Microbiome network analysis of co-occurrence patterns in anaerobic co-digestion of sewage sludge and food waste

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ABSTRACT

Addition of food waste (FW) as a co-substrate in anaerobic digesters of wastewater treatment plants is a desirable strategy towards achievement of the potential of wastewater treatment plants to become energy-neutral, diverting at the same time organic waste from landfills. Because substrate type is a driver of variations in phylogenetic structure of digester microbiomes, it is critical to understand how microbial communities respond to changes in substrate composition and concentration. In this work, high throughput sequencing was used to monitor the dynamics of microbiome changes in four parallel laboratory-scale anaerobic digesters treating sewage sludge during acclimation to an increasing amount of food waste. A co-occurrence network was constructed using data from 49 metagenomes sampled over the 161 days of the digesters' operation. More than half of the nodes in the network were clustered in two major modules, i.e. groups of highly interconnected taxa that had much fewer connections with taxa outside the group. The dynamics of co-occurrence networks evidenced shifts that occurred within microbial communities due to the addition of food waste in the co-digestion process. A diverse and reproducible group of hydrolytic and fermentative bacteria, syntrophic bacteria and methanogenic archaea appeared to grow in a concerted fashion to allow stable performance of anaerobic co-digestion at high FW.

Key words | anaerobic digestion, co-digestion, food waste, microbiome, network analysis, sewage sludge

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INTRODUCTION

The activated sludge process has been successfully applied worldwide for municipal and industrial wastewater treatment for more than 100 years (Jenkins & Wanner 2014). Yet achieving effluent with a low concentration of organic matter and nutrients and low suspended solids comes at the expense of high energy consumption, mainly used for aeration. Part of the spent energy can be retrieved from the biogas generated during anaerobic digestion of the excess sludge produced as a by-product of the activated sludge process. However, sewage sludge has low digestibility; therefore, the generation of electricity from biogas in a combined heat and power plant recovers only a modest fraction of the energy present in the raw wastewater (McCarty et al. 2011). Addition of food waste

(FW) as a co-substrate in anaerobic digesters is gaining attention as one of the most promising strategies towards achievement of the potential of wastewater treatment plants to become energy-neutral (Gu et al. 2017; Nghiem et al. 2017; Mehariya et al. 2018). An additional environmental benefit of co-digesting food waste in anaerobic digesters is the diversion of organic matter from landfills, therefore reducing a major anthropogenic source of methane emission.

All the same, food waste is a challenging substrate that poses operational constraints to the anaerobic digestion process. The variable composition of readily biodegradable matter containing high volatile solids (VS) and high chemical oxygen demand may exacerbate the tendency to cause

digester foaming (Kougias *et al.* 2014) and to increase the risk of volatile fatty acids (VFA) accumulation, with a consequent inhibition of methanogens that leads eventually to reactor upset (Carballa *et al.* 2015).

Because substrate type is the major determinant that drives variations in phylogenetic structure of digester microbiomes (Ibarbalz *et al.* 2013; Zhang *et al.* 2014), it is critical to understand how microbial communities respond to changes in substrate composition and concentration. Whereas several studies suggested that food waste can be better managed when treated in co-digestion with sewage sludge and other substrates compared with mono-digestion, there is no agreement on what is the best mixing ratio for good performance and long term stability. Therefore, gaining deeper insight into the changes that occur within the structure of the anaerobic digester microbiome upon addition of food waste as a co-substrate of sewage sludge may contribute to better understanding and thereby improved control of process stability and biogas production of co-digestion.

In anaerobic digesters, different microbial guilds responsible for hydrolysis, acidogenesis, acetogenesis and methanogenesis interact by means of a sequential and concerted process to achieve the degradation of complex organic matter to methane and carbon dioxide (De Vrieze & Verstraete 2016). High throughput sequencing is an effective tool to characterize taxonomic profiles of microbial communities in complex environments. In combination with network analysis it can provide valuable insight into potential interactions occurring between functional guilds and also into community dynamics; that is, taxa replacement under changing environmental conditions (Widder *et al.* 2016). In this work, we investigated the changes that take place at microbial level when food waste is incorporated as a co-substrate to digesters adapted to sewage sludge. Specifically, we searched for 16S rRNA genes directly in shotgun metagenomic data obtained from a laboratory-scale experiment designed to analyze the acclimation of microbial communities treating sewage sludge to increasing amounts of food waste. Compared with 16S sequencing, shotgun sequencing is less prone to biases because it does not rely on targeted primers to polymerase chain reaction (PCR) amplify a marker gene (Engelbrektson *et al.* 2010). The substrate-dependent microbial associations revealed through co-occurrence networks provided clear evidence of a shift between different modules of co-existing species, which appeared to be coupled to the stable performance of anaerobic co-digestion at different organic loading conditions.

