environmental stresses.  
349 Although it is estimated that plants could contribute around 3–24% to the global CH<sub>4</sub> budget, an estimate of CH<sub>4</sub>  
350 production by animals and fungi is still lacking. Consequently, Eukarya are not considered as a CH<sub>4</sub> source by  
351 the Intergovernmental Panel on Climate Change (IPCC), and their role in biogas production could be useful for  
352 better quantitation of the global CH<sub>4</sub> budget. The influence of rumen fungi for improvement of biogas production  
353 from animal manure on anaerobic digesters have gained attention as a biological pre-treatment option of various  
354 polymeric substances. These microorganisms are able to effectively digest lignocellulosic compounds, providing  
355 energy due to symbiotic associations with rumen microorganisms (Yıldırım et al. 2017). For instance, Gorrasi et

356 al. (2014) demonstrated the potential application of chitinolytic fungi to obtain H and Ma et al. (2015)  
357 determined that rumen microorganisms have higher hydrolytic and acidogenic activity than other microbial  
358 species using lignocellulosic biomass as substrates.

359

### 360 **Future implications and prospective biotechnologies**

361 New advances in monitoring AD will require the application of control strategies to redirect the microbiome to  
362 reach a stable functionality. Until now, microbial process control actions have usually taken place by altering  
363 basic operational parameters, such as pH and temperature. For example, increases in AD efficiency were done  
364 using different ways: bioaugmentation, as a suitable alternative to increase VFA removal (Town and  
365 Dumonceaux 2016) or hydrolysis (Martin-Ryals et al. 2015); microwave (MW) pre-treatment, as an effective  
366 way of enhancing biogas production and solids removal (Coelho et al. 2011). However, to engage direct steering  
367 of the microbiome to sustain process stability, this knowledge has to be integrated into advanced monitoring and  
368 control strategies. For example, the ratio of syntrophic acetate-oxidizing bacteria or methanogenic archaea to  
369 total bacteria has been suggested as a possible microbial community monitoring strategy for AD (De Vrieze et  
370 al. 2012). This has to be based on specific genes and/or their transcripts, such as the methyl co-enzyme M  
371 reductase (*mcrA*) gene for methanogens (Wilkins et al. 2015) and the formyl tetrahydrofolate synthetase  
372 (FTHFS) gene for syntrophic acetate-oxidizing bacteria (Akuzawa et al. 2011; Hori et al. 2011).

373 The study of biogeochemical cycles in natural ecosystems can drive innovation in bioenergetics applications  
374 to support improvements of AD. In this sense, Izzo et al. (2014) explored the potentials offered by the structural  
375 and functional microbial biodiversity in hypertrophic lagoons characterised by rapid and huge biomass blooms  
376 and decomposition. They selected the microbial communities as inoculum and successfully tested for hydrogen  
377 production on different kinds of organic wastes.

378 To decrease the cost of the treatment is of vital importance in AD. This can be achieved by using raw  
379 material with lower water content and running the process with a higher dry matter content. The biogas produced  
380 can often be utilized to cover the need for process energy. Thus, the economy of a biogas plant is directly linked  
381 to the amount of biogas produced per unit of raw material treated in the plant. In short, advanced and direct  
382 monitoring of the microbiome is possible through the application of different microbial techniques. Accurate and  
383 quick decision tools have to be developed. The integration of existing physicochemical techniques and  
384 microbiome-based monitoring is necessary to increase product recovery and the overall energy efficiency of  
385 microbial processes.

386

### 387 **Compliance with ethical standards**

388 **Conflict of interest** The authors declare that they have no conflict of interest.

389 **Ethical approval** This article does not contain any studies with human participants or animals performed by  
390 any of the authors.

391

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715

716 **Table 1** Main eukaryal phylotypes found in anaerobic digesters. Data were taken from Matsubayashi et al. (2017).

<i>Kingdom/ Superphylum</i>	<i>Phylum</i>	
<i>Alveolata</i>	<i>Perkinsozoa</i>	<i>A31</i>
<i>Amoebozoa</i>	<i>Discosea</i>	<i>Order Dactylopodida</i>
	<i>Gastrotricha</i>	<i>Chaetonotus cf.</i>
<i>Animalia</i>	<i>Gastrotricha</i>	<i>Chaetonotus cf.</i>
<i>Archaeplastida</i>	<i>Chlorophyta</i>	<i>ANI-3</i>
	<i>Chlorophyta</i>	<i>Family Chlorellaceae</i>
	<i>Chlorophyta</i>	<i>Prototheca zopfi</i>
	<i>Ciliophora</i>	<i>Acaryophrya sp.</i>
	<i>Ciliophora</i>	<i>Vorticellides aquadulcis</i>
<i>Fungi</i>	<i>Arthropoda</i>	<i>Allonothrus sp.</i>
	<i>Arthropoda</i>	<i>Boletoglyphus sp.</i>
	<i>Arthropoda</i>	<i>Naidacarus arboricola</i>
	<i>Arthropoda</i>	<i>Rhizoglyphus sp.</i>
	<i>Ascomycota</i>	<i>Candida sp.</i>
	<i>Ascomycota</i>	<i>Exophiala equine</i>
	<i>Ascomycota</i>	<i>Family Dipodascaceae</i>
	<i>Ascomycota</i>	<i>Penicillium chrysogenum</i>
	<i>Ascomycota</i>	<i>Phoma sp.</i>
	<i>Ascomycota</i>	<i>Xenobotrytis sp.</i>
	<i>Basidiomycota</i>	<i>Lentinus sp.</i>
	<i>Basidiomycota</i>	<i>Trichosporum cutaneum</i>
	<i>Cryptomycota</i>	<i>LKM11</i>
<i>Cryptomycota</i>	<i>LKM15</i>	
<i>Metazoa</i>	<i>Platyhelminthes</i>	<i>Gierysztoria sp.</i>
	<i>Rotifera</i>	<i>Brachionus calyciflorus</i>
<i>Rhizaria</i>	<i>Cercozoa</i>	<i>Rhogostoma minus</i>
<i>Stramenopiles</i>	<i>Hyphochytriomycetes</i>	<i>Rhizidiomyces apophysatus</i>

717

718 **Figure legends**

719

720 **Fig. 1** Multivariate redundancy analyses relating performance parameters (dried sludge, volatile dried sludge,  
721 pH, acid/alkalinity ratio AC/AL, O<sub>2</sub>, CO<sub>2</sub> CH<sub>4</sub> and biogas production) with changes in diversity or abundance of  
722 the most representative archaeal **(a)** and bacterial **(b)** phylotypes in anaerobic digestion. Data were taken from  
723 González-Martínez et al. (2016b)