1 **Comparing the inhibitory thresholds of dairy manure co-digesters**
2 **after prolonged acclimation periods: part 2 - correlations between**
3 **microbiomes and environment**

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Abstract:

Here, we studied the microbiome succession and time-scale variability of four mesophilic anaerobic reactors in a co-digestion study with the objective to find links between changing environmental conditions and the microbiome composition. The changing environmental conditions were ensured by gradual increases in loading rates and mixing ratios of three co-substrates with a constant manure-feeding scheme during an operating period longer than 900 days. Each co-substrate (*i.e.*, alkaline hydrolysate, food waste, and glycerol) was co-digested separately. High throughput 16S rRNA gene sequencing was used to examine the microbiome succession. The alkaline hydrolysate reactor microbiome shifted and adapted to high concentrations of free ammonia, total volatile fatty acids, and potassium to maintain its function. The addition of food waste and glycerol as co-substrates also led to microbiome changes, but to a lesser extent, especially in the case of the glycerol reactor microbiome. The divergence of the food waste reactor microbiome was primarily linked to increasing free ammonia levels in the reactor; though, these levels remained below previously reported inhibitory levels for acclimated biomass. The glycerol reactor microbiome succession included an increase in Syntrophomonadaceae family members, which have previously been linked to long-chain fatty acid degradation. The glycerol reactor exhibited rapid failure and limited adaptation at the end of the study.

Keywords: Bacteroidetes; Clostridia; co-digestion; ammonia; volatile fatty acids

Abbreviations: ANOVA, Analysis of Variance; COD, Chemical Oxygen Demand;
HRT, Hydraulic Retention Time; LCFA, Long Chain Fatty Acids; OLR, Organic
Loading Rate; OTU, Operational Taxonomic Unit; QIIME, Quantitative Insights into
Microbial Ecology; PCoA, principal coordinate analysis; db-RDA, constrained
distance-based redundancy analysis; TKN, Total Kjeldahl Nitrogen; VFA, Volatile Fatty
Acids; VIF, Variance Inflation Factor; AD, anaerobic digester; CT, co-digestion
treatment; AH, alkaline hydrolysate; FW, food waste; GY, crude glycerol; MN, manure

1. Introduction

The application of anaerobic co-digestion technology for the treatment of organic wastes has increased in recent years. Benefits of co-digestion over mono-substrate anaerobic digestion include a better nutrient balance, a shortened payback period due to a more efficient use of the reactor volume, and a reduction of the negative effects of toxic compounds (Mata-Álvarez et al. 2000). Several co-digestion studies have noted distinct differences in the methane yields and other environmental parameters based on the type of substrate fed (Cirne et al. 2007, Weiland et al. 2010).

Studies have also found that the microbiota composition in anaerobic reactors is greatly influenced by the composition of the input substrates (Regueiro et al. 2014, Zhang et al. 2014). Sundberg et al. (2013) found differences in the microbial communities between full-scale mono-substrate reactors treating sewage sludge *vs.* co-digestion of wastes from a combination of sources. The latter study only used a single sampling point for each reactor. The emergence of next-generation sequencing technologies has enabled researchers to carry out large-scale sequencing surveys in a cost-effective way (Caporaso et al. 2012). As a result, researchers have begun to utilize these technologies to perform time-series studies of multiple anaerobic reactors (Vanwonterghem et al. 2014, Werner et al. 2011) to monitor microbiota changes during the operating period.

Fernández et al. (1999) suggested that microbial communities change even during stable operating periods when no apparent changes in the operating conditions are made. In addition, changes in operating and environmental parameters besides the organic composition have been shown to substantially influence the microbial communities present in a reactor (Regueiro et al. 2014, Town et al. 2014). By using statistical

methods, Werner et al. (2011) found that differences in the bacterial community composition of full-scale anaerobic reactors were correlated with operating conditions (*i.e.*, reactor temperature and feed-to-biomass rates) and function (*i.e.*, substrate removal efficiency and methanogenic activity). Importantly, finding this correlation was only possible because of a time-series study with multiple reactors, resulting in a sufficient amount of environmental metadata in parallel to an in-depth microbiome characterization by DNA sequencing.