METHODS

Inoculum and substrates

The inoculum used for start-up of the laboratory-scale digesters was collected from the anaerobic digester of a municipal sewage treatment plant in the Buenos Aires area (Argentina) operating at mesophilic temperature with a 21-day hydraulic retention time (HRT). The total and volatile solids of the inoculum were 2,920 and 1,920 mg L^{-1}, respectively. The total alkalinity was 2.71 g CaCO$_3$ L^{-1}, the VFA was 337.50 mg L^{-1} and the pH was 7.12. Mixtures of settled primary sludge and waste activated sludge used to feed the laboratory-scale digesters (called hereafter sewage sludge, SS) were collected weekly from the sludge mixing tank that feeds the same full-scale digester. The proportion of primary sludge to waste activated sludge was approximately 4:1. Total solids and volatile solids of sewage sludge were 4,650 and 3,250 mg L^{-1}, respectively. The total Kjeldahl nitrogen was 2,100 mg L^{-1}. For co-substrate, synthetic mixtures mimicking the typical composition of restaurant food waste (FW) were prepared using food products from vegetal (68.4%) and animal (17.3%) origin, plus 14.3% of bakery leftovers. Food waste mixtures were prepared weekly and suspended on water with pH adjusted to 7. The final synthetic mixture contained 17% of total solids (90% volatile). Contents of proteins, fats and carbohydrates were 3.2%, 0.6% and 12.6% of dry weight. The total Kjeldahl nitrogen in the food waste was on average 5.1 g kg^{-1}.

Semi-continuous digesters

Four laboratory-scale anaerobic digesters with a working volume of 5 L run during 161 days in semi-continuous stirred tank reactors at mesophilic conditions (35 °C) with an HRT of 21 days. The inoculum used for the four laboratory-scale anaerobic digesters was obtained from the anaerobic digester of a municipal sewage treatment plant. The digesters were fed daily and stirred intermittently with cycles of 30 minutes on and 60 minutes off. Feeding started gradually with a mixture of settled primary sludge and waste activated sludge collected from the same wastewater treatment plant (WWTP) until the organic loading rate was 1.5 g VS L^{-1} d^{-1} in all digesters. Subsequently, digesters 2 to 4 were acclimated to gradual additions of FW until organic loading rates (OLR) were 2.5, 3.5 and 4.5 g VS L^{-1} d^{-1}, respectively. At day 125, all digesters (including the control digester 1) received an additional pulse of 2 g VS L^{-1} d^{-1} of FW for two weeks, followed by a return to previous feeding conditions.

Chemical analyses

Total solids (TS), total volatile solids (VS), and total VFA in the inoculum, in the digester slurries, and in the substrates were determined according to *Standard Methods*, sections 2540B, 2540E, and 5560C, respectively (APHA 1998). Biogas volume was measured with a drum-type gas meter Ritter TG 0,5/5, and biogas composition was determined using a Landtec Systems Gas Analyzer Biogas 5000.

Sample collection, DNA extraction and shotgun sequencing

Sludge samples were taken from each digester on a daily basis for the measure of performance. For microbiome analysis, 2 mL of sludge were sampled at different times across the 161 days of digester operation (days 8, 20, 30, 34, 69, 83, 114, 125, 128, 135, 140, 161) and stored at $-70\,°C$. Metagenomic DNA was extracted using the FastDNA Spin kit for soil (MP Biomedicals, USA), according to manufacturer's instructions. DNA sequencing was performed on an Illumina HiSeq platform at INDEAR (Rosario, Argentina). Raw reads from 16S rRNA genes obtained in this study have been deposited in the Sequence Read Archive from the National Center for Biotechnology Information (NCBI; BioProject ID: PRJNA544497).

Data analyses

Paired-end metagenomic sequences (2×250 bp) were filtered and trimmed to remove ambiguous bases (N), ensuring a minimum average quality value (Q) of 25. Metagenomic sequences corresponding to ribosomal rRNA genes were identified from raw reads using Metaxa2 (Bengtsson-Palme *et al.* 2015). Almost full-length small subunit ribosomal genes (average size: 1,296 nt) were assembled from the short reads of the metagenomes and clustered at a 97% level, using the program EMIRGE (Miller *et al.* 2011). OTUs were classified using the Silva database v. 132 using 88% minimum identity with the query sequence. Filtered PE reads were mapped back to the reconstructed SSU rRNAs using Bowtie2 for the estimation of the relative abundance of each OTU in the communities.

