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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			
3	Authors:		
4	Antonio Castellano-Hinojosa <sup>1,‡</sup> , Caterina Armato <sup>2,3,‡</sup> , Clementina Pozo <sup>1</sup> , Alejandro González-Martinez <sup>4</sup> , Jesús		
5	González-López <sup>1,*</sup>		
6			
7	Affiliations:		
8	<sup>1</sup> Department of Microbiology and Institute of Water Research, University of Granada, Granada, Spain		
9	<sup>2</sup> Department of Public Health and Pediatrics, University of Torino, Torino, Italy		
10	<sup>3</sup> Centre for Sustainable Future Technologies (CSFT@PoliTo), IstitutoItaliano di Tecnologia, Torino, Italy		
11	<sup>4</sup> Department of Built Environment, School of engineering, Aalto University, Espoo, Finland		
12			
13	<sup>†</sup> Antonio Castellano-Hinojosa and Caterina Armato contributed equally to this work.		
14			
15	* Corresponding author:		
16	Department of Microbiology and Institute of Water Research, University of Granada, Granada, Spain		
17	E-mail address: jgl@ugr.es (J. González-López).		

# 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

#### 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  $CH_4$  (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).

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

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#### 64 Digester configurations: advantages and disadvantages

65 Since the appearance of AD, a wide variety of digester configurations has been tested such as thermophilic/mesophilic digestion, dry/wet digestion, one-phase/two-phase digestion or one-stage/two-stage 66 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.

The simplest possible configuration is the one-stage AD batch reactor, in which the tank is filled with the feedstock and let stand for a period after which it is emptied (Khalid et al. 2011). Although this kind of system has very low operational cost, it exhibits some limitations such as high fluctuations in gas production, biogas losses during emptying the bioreactors and restricted bioreactor heights (Khalid et al. 2011; Zhang et al. 2015; Sunyoto et al. 2016). A more widely used type of one-stage AD bioreactor is commonly defined 'one-stage 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 (Achinas 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 eukaryotic groups (see Section 2). The groups of microbes that have been identified for AD are mesophilic and 116 117 thermophilic strains. While great diversity exists between mesophilic and thermophilic bacteria, with the latter showing both higher specific growth and decay rates, methanogen growth is mostly favoured by both mesophilic

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).

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# 133 Biogas production

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

As an example, the Korean government recently solicited the use as an AD substrate of organic waste from ocean dumping or landfill, with the aim to produce renewable energy; this raises the issue of efficiently degrading septage and sewage sludge, and the consequent investigation of different mixtures for co-digestion approaches (Park et al. 2016). Otherwise, good availability of a specific kind of waste can turn it into a candidate substrate. In Colombia, for example, the massive production of coffee generates a large amount of coffee mucilage, a crop residue rich in carbohydrates. This organic matter has been successfully used in co-digestion 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 of158 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).

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#### 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.

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# 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 methanogenic organisms involved in AD, biomonitoring of the system could be an important feature for 182 183 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 (Kaever 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.

The AD process comprises four interdependent steps in which microorganisms responsible for a specific stage provide the intermediates for the next. Microbial community structure and dynamics are important to sustain functional redundancy and to maintain a well-balanced process (Allison and Martiny 2008; Ziganshin et al. 2013). Archaea, Bacteria and Eukarya communities form the microbiome of the anaerobic digester and 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 *Methanothrix*) 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  $CO_2$  or methyl compounds to synthesize  $CH_4$  (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  $L^{-1}$  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  $\leq$  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  $NH_4^+$  (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.

The presence of bacterial genera such as *Desulfotomaculum*, *Desulfovibrio*, *Syntrophobacter*,
 *Syntrophomonas*, *Syntrophospora*, *Clostridium*, *Bacteroides*, *Bifidobacterium*, *Butyrivibrio*, *Pseudomonas*,
 *Bacillus*, *Streptococcus* and *Eubacterium* has been related to acid formation and hydrogen release (Yamada et al.
 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-Martinez et al.

(2016b) studied archaeal and bacterial community dynamics of a bench-scale two-stage anaerobic digester. An
overview of the response of key archaeal and bacterial phylotypes to changes in performance parameters is
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.

Very limited information on the physiology of anaerobic or facultative anaerobic eukaryotic organisms is available to date. Some of the Fungi found in AD contribute to the degradation of some organic matter in anaerobic environments and they could be implicated in the hydrolysis of organic matter in anaerobic sludge digestion processes. Previous studies have demonstrated that phylotypes in Plantae, Animalia and Fungi can 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.

The dynamics of prokaryotic organisms have been described during the start-up stage of AD (Gonzalez-Martinez et al. 2016b) as showing substantial changes under unstable conditions. Thus, a challenge exists to develop a useful biomonitoring tool for environmental engineers. Many studies have indicated that *Methanosaeta* and *Methanosarcina* are competitive genera in the AD process. During the acidification phase, *Methanosaeta*, an acetoclastic methanogen, is the dominant genus but its abundance decreases significantly during the methane production phase. In the latter phase, the acetoclastic methanogen *Methanosarcina* increases significantly. *Methanosarcina* is more tolerant to inhibitors than *Methanosaeta* (Cho et al. 2013). At the end of
AD, *Methanoculleus*, a hydrogenotrophic methanogen, becomes dominant because of the exhaustion of acetic
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,  $CO_2/H_2$ , 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 hydrolysation 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

al. (2014) demonstrated the potential application of chitinolytic fungi to obtain H and Ma et al. (2015)
determined that rumen microorganisms have higher hydrolytic and acidogenic activity than other microbial
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).

The study of biogeochemical cycles in natural ecosystems can drive innovation in bioenergetics applications to support improvements of AD. In this sense, Izzo et al. (2014) explored the potentials offered by the structural and functional microbial biodiversity in hypertrophic lagoons characterised by rapid and huge biomass blooms and decomposition. They selected the microbial communities as inoculum and successfully tested for hydrogen 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 by390 any of the authors.

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

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

## 718 Figure legends

- 720 Fig. 1 Multivariate redundancy analyses relating performance parameters (dried sludge, volatile dried sludge,
- 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
- the most representative archaeal (a) and bacterial (b) phylotypes in anaerobic digestion. Data were taken from
- 723 González-Martínez et al. (2016b)