Here, our main objective was to examine how the reactor microbiomes changed in response to co-substrate addition, especially when the addition of the co-substrate led to potentially inhibitory environmental conditions in the reactor. The controlled conditions that are necessary to achieve this objective were ensured by gradual increases in loading rates and mixing ratios of three co-substrates in separate reactors and a control reactor with only the base substrate (dairy manure). We tested three co-substrates: alkaline hydrolysate, food waste, and crude glycerol with a constant manure-feeding scheme during an operating period exceeding 900 days at mesophilic conditions. 180 biomass samples from the time series were sequenced *via* Illumina MiSeq paired-end sequencing of the 16S rRNA gene to investigate changes in the reactor microbiome composition. Constrained ordination was used to identify which of the operating and environmental parameters significantly correlated with microbial community succession. Specific indicator populations were linked to individual reactors *via* machine learning.

2. Materials and Methods

2.1 Lab-scale anaerobic reactor operation

Four mesophilic continuously-stirred anaerobic reactors with a working volume of 4.5 L

and a constant hydraulic retention time (HRT) of 25 days were operated for more than 900 days. One reactor was fed only dairy manure (MN) and was used as a control reactor (manure only), while the remaining three reactors received alkaline hydrolysate (AH), food waste (FW), or crude glycerol (GY) as co-substrates. All manure and co-substrates batches were homogenized and stored at -23°C until use. The reactor biomass was conditioned to operate at an initial target organic loading rate (OLR) of 2.0 g VS L·Day⁻¹ and a hydraulic retention time (HRT) of 25 days. Reactors were inoculated with anaerobic digester (AD) effluent from a local dairy farm and fed the same manure semi-continuously (*i.e.*, once every two days). Following start-up (Days 1-295), steady state conditions (SS) were established over three HRT periods (Days 296-370, SS). On Day 371, the co-digestion experiment began with seven co-digestion treatment (CT) periods based on gradually increasing the mixing ratios of co-substrate (CT-I – CT-VII). Alkaline hydrolysate, food waste, and crude glycerol were incrementally added to the manure-fed base operating conditions such that the OLR was increased by 10% (VS basis) for each increment, while maintaining a 25-day HRT. Three batches of manure (Batch #1: Days 145-300, Batch #2: Days 300-697, and Batch #3: Days 697-932), two batches of alkaline hydrolysate (Batch #1: Days 371-603 and Batch #2: Days 603-865), one batch of food waste, and one batch of crude glycerol were used in this study. Reactors treating hydrolysate and glycerol were terminated on Days 865 and 895 of the operating period, respectively. The average values for the environmental parameters monitored in the reactors are reported with the associated 95% confidence interval error.

2.2 Sampling of anaerobic reactors

We selected 180 samples from the four reactors during the entire operating period for DNA extraction. The samples were selected at least once per HRT per reactor, with

additional samples selected during times of system disturbance. Detailed information of the 180 selected samples is displayed in **Fig. S1** along with the OLR applied in each reactor. After collection, samples were centrifuged, and approximately 3 g of the biomass was stored at -80°C until further processing.

2.3 DNA extraction, PCR, and sequencing

Genomic DNA was extracted from the 180 reactor biomass samples using the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the protocol of the manufacturer. The extracted DNA was PCR amplified using 515-forward primers and 806-reverse Golay barcoded primers targeting the V4 region of the 16S rRNA gene according to the protocols of Gilbert et al. (2010).

Duplicate 50 µL PCR reactions were performed using: 0.5 µL of 10 µM 515f forward primer, 0.5 µL of 10 µM 806r barcoded reverse primer, 20 µL of 5PRIME HotMaster Mix (5 Prime Hot Master Mix, 5 Prime, Fisher Scientific, USA), 28 µL of molecular grade H₂O, and 1 µL of DNA template. The PCR cycle conditions consisted of 94°C for 3 min for initial denaturation; 25 cycles of denaturation at 94°C for 45 s followed by annealing at 50°C for 60 s, and elongation at 72°C for 90 s; and finally elongation for 10 min at 72°C. The duplicate PCR products were then pooled and confirmed by gel electrophoresis. The PCR products were cleaned using the MagBind E-Z pure cleaning kit (Omega Bio-tek Inc., Norcross, GA, USA). The DNA concentration of the amplicons was quantified using Invitrogen Quant-It Pico Green DNA quantification kit (Life technologies, USA), and samples were subsequently pooled at equimolar ratios (approximately 100 ng of each sample). Libraries were sent for paired-end sequencing (2x250bp) on the MiSeq platform (Illumina, San Diego, CA, USA) at the Cornell University Biotechnology Resource Center (Ithaca, NY, USA). Sequences were

submitted to the EBI database under the following accession number TBD. Sequences and study metadata is made publically available at QIITA, which is an open-source microbiome storage and analysis resource, under the study number 10137.