The ordination technique Constrained Analysis of Principal Coordinates (CAP) was performed with the 'vegan' package version 2.0-10 (Department of Statistics, Iowa State University, Ames, IA, USA) in R 3.0.2, using Bray Curtis dissimilarity index and default parameters.

Network correlations

A co-occurrence network was constructed using 49 samples from the four digesters to determine potential associations between microbial OTUs. Pearson's correlation was calculated between each pair of OTUs using rarefied OTU tables. Pearson's coefficient correlations with a score lower than -0.7 and higher than 0.7, and q-value <0.01 were considered significant. The interactive platform Gephi was used to visualize the network. Modularity was calculated by the 'Louvain method' (Blondel *et al.* 2008) using the clustering algorithm implemented in Gephi. Modules were filtered to remove nodes with a degree less than 2.

RESULTS AND DISCUSSION

The four laboratory-scale anaerobic digesters were inoculated with sludge from an anaerobic digester of a municipal sewage treatment plant and fed initially with sewage sludge; that is, a mixture of settled primary sludge and waste activated sludge collected from the same WWTP. Subsequently, each digester, except the control digester, received food waste in addition to the sewage sludge, increasing the volatile solids loading at a rate of 10% per day. Using this strategy, digesters showed stable operation with short acclimation time. Once the digesters reached each desired OLR, the feeding regime was maintained for several hydraulic retention times (Figure 1). An additional phase of the experiment was initiated at day 125, in which all digesters (including the control digester) received a larger increase in FW during two weeks, following by a return to previous feeding conditions (Figure 1).

Methane yield increased linearly with increasing percentage of organic loading in all digesters. Despite the relatively high variability of the pooled VFA values, VFA concentration remained low in all digesters, and no accumulation was observed throughout the study. The values of methane production were in agreement with previous experience of anaerobic digestion of similar substrates (Kim *et al.* 2003). Synergy between substrates is usually observed in anaerobic co-digestion of FW and SS, resulting in higher methane production, in comparison with mono-digestion (Zhang *et al.* 2017a). It has been shown at full-scale that by doubling the organic load of sludge using an organic co-substrate, the biogas production may increase by a factor of three (Aichinger *et al.* 2015). In our experiments the specific biogas production increased with increasing content of FW, albeit slightly (Table 1). This is consistent with the
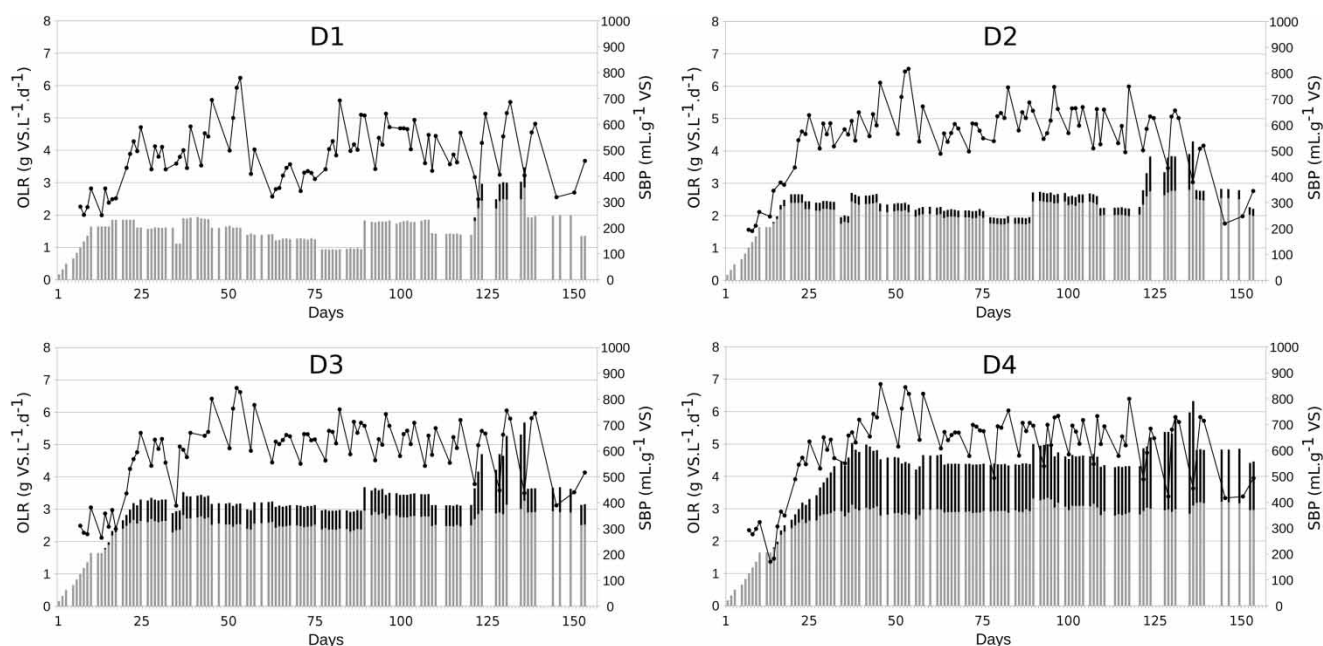
Figure 1 │ Volatile solids fed to the digesters, from sewage sludge (gray) and from food waste (black). The line indicates specific biogas production (SBP), in mL biogas g^{-1} VS added.

