

Effect of a partial substitution of energy crop with agricultural waste on the dynamics of bacterial communities in a two stage anaerobic digester

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The two-stage (hydrolytic-methanogenic) anaerobic digestion has been largely used as a reliable alternative to single reactor, for being more efficient in preventing the biogas production failures, due to e.g. pH inhibition and metabolic unpairing of the microbial community. Concerning the various possible substrates, the substitution of dedicated crops with lower cost and environmental impacting food and agricultural waste is considered a priority for both economic and sustainability reasons. Despite their great potential, the change of feeding mix in AD might result in lower energy production, related for example to nutrient imbalance or the increase of the total ammonia nitrogen (TAN) content. The causes of AD system failures can be related to the unpredicted shift in the microbial communities and metabolic chain. Therefore, many studies have investigated and identified the microorganisms involved in the removal of nutrients like phosphorus and nitrogen, and other novel groups such as sulphate-reducing bacteria (SRB), methanotrophic bacteria (Reyes *et al* 2014), or syntrophic acetate oxidizing (SAO) bacteria, performing the oxidation of acetate to hydrogen and carbon dioxide whose dynamics in the AD process still needs to be fully elucidated. Recent findings supported the thesis that shifts from acetoclastic methanogenesis to syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenesis may be caused by high TAN concentration (Westerholm *et al* 2016). In a previous study, we investigated the influence of the slowly replacement of the energy crops with poultry manure litter (PML) in the feeding of a full-scale two-stage biogas plant on microbial community structure and the results disclosed that the change in the digester feeding and in the carbon:nitrogen (C:N) ratio had been key factors in determining the bacterial and archaeal communities (Bellucci *et al* 2019). To gain a deep understanding of the response of the microbial community, in the present study extended analysis of the bacterial populations which could have competed or shifted also at relatively low level (e.g. SAO, sulfate-reducing bacteria) by means of 454-pyrosequencing and LEfSe method were performed.

Materials and Methods

A two-stage biogas plant, fed once a week with cow manure and energy crops, located in Manfredonia (Foggia, Italy), was monitored for five months as already described by Bellucci *et al* 2019. The feedstock mixture was changed over time for partially replacing energy crops with chicken manure, consequently reducing the C/N ratio. PCR amplification of 16S rRNA genes for bacteria was realized with a primer set amplifying the V4-V6 variable regions (primers 518F 5'-CCAGCAGCYGCGGTAAN-3' and 1046R 5'-CGACRRCCATGCANCACT-3'). Samples were sequenced using the Roche 454 GS-FLX system, titanium chemistry, accordingly to the company protocols. Pyrosequencing data were analyzed with Qiime 2 and statistical Lefse analysis (Segata *et al* 2011) was used to evaluate the most dynamical OTUs in the two bioreactors.

Results and Discussion

Deep sequencing analysis focused on bacterial community revealed a shift in the composition in response to the diet change and according to compounds availability. The phyla *Firmicutes*, *Actinobacteria* and *Bacteroidetes* were the dominant phyla in both reactors with a low variability over time but significant variations in diversity in lower taxonomic levels were observed. LEfSe analysis highlighted how the more dynamic bacterial taxa differ according to several times and to the change of feed.

In the Hydrolysis reactor, *Mogibacteriaceae*, *Brucellaceae* and *Corynebacterium* were among the most differentially abundant taxa at day 1, while *Thermacetogenium* genus or *Syntrophobacterales* order at 64 days differentiated, then *Desulfobacterales* order was present at 85 days and *Clostridium* genus and *Actinobacteria* phylum at day 128. Noteworthy was the presence of the genus *Thermacetogenium*, whose members (e.g. *Thermacetogenium phaeum*) are known to be strictly anaerobic, thermophilic and involved in the syntrophic acetate oxidation (SAO) (Westerholm *et al* 2016). This finding could confirm that the high TAN levels for the feed change may have led to the shift from acetoclastic methanogenesis to syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenesis. *Syntrophobacterales* is a well-known syntrophic group (Azman *et al* 2017) while *Desulfobacterales* order includes sulfate reducing bacteria (SRB) belonging to three main families. *Desulfobacteriaceae* family for example, exploits SO_4 , O_2 , and NO_3 as terminal electron acceptors to oxidize H_2 and organic compounds, including acetate (Reyes *et al* 2014).

In the Methanogenesis reactor, the dynamics are less prominent, due to longer hydraulic retention times (HRT). At the first day and at the family level, *Clostridiaceae*, *Mogibacteriaceae*, *Lactobacillaceae* and *Bacillaceae* (*Anoxybacillus kestanbolensis* in particular) families were identified to be differentially abundant, followed by *Planctomycetaceae*. At 41 days, interesting was the differentiation of the order *Thiotricales*, as the *Planctomycetaceales* at 50 days and *Dethiobacter* genus at 64 days. At the last day (128) *Xanthobacteraceae* family only. *Anoxybacillus kestanbolensis*, a facultative anaerobe thermophilic bacillus isolated from mud and water samples of Kestanol, is known for the Arabinofuranosidase activity in the conversion of hemicelluloses (Canakci *et al* 2008). The selection of this cellulose degrading bacteria could be explained by the presence of the straw in the poultry litter feedstock. Noticeable is also the order *Thiotricales* which may include the filamentous sulfur-oxidizing bacteria (SOB) such as *Beggiatoa*, *Thioploca* and *Thiotrix*. Other SOB described, belong to the genera *Xanthobacter* also found in the reactor. Then *Dethiobacter* use sulfur compounds (thiosulfate and polysulfide but not sulfate) as electron acceptor, ensuring the methanogenic trophic chain as previously described by Bellucci *et al* 2019. The chicken manure contains high concentration of S owed to the high demand of methionine and cysteine (sulfuric amino acids) of poultry, which can explain the presence of these communities. Regarding *Planctomycetales*, Park *et al* 2010 found *Candidatus "Brocadia sp. 40"* belonging to this order, as the dominant anammox population present in a bioreactor fed with actual anaerobic digestion centrate from a full-scale operational wastewater treatment facility. It is widely known that anammox bacteria play a substantial role in the global nitrogen cycling and so we might hypothesize their involvement in the nitrogen removal through anammox process in this step.

Conclusions

The change in the diet affected the bacterial community dynamics in both reactors, with interesting specific features. Hydrolysis reactor showed major bacterial community dynamics related to sulphate reducing and syntrophic acetate oxidizing taxa, while the methanogenic bioreactor more dynamical OTUs were the ones related to cellulose degradation and potential anaerobic ammonia oxidation.

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