2.4 Amplicon sequence analysis

Computational analysis of the reactor sequencing reads was performed using the Quantitative Insights into Microbial Ecology (QIIME v1.7) platform (Caporaso et al. 2010). Paired-end reads were joined using the fastq-join program created by Aronesty (2011) with the default options. Quality filtering and demultiplexing was performed (*via* the `split_libraries_fastq.py` script in QIIME; default values were used with the exception that the minimum acceptable Phred quality score was set to 25). In total, for the 180 samples extracted we obtained 11,213,454 sequences after filtering out low quality sequences (*i.e.*, approximately 14% of the total number of sequences). The number of assigned reads per sample ranged between 12,154 and 135,747 with an average of 52,144 reads per sample and a median read length of 253 bp. Closed-reference operational taxonomic unit (OTU) picking against the Greengenes reference database (May 2013) was performed (McDonald et al. 2012). The default method `uclust` (Edgar et al. 2010) was used to group sequences into OTUs at 97% identity. Representative sequences selected for each OTU were assigned taxonomy with the RDP classifier. After eliminating singleton OTUs (179 OTUs) and samples from the initial operating period (we eliminated 13 samples until Day 267) when only two reactors were running, the number of unique OTUs was reduced to 4973.

To analyze the alpha diversity (OTU diversity within each sample), the sample libraries were rarified 10 times at a depth of 10,000 reads per sample, collated, and analyzed by

the total observed OTUs (*i.e.*, a count of the unique OTUs present in a sample), Chao1 (*i.e.*, an estimate of OTU richness in a sample), and J index (*i.e.*, Pielou's measure of species evenness) (Wilkins et al. 2015). Beta diversity (diversity between samples) based on the weighted UniFrac distance metric was used to compare the microbial communities between samples. Weighted UniFrac is a phylogenetic measure of the pairwise dissimilarity in microbial community composition (Lozupone et al. 2005, et al. 2011), which takes into account the relative abundance of different microbial community members. An even sampling depth of 10,000 reads per sample was used to calculate beta diversity. Principle coordinate analysis (PCoA) plots were generated based on the weighted Unifrac distance matrices. To visualize the divergence of each of the co-substrate reactor microbiomes from the control reactor microbiome, time series analysis plots of the first (and second in the case of the alkaline hydrolysate reactor) principal coordinate axes scores *vs.* time were created. The top 25 OTUs with the highest average relative abundance across the co-digestion period were represented on the time series analysis plots in relation to their importance in driving the observed changes in the plot (*i.e.*, a taxa biplot). The average relative abundance values for the taxa mentioned in the text are reported with their standard deviation error.

2.5 Statistical analysis

Constrained redundancy analysis was performed using the vegan package (Oksanen et al. 2011) in R (2.13.1). Specifically, distance-based redundancy analysis, or db-RDA, *via* the capscale function in vegan was performed to find the environmental parameters (*i.e.*, total volatile fatty acids (VFA), total ammonium, free ammonia, methane content, chemical oxygen demand (total and soluble), potassium, solids (total and volatile), and alkalinity (total and bicarbonate)) that best recreated the beta diversity observed in the

unconstrained PCoA plot. It should be noted that long-chain fatty acid (LCFA) concentrations were not used as a parameter in the db-RDA due to a limited number of measurements. We used Analysis of Variance (ANOVA) to determine if each constraining environmental parameter added a significant amount of information to the constrained model ($p < 0.05$), or if it could be left out. The Variance Inflation Factors (VIF) for the constraining parameters were also evaluated to determine if the constraints were redundant (VIF score > 10). Redundant or non-significant parameters were discarded in an iterative process until all the constraining variables in the db-RDA were significant ($p < 0.05$) and non-redundant (VIF score < 10) (Agler et al. 2012).

Machine learning was performed using the pamR (Hastie et al. 2013) package in R, respectively. pamR was used to select the most predictive OTUs related to one type of reactor, based on the nearest shrunken centroid classification method. We selected a threshold value for the classifier that gave a low misclassification error of 19% (*i.e.*, percentage of misclassified samples).