Table 1 │ Summary of process performance

	SBP (mL·g VS^{-1} added)	Average methane content (%)	Average VFA mg L^{-1}	Average VFA/ALK ratio
Digester 1	568	66.3 ± 2.9	151.2 ± 71.1	0.04 ± 0.02
Digester 2	637	56.9 ± 3.1	152.6 ± 44.7	0.04 ± 0.01
Digester 3	665	57.8 ± 1.9	208.2 ± 104.6	0.05 ± 0.03
Digester 4	683	55.9 ± 1.5	226.9 ± 93.7	0.05 ± 0.02

fact that synergy is weak or lacking when sewage sludge and food waste are co-digested at low solid concentration (Liu *et al.* 2016b).

Phylogenetic characterization of the microbial community structure in the anaerobic digesters was carried out by assembling 295,487 reads (average per sample: 6,030, median: 6,025), to reconstruct a total of 539 small subunit rRNA genes longer than 800 bp from the 49 metagenomes, with a median of 298 (maximum: 347, minimum: 212). Asymptotic rarefaction curves suggested that sequencing depth adequately covered the most abundant microbial community members (data not shown). To explore the response of the overall microbial community to changes in substrate composition, profiles of 16 rRNA gene abundance were analyzed using Constrained Analysis of Principal Coordinates (CAP) (Figure 2). Each point in the CAP ordination represents the community structure of one sample at a
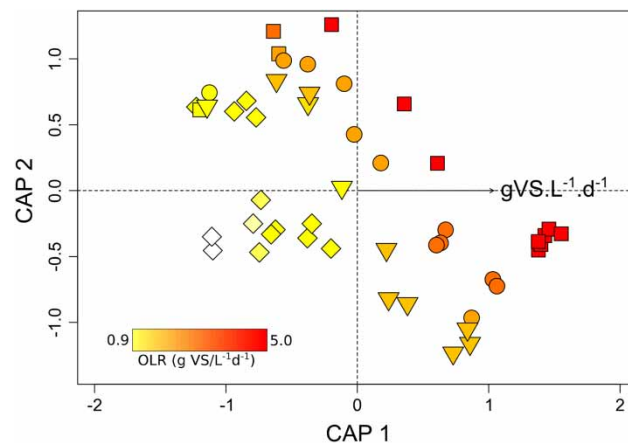
Figure 2 │ Constrained Analysis of Principal Coordinates (CAP) using the pairwise Bray-Curtis dissimilarity index. Digesters are denoted by symbols: D1: diamonds; D2: triangles; D3: circles; D4: squares. Intensity of symbols vary along a gradient of the organic loading rate at the time of sampling.

single time point. The colors of the symbols vary along a gradient of organic loading rate at the time of sampling. In all digesters, higher concentration of volatile solids from food waste in the feed was accompanied by a shift in microbial communities. This was also the case for the additional pulse of FW started at day 125, which replicated the direction of changes in all digesters (Figure 2).

Insight into how changes of substrate type and concentration affected the network of interactions between members of the microbial consortia in the anaerobic
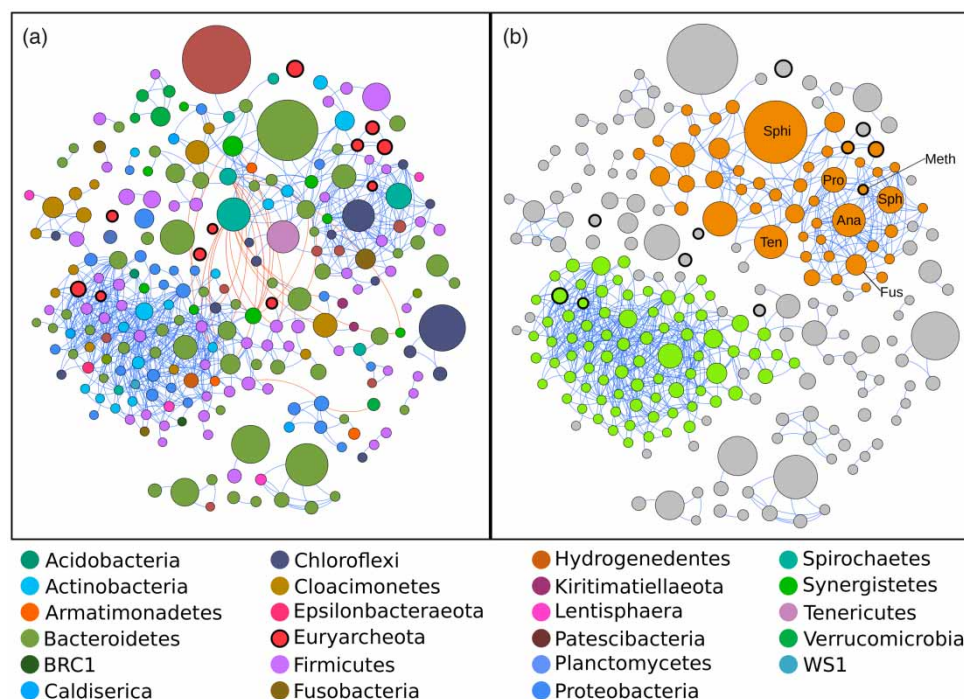
digesters was gained by means of co-occurrence network analysis. A global network was constructed using the data from all of the digesters taken over the time course of the experiment. The network had 231 nodes with a large number of strong positive correlations and much fewer negative correlations. Each node represents a taxon, with a node size proportional to its relative abundance in the sample (Figure 3(a)). The node degree distribution was well fitted by a power-law model ($R^2 = 0.87$); that is, the probability $P(k)$ that a node in the network interacts with k other node decays following $P(k) \sim k^{-\gamma}$, with $\gamma = 1.15$. This property is indicative of a scale-free network, where nodes with small degrees are most frequent but there are also highly connected nodes (hubs) (Albert 2005).

The network exhibited a modular structure, with a modular index 0.475. A module in the network is a subnetwork that has more internal edges than external edges; that is, a group of highly interconnected OTUs that had much fewer connections with OTUs outside the group. They represent groups of microbial taxa that potentially interact or share the same ecological niche without direct interaction. The two major modules are highlighted in Figure 3(b). The association of assemblages clustered in modules to distinct

operational regimes can be better illustrated by extracting the interacting taxa from the global network and visualizing them separately in each digester at each sample point (Figure 4). We plotted subnetworks during the period where the cumulative biogas production was linear, from start up until day 114; that is, before the digesters were subjected to the jump in organic loading.

The time-course analysis of the network allowed us to clearly visualize the shift in microbiome dynamics accompanying the changes in the digesters' feeding. We note that there are also changes in the control digester, which we interpret as an effect of immigration produced by the (weekly) change in the composition of the sewage sludge, or alternatively in terms of the dynamics of acclimation to laboratory-scale conditions, as the experiment was started shortly after the inoculum was transferred to laboratory settings. Nevertheless, these changes do not interfere with the interpretation of the main effect caused by the changes in feeding regime. The first module (average degree = 11.34, lower left in Figure 3(b)) is present during the initial operation of laboratory-scale digesters, and was associated to groups of microorganisms adapted to the habitat-specific characteristics of the full-scale digester from where the inoculum was

● Acidobacteria   ● Chloroflexi   ● Hydrogenedentes   ● Spirochaetes
● Actinobacteria   ● Cloacimonetes   ● Kiritimatiellaeota   ● Synergistetes
● Armatimonadetes   ● Epsilonbacteraeota   ● Lentisphaera   ● Tenericutes
● Bacteroidetes   ● Euryarcheota   ● Patescibacteria   ● Verrucomicrobia
● BRC1   ● Firmicutes   ● Planctomycetes   ● WS1
● Caldiserica   ● Fusobacteria   ● Proteobacteria

Figure 3 │ Co-occurrence network constructed using 49 metagenomic samples from the four digesters. Each node represents a taxon, with a size proportional to its relative abundance across all samples, while blue and red edges between the nodes represent positive and negative correlations between taxa. Pearson coefficient correlations with a score lower than −0.7 and higher than 0.7, and q-value <0.01 were considered significant. (a) Nodes (OTUs) are colored by phylum. (b) Nodes are colored according to their belonging to the two major modules. Abbreviations: Sphi: Sphingobacteriales ST-12K33 Ana: Anaerolinaceae; Sph: *Sphaerochaeta*; Pro: *Proteiniphilum*; Ten: Tenericutes; Fus: Fusobacteria; Meth: Methanomassiliicoccales. The full color version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wst.2019.194.
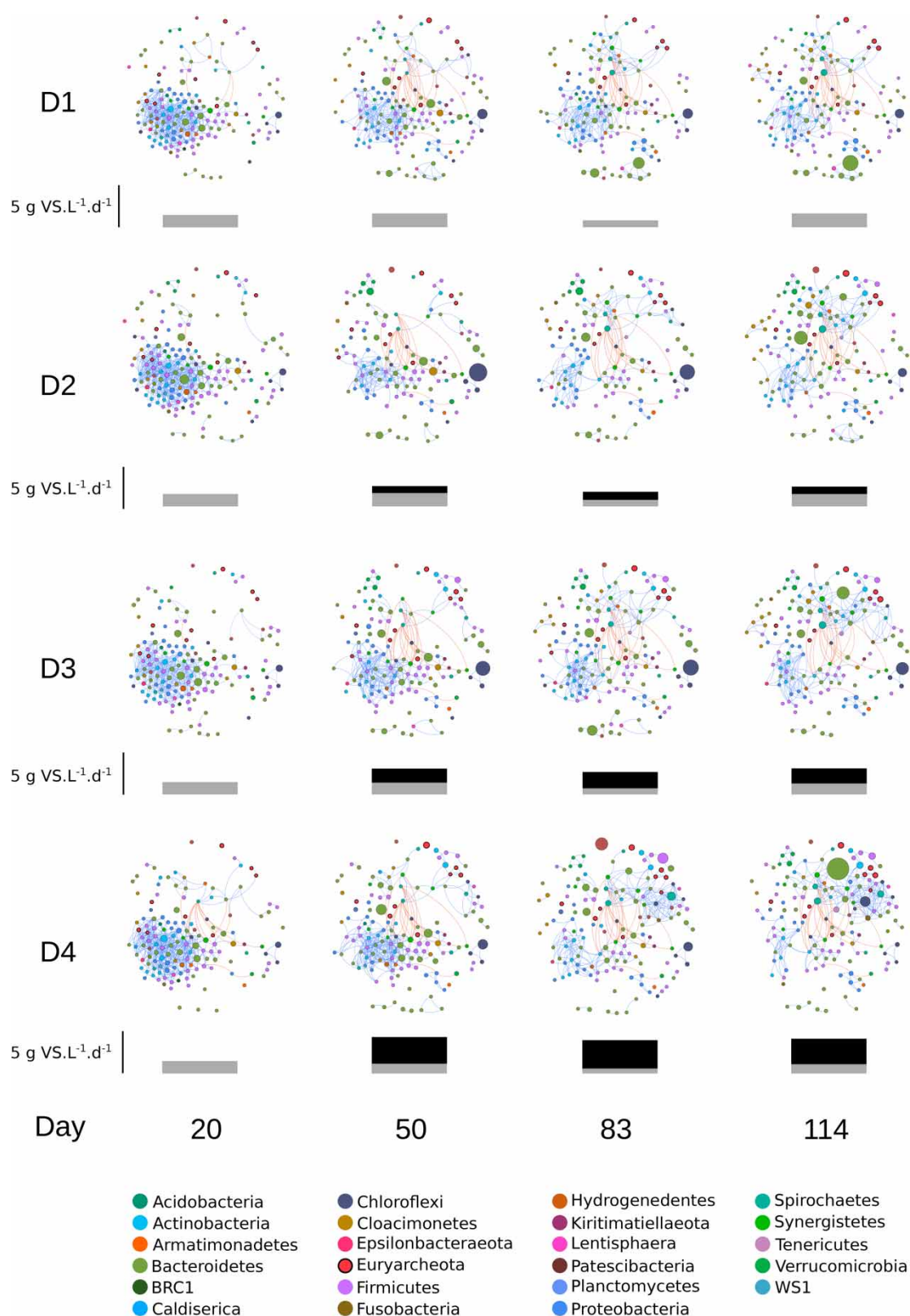
Figure 4 | Dynamics of network connections of co-occurring OTUs. The four digesters are named D1 to D4. Subnetworks were extracted from the global network. The size of nodes is proportional to the relative abundance of the OTUs in each individual sample. Stacked columns are VS from sewage sludge (in gray) and food waste (in black) that was fed daily to the digesters at the time of sampling.

obtained, namely sewage sludge at low VS concentration. The second module of coexisting microorganisms (average degree = 8.08, upper right in Figure 3(b)) accompanied the increase of FW content in the feed. Microorganisms in this module were a minor part of the control digester and of the other three digesters at low VS concentration, and increased in concert with increasing food waste (Figure 5).

There are 49 taxa in the module associated to the addition of FW as co-substrate, including hydrolytic and fermentative bacteria belonging to the phyla Chloroflexi, Spirochaetes, Fusobacteria, Patescibacteria, and Firmicutes (Figure 3). The module also includes syntrophic bacteria belonging to the phylum Synergistetes and four methanogenic archaea. A distinct feature of this module is the disruption of the species evenness observed at low VS. A similar observation was reported upon increasing the load of high-strength food wastewater in anaerobic co-digestion of waste activated sludge (Jang et al. 2016). Even though process stability was maintained throughout this study, systems where microbial communities rely more on dominant groups could potentially be more prone to process instability due to external disturbances. Dominance of Sphingobacteriales ST-12K33 was previously associated with the anaerobic

digestion of food waste (Li et al. 2015), with co-digestion of the organic fraction of municipal solid waste with different co-substrates (Ziels et al. 2018), as well as with high-solids anaerobic digestion (Liu et al. 2016a). As of today, there is no available information about the metabolic capabilities of this taxon, which appears to thrive in anaerobic digesters under high organic loading conditions.

A prominent member of the module, especially abundant in the digester with the highest concentration of FW, is a highly interconnected bacterium of the family Anaerolineaceae (phylum Chloroflexi), with the known capacity of fermenting the carbohydrate fraction of food waste in anaerobic digestion (Narihiro et al. 2009; Yi et al. 2014; Zamanzadeh et al. 2016; Tonanzi et al. 2018).

Another bacterium particularly abundant at very high FW is *Sphaerochaeta* (in phylum Spirochaetes). The abundance of members in phylum Spirochaetes and their interactions with methanogens in anaerobic co-digestion of mixed-substrates has been highlighted in a study of the anaerobic co-digestion of glycerin and sewage sludge (Razaviarani & Buchanan 2015). Genome analysis has shown that the *Sphaerochaeta* genome is enriched in genes that may have been acquired from Clostridia through
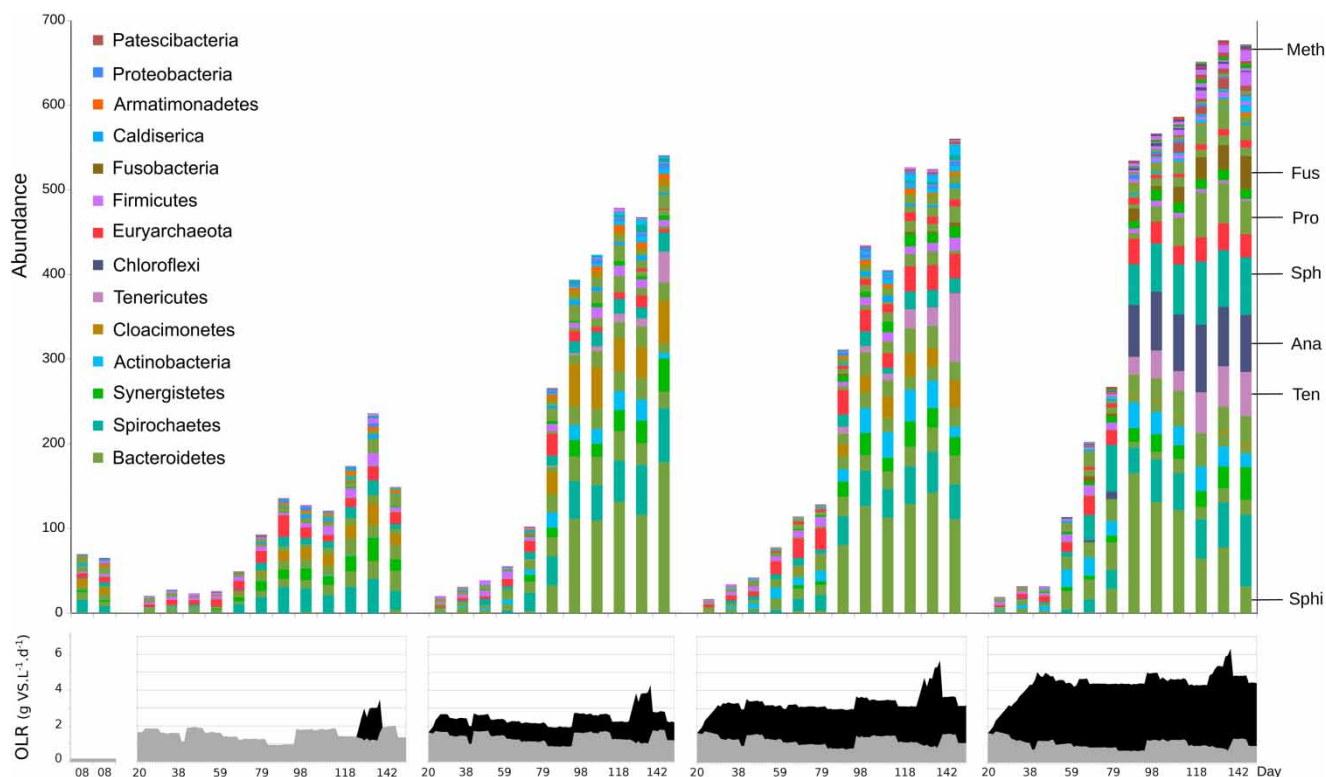
Figure 5 | Abundance distribution of the OTUs belonging to the major module in the co-occurrence network responsive to high food waste across digesters and time. Abbreviations are as in Figure 3. The graph at the bottom corresponds to the volatile solids loading rate, from sewage sludge (gray) and from food waste (black).