3. Results and Discussion

3.1 Inhibitory conditions were the main cause of reactor microbiome divergence

From the PCoA plot (**Fig. 1A**) representing the phylogenetic variation between reactor microbiome samples (*i.e.*, based on the weighted Unifrac distance metric), it can be seen that the alkaline hydrolysate reactor microbiome diverged considerably during the operating period from the other reactor microbiomes. Constrained redundancy analysis (*i.e.*, db-RDA) identified free ammonia, total volatile fatty acid (VFA), and potassium concentrations as the parameters that best explained the variation in microbial community structure observed in the PCoA plot (**Fig. 1B**). These constraining

parameters were selected from all the measured environmental parameters because they were both significant (ANOVA test with $p < 0.05$) and non-redundant ($VIF < 10$) in the db-RDA. The first two axes of the db-RDA (*i.e.*, RDA1 (29%) and RDA2 (9%)) explain approximately 83% of the phylogenetic variation shown by the first two axes of the unconstrained PCoA plot (*i.e.*, PC1 (32%) and PC2 (14%)).

During anaerobic digestion, ammonia is formed from the degradation of proteins, nucleic acids, and urea, which are present in high concentrations in animal wastes. Alkaline hydrolysate is a protein-rich waste stream produced by the hydrolysis of animal tissue under alkaline conditions and high temperatures and pressures (Das et al. 2008). The unionized form of ammonia (NH_3), or free ammonia, is more toxic to microbes than the ionized form (NH_4^+), because free ammonia can more easily permeate the cell membrane. Free ammonia has previously been shown to be inhibitory to unacclimated anaerobic digester biomass at concentrations as low as 80-150 mg $\text{NH}_3\text{-N}\cdot\text{L}^{-1}$ (Braun et al. 1981, De Baere et al. 1984). However, for ammonia-adapted biomass, this threshold can be as high as 700-1100 mg $\text{NH}_3\text{-N}\cdot\text{L}^{-1}$ (Angelidaki and Ahring et al. 1994, Hansen et al. 1998). In the alkaline hydrolysate reactor, the concentration of free ammonia increased from an average of 47 ± 6 mg $\text{NH}_3\text{-N}\cdot\text{L}^{-1}$ prior to co-digestion (SS) to 457 ± 24 mg $\text{NH}_3\text{-N}\cdot\text{L}^{-1}$ in the final period CT-V. Average free ammonia concentrations in CT-I, CT-II, CT-III, and CT-IV were 100 ± 13 , 207 ± 29 , 344 ± 46 , and 338 ± 30 mg $\text{NH}_3\text{-N}\cdot\text{L}^{-1}$, respectively.

High free ammonia concentrations have previously been shown to inhibit acetoclastic methanogens (Angenent et al. 2002, Werner et al. 2014), which can lead to a build-up of acetate. The buildup of acetate can make beta-oxidation of higher-chain VFAs less

thermodynamically favorable and lead to an accumulation of higher-chain VFAs in the reactor. In period CT-V, total volatile fatty acid levels began to increase considerably in the alkaline hydrolysate reactor (*i.e.*, from an average of $300 \pm 70 \text{ mg} \cdot \text{L}^{-1}$ in CT-IV to an average of $3410 \pm 80 \text{ mg} \cdot \text{L}^{-1}$ in the final 25 days of the reactor). Acetate initially accumulated; followed by higher order short-chain and long-chain fatty acids. The reactor entered a new steady state in period CT-V in which the VFAs and ammonia concentrations counteracted each other to maintain the pH of the system.

The alkaline hydrolysate waste batches also contained a relatively high concentration of potassium salt (*i.e.*, Batch #1: $7.65 \pm 0.46 \text{ g K}^+ \cdot \text{L}^{-1}$ and Batch #2: $8.33 \pm 0.6 \text{ g K}^+ \cdot \text{L}^{-1}$) compared to the food waste and cow manure batches. Though potassium is an important macronutrient in biological systems, previous studies have demonstrated that high concentrations of potassium salts can lead to inhibition in anaerobic digesters. For example, Fang et al. (2011) found an IC_{50} value of $28 \text{ g K}^+ \cdot \text{L}^{-1}$ for anaerobic digesters co-digesting cow manure and desugared molasses. Studies have shown that anaerobic digesters can adapt to treating high salt content wastes, if given sufficient acclimatization time (De Baere et al. 1984). In the alkaline hydrolysate reactor, potassium concentrations steadily increased from an average of $1.62 \pm 0.10 \text{ g K}^+ \cdot \text{L}^{-1}$ prior to co-digestion (SS) to a maximum concentration of $7.7 \text{ g K}^+ \cdot \text{L}^{-1}$ on Day 841 (CT-VI).

Potentially inhibitory levels of environmental parameters clearly led to differences between the reactor microbiomes, especially in the case of the alkaline hydrolysate reactor. However, when considering the co-digestion period as a whole (*i.e.*, Days 371 to 935), we did not observe significant differences between the co-digestion reactors in

terms of average OTU richness (*i.e.*, as measured by alpha diversity metrics: total observed OTU ($p=0.13$) and Chao1 ($p=0.11$)) (Table 1).

3.2 Adaptation of alkaline hydrolysate reactor microbiome under stress

As noted above, the alkaline hydrolysate reactor microbiome diverged considerably from the other reactor microbiomes. Gradual increases in the organic loading rate of the alkaline hydrolysate co-substrate to the reactor allowed the reactor microbiome to change and adapt. Despite potentially inhibitory levels of free ammonia and total volatile fatty acids, the reactor mostly maintained its function (*i.e.*, methane yield decreased by $<10\%$) in the final period of the study (CT-V). During the entire co-digestion period, broad shifts in the alkaline hydrolysate reactor microbiome were noted: 1) an increase in Bacteroidetes members in the early periods (*i.e.*, CT-II) of co-digestion as well as a later decline; and 2) a shift to syntrophic acetate oxidation (based on indirect evidence) when volatile fatty acids levels increased in the reactor during CT-V and CT-VI.

The introduction of the alkaline hydrolysate to the reactor system led to a small increase initially (*i.e.*, from $45.8\pm5.5\%$ average in SS period to $62.4\pm2.1\%$ average in CT-III) in the relative abundance of the Bacteroidetes phylum (**Fig. S2C**), whose members have previously been linked to the breakdown of protein-rich waste materials (Kampmann et al. 2012, Kindaichi et al. 2004). Within the Bacteroidetes phylum, the relative abundance of one OTU belonging to the Bacteroidaceae family (**Fig. S3C**) rapidly increased in the alkaline hydrolysate reactor during CT-II, which led to a significant decrease in the evenness of the reactor microbial community (**Fig. S4**). Previously, Werner et al. (2014) noted that perturbations due to ammonia stress led to an initial

decline in community evenness in swine manure anaerobic digesters. As total and free ammonia levels increased in the reactor during CT-II, the microbiome clearly diverged from the other reactor microbiomes along PC2 in the PCoA (**Fig. 1A**), and this divergence was principally due to the one prominent OTU belonging to the Bacteroidaceae family (**Fig. 2B**).

Even though the OTU belonging to the Bacteroidaceae family (Bacteroidales order) initially increased with introduction of the protein-rich waste, the Bacteroidaceae family population considerably decreased in the latter half of the study (**Fig. S3C**) during which the free ammonia, total volatile fatty acid, and potassium concentrations increased. A previous study noted that Bacteroidales are often the dominant order in mesophilic anaerobic digesters subjected to relatively low levels of total volatile fatty acids, ammonia, and salts (De Vrieze et al. 2015). Similar to this previous study, our study observed a negative correlation between the relative abundance of Bacteroidales order members and the relative abundance of Firmicutes phylum members. Firmicutes are able to handle higher levels of dissolved salts (Goberna et al. 2009), and as gram-positive bacteria they have characteristically thicker cell walls than gram-negative bacteria such as Bacteroidetes.

At the high free ammonia, total VFA, and potassium concentrations, which we observed in the final periods of the study (CT-IV and CT-V), we can possibly infer from previous knowledge, and from indirect evidence that the alkaline hydrolysate reactor microbiome had shifted towards syntrophic acetate oxidation. High free ammonia concentrations have previously been shown to inhibit acetoclastic methanogens, which can lead to a build-up of acetate. Reactors that adapted to high free ammonia concentrations