horizontal gene transfer events, allowing the bacterium to have a fermentative lifestyle (Caro-Quintero *et al.* 2012). Bacteria affiliated to *Proteiniphilum* (phylum Bacteroidetes) might play an important role by contributing to the conversion of organic compounds into acetic acid and CO_2 (Chen & Dong 2005). *Proteiniphilum* has been previously found in high numbers in other anaerobic digesters (Nelson *et al.* 2011), including one digesting FW, where it increased with increasing total solids contents (Yi *et al.* 2014), and in a three-stage anaerobic digester co-digesting food waste and horse manure (Zhang *et al.* 2017b).

The abundant Tenericutes belongs to the class Mollicutes (order Izimaplasmatales). A distinctive characteristic of this phylum of fermentative bacteria is the ability to hydrolyze arginine and urea, and the dependence on cholesterol for growth (Razin 2006). Described species from the phylum Fusobacteria can produce a variety of organic acids, including acetic, formic and butyric acid via fermentation of carbohydrates, amino acids or peptides (Potrykus *et al.* 2007). A putative role for the family Synergistaceae is the syntrophic degradation of volatile free acids via the H_2 interspecies transfer pathway (Xu *et al.* 2016).

The methanogen Methanomassiliicoccales is correlated with the aforementioned taxa within the module related with high food waste. The order Methanomassiliicoccales is the 7th order of Archaea, and has been identified in diverse anaerobic environments including the gastrointestinal tracts of humans and other animals (Borrel *et al.* 2014), as well as in environmental and non-human gastrointestinal samples (Parks *et al.* 2017). Energy for growth derives in Methanomassiliicoccales from the hydrogen-dependent reduction of methyl compounds to methane (Speth & Orphan 2018). Less connected methanogenic archaea affiliated to the genera *Methanospirillum*, *Methanosaeta* and *Methanoculleus* also belong to the high FW module. Yet it is worth mentioning other abundant methanogenic archaea in the genera *Methanosaeta* and *Methanospirillum* that were not exclusive to high organic loading conditions, and hence did not form part of the network of co-occurrence, were relatively abundant and may also be involved in the biogas production during this period.

CONCLUSIONS AND FINAL REMARKS

We successfully reconstructed 16S rRNA genes from shotgun metagenomic data corresponding to the dominant member of anaerobic biodigesters and analyzed the data by using a network-based analysis approach, to understand how anaerobic digester microbiomes were affected by changes in substrate composition and concentration. The co-occurrence network indicated a clear shift in the microbiome composition that occurred upon the addition of food waste as a co-substrate in the anaerobic digestion process. A diverse and reproducible group of hydrolytic and fermentative bacteria, syntrophic bacteria and methanogenic archaea appear to grow in a concerted fashion to allow stable performance of anaerobic co-digestion at high FW.

Despite the revived interest in co-digestion, few studies have evaluated how co-digestion impacts the anaerobic digestion (AD) microbiome, especially in a semi-continuous regime. Previous studies also observed that microbial communities were strongly altered by the loading of FW (Xu *et al.* 2017; Zhang *et al.* 2017a). However, the response of the microbial composition varied greatly between different studies, depending on the specific conditions. Therefore, determining how stability is influenced and which functions are affected by changes in community composition remains a challenge.

Co-occurrence networks represent associations between two microorganisms, but do not prove that there is a direct interaction or association between them. Ecological interpretation of the observed associations is currently being investigated by assembling the individual genomes from the metagenomes to obtain a better understanding of the physiological capabilities of the microorganisms in the system and to reach a full overview of the metabolic pathways that lead to methane production.

ACKNOWLEDGEMENTS

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