exhibited a shift to syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis to utilize this excess acetate (Werner et al. 2014). We do not have direct evidence, but we observed a strong positive correlation (*i.e.*, $R^2=0.74$) between the total volatile fatty acid concentration in the alkaline hydrolysate reactor and the relative abundance of Clostridia class members in the phylum Firmicutes (**Fig. S5**), which have been previously implicated as syntrophic acetate oxidizers (Ziganshin et al. 2013). OTUs belonging to the class Clostridia, specifically OTUs classified to *Tepidimicrobium*, and *Gallicola* genus and Aerococcaceae family, were important taxa within the hydrolysate reactor microbiome between Days 750 to 800 (CT-V) (**Fig. 2A**). Within the Clostridia class, two OTUs belonging to the halophilic order Natranaerobiales (Mesbah et al. 2007) were also found to be highly predictive of the alkaline hydrolysate reactor microbiome *via* machine learning (**Fig. S6**). In regards to the methanogenic populations, no clear switch from acetoclastic to hydrogenotrophic methanogenesis was noted in the hydrolysate reactor microbiome, which would have been a direct evidence of the emergence of syntrophic acetate oxidation. However, the low sequence counts for the methanogen populations, likely due to primer bias, did not provide enough evidence to disprove or proof a shift towards syntrophic acetate oxidation.

The evenness of the alkaline hydrolysate reactor microbiome also recovered to pre-co-digestion levels with the presumed shift of the reactor towards syntrophic acetate oxidation (**Fig. S4**). As noted above, Werner et al. (2014) previously observed a decrease in community evenness with initial ammonia stress in swine waste anaerobic digesters, which also negatively affected reactor performance. However, performance eventually recovered, and this recovery coincided with a shift in the microbiome

towards syntrophic acetate oxidation and greater community evenness. Indeed, in an earlier study, Werner et al. (2011) found that, in general, anaerobic digester microbiomes with greater community evenness are more functionally stable than those with less even communities.

WWE1 members, principally composed of the genus *Candidatus Cloacomonas*, also declined ($8.6 \pm 5.2\%$ average in SS period to $0.52 \pm 1.11\%$ average in CT-V) in the later periods of the alkaline hydrolysate reactor (**Fig. S2C**). Pelletier et al. (2008) sequenced the genome of *Candidatus Cloacomonas Acidaminovorans* and concluded from *in-silico* proteome analysis that this species of the WWE1 phylum is capable of fermenting amino acids, sugars, and carboxylic acids. Some members of the Firmicutes phylum can perform these same functions (Kratat et al. 2011), and may have filled this metabolic niche in the hydrolysate reactor. A previous study also found a considerable decrease in *Candidatus Cloacomonas acidaminovorans* upon feeding a protein-rich substrate and hypothesized this was due to a reduction of the co-factors it needed for growth in the reactor environment (Kovács et al. 2013). In contrast, in the control reactor, the presence of the WWE1 phylum was maintained throughout the study, and no significant differences in the relative abundance between co-digestion periods was found (p-values >0.1) (**Fig. S2A**).

Finally, it should be noted that a new batch of alkaline hydrolysate (Batch #2) was introduced on Day 603 (CT-IV), and despite having similar proportions of organic-N and potassium as Batch #1 (*i.e.*, Batch #1: 0.95 Org-N:K^+ ; Batch #2: 1.08 Org-N:K^+), Batch #2 had a much lower initial ammonium concentration (*i.e.*, Batch #1: $0.94 \text{ g NH}_3\text{-N}\cdot\text{L}^{-1}$; Batch #2: $0.07 \text{ g NH}_3\text{-N}\cdot\text{L}^{-1}$). Thus, some of the population shifts in the alkaline

hydrolysate reactor microbiome observed in CT-IV may be partially attributable to this change in substrate batch.

3.3 Food waste reactor microbiome changes linked to free ammonia levels

The PCoA and db-RDA of all the reactors together clearly shows that changes in the environmental conditions in the reactors as a result of co-substrate addition were more important drivers of microbiome composition than the co-substrate by itself. Compared to the alkaline hydrolysate microbiome, the food waste microbiome did not diverge as significantly from the control microbiome. However, when examining the PCoA of the food waste, glycerol, and control reactor microbiome samples (no alkaline hydrolysate samples) (**Fig. 3A**) as well as the time series plot of the food waste and control reactor microbiome (**Fig. 2C**), it is clear that the food waste microbiome changed with co-substrate addition.

The divergence of the food waste reactor microbiome was linked to an increase in free ammonia levels in the reactor. The db-RDA analysis (**Fig. 3B**) of the food waste, glycerol, and control reactor samples revealed that free ammonia was correlated with the change in the food waste microbiome (*i.e.*, the direction of the free ammonia vector clearly corresponds to the divergence of the food waste microbiome with time, primarily along the PC1 axis). During the co-digestion phase (*i.e.*, Day 371 – 935), free ammonia levels in the food waste reactor increased 136% over background levels, from an average of $45 \pm 6 \text{ mg NH}_3\text{-N}\cdot\text{L}^{-1}$ prior to co-digestion (SS) to $106 \pm 15 \text{ mg NH}_3\text{-N}\cdot\text{L}^{-1}$ in the final HRT of the study (CT-VII). However, these ammonia levels were below levels previously reported as inhibitory for acclimated biomass (Braun et al. 1981, De Baere et al. 1984, Hansen et al. 1998, Kovács et al. 2013), and the food waste reactor exhibited

no major reductions in performance throughout the study period.

At the relatively low levels of free ammonia seen in the food waste reactor, Bacteroidetes members appeared to be acting as protein degraders. As observed in the earlier stages of the alkaline hydrolysate reactor, the relative abundance of Bacteroidetes members increased in the food waste reactor as free ammonia levels increased (**Fig. S2B**). Specifically, Bacteroidetes increased from an initial average relative abundance of $45 \pm 3\%$ during CT-I to a final average relative abundance of $59 \pm 4\%$ in the last co-digestion period, CT-VII. An OTU belonging to the Bacteroidales order within the phylum Bacteroidetes was found to be predictive of the food waste reactor *via* machine learning (**Fig. S6**).

Other parameters or characteristics of the food waste substrate that were not monitored may have played a role in driving the observed microbiome changes. As noted previously, studies have found that the microbiota composition in anaerobic reactors is greatly influenced by the composition of the input substrates (Regueiro et al. 2014, Zhang et al. 2014). The first two axes of the db-RDA (*i.e.*, RDA1 (19.9%) and RDA2 (8.4%)) only explained approximately 65% of the phylogenetic variation shown by the first two axes of the unconstrained PCoA plot (*i.e.*, PC1 (27.8%) and PC2 (15.5%)) (**Fig. 3**). This is much lower than in the PCoA and db-RDA analyses of all the reactor samples (*i.e.*, including the alkaline hydrolysate reactor) where the first two axes of the db-RDA explained approximately 83% of the phylogenetic variation shown by the first two axes of the unconstrained PCoA plot (**Fig. 1**).

3.4 Crude glycerol reactor exhibited sudden failure and little adaptation

The crude glycerol reactor achieved the highest methane yields of any of the co-substrates. However, crude glycerol caused sudden and acute instability at its maximum mixing ratio with manure (*i.e.*, 49:51, glycerol:manure; VS basis). The final 10% increase in OLR led to a rapid accumulation of VFAs (*i.e.*, to a maximum concentration of 4140 mg·L⁻¹ on Day 895) and excessive foaming, which ultimately led to system failure (*i.e.*, as indicated by a large drop in methane production). This response was attributed to the high lipid content of this material, which due to rapid hydrolysis had caused an accumulation of long-chain fatty acids (LCFAs) to inhibitory levels (*i.e.*, to an average concentration of 324.9±23.6 mg COD·g TS⁻¹ for the solid-phase associated LCFAs and 1633.9±156.0 mg COD·L⁻¹ for the liquid-associated LCFAs on the final day of operation, Day 895).

Due to the rapid failure of the glycerol reactor, only one microbiome sample is available from the time of failure (*i.e.*, Day 895 sample). Shifts in the taxonomy of this microbiome sample can clearly be observed in the phyla and family level taxa plots (**Fig. S2D and S3D**). Due to the single timepoint, it is difficult to draw conclusions regarding the trajectory of the microbiome shift under these final inhibitory conditions.

From the PCoA of the food waste, glycerol, and control reactor microbiomes, it can be seen that the glycerol reactor microbiome exhibited the least divergence from the control reactor microbiome (**Fig. 3A**). However, the PCoA of only the glycerol and control reactor microbiomes (**Fig. 4**) does show that the glycerol reactor microbiome diverged somewhat in the later periods, primarily along the PC2 axis. One control reactor microbiome sample is also clustering with these later glycerol reactor time points, potentially due to mislabeling or sample handling issues. In general, the control

reactor microbiome diverged from its earlier community primarily along the PC1 axis (Fig. 2D, Fig. 4). We hypothesized that a change in the both reactor microbiomes may have occurred due to the switch to a new batch of manure on Day 697. Yet, it is clear from the PCoA that the shift occurred due to an earlier unmonitored change in the reactor environments, because several microbiome samples prior to the manure batch change cluster with the time points from the later manure batch. As mentioned previously, Fernández et al. (1999) observed changes in anaerobic digester microbiomes during stable operating periods when no apparent changes in the operating conditions were made.

In the db-RDA of the food waste, glycerol, and control reactor microbiomes (Fig. 3B), methane content and total VFA concentrations were the environmental parameters linked with the divergence of the glycerol reactor microbiome. As mentioned previously, due to limited measurements, LCFA concentrations were not included as a parameter in the db-RDA analysis. Compared to the food waste and control reactors, the glycerol reactor exhibited a higher methane content in its biogas for the majority of the co-digestion periods. As mentioned above, total volatile fatty acid concentrations rapidly rose in the glycerol reactor during its final days of operation. However, it should be emphasized again that the glycerol reactor did not exhibit a large divergence from the control reactor microbiome.

One reason for the smaller divergence observed in the glycerol reactor microbiome, when compared to the other co-substrates, could be due to the smaller volume of glycerol co-substrate that was needed to achieve the same OLR as the other co-digestion reactors. This translated to a generally lower level of potentially inhibitory compounds

in the glycerol reactor. For example, the glycerol co-substrate by itself had a higher potassium concentration than the alkaline hydrolysate co-substrate. However, due to the significant dilution of the energy-rich glycerol co-substrate, the potassium concentration in the substrate entering the glycerol reactor was lower than the concentration entering the alkaline hydrolysate reactor. As a consequence, the potassium concentration reached relatively low levels in the glycerol reactor (*i.e.*, $2.6 \pm 0.3 \text{ g K}^+ \cdot \text{L}^{-1}$ in period CT-VI) compared to the alkaline hydrolysate reactor.

Some differences were observed in the glycerol reactor microbiome compared to the other reactor microbiomes. Members of the Syntrophomonadaceae family (class Clostridia) have been previously linked to LCFA degradation (Hatamoto et al. 2007, Sousa et al. 2007). At the family level, Syntrophomonadaceae abundance was considerably higher in the glycerol reactor ($12.4 \pm 3.9\%$ on average across the entire co-digestion period) compared to the other reactors ($6.8 \pm 2.5\%$ in hydrolysate, $2.2 \pm 0.9\%$ in the food waste and $3.3 \pm 2.5\%$ in the manure-only reactor) (**Fig. S3D**). In both the hydrolysate and glycerol reactors, LCFAs accumulated in the liquid phase, but those in the solid phase only accumulated in the glycerol reactor (*i.e.*, up to $325 \text{ mg COD} \cdot \text{gTS}^{-1}$). In the final timepoint in the glycerol reactor, the relative abundance of the Syntrophomonadaceae family declined.

4. Conclusions

- Inhibitory conditions appeared to be more important drivers of microbiome succession than the co-substrate itself; though, the co-substrate type did play a role.

- Gradual introduction of the alkaline hydrolysate co-substrate allowed the reactor microbiome to shift and adapt to the high levels of free ammonia, potassium, and volatile fatty acids to which it was exposed.
- Bacteroidales members increased in the alkaline hydrolysate and food waste reactors upon increased free ammonia levels. However, inhibitory levels of ammonia and VFAs drove the increase of Clostridia class members in the alkaline hydrolysate at the expense of Bacteroidales members.
- The glycerol reactor microbiome experienced an increase in the relative abundance of Syntrophomonadaceae family members, which have previously been associated LCFA degradation.

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674 **Table legends**

675 **Table 1.** Averaged total observed species (observed OTUs) and predicted species

676 (Chao1) values for the four reactor types across the co-digestion period.

677

Figure captions

Figure 1. Reactor microbiome succession and its correlation to environmental conditions. **A)** 2D PCoA of the major principal coordinates based on the pairwise weighted UniFrac distances matrix. **B)** Constrained Distance-Based Redundancy Analysis (db-RDA) showing the environmental parameters that best explain the variation seen in microbial community structure based on weighted UniFrac distances. Red circles represent data from control manure-only digestion (Manure); green circles represent data from food waste co-digestion (Food waste); blue circles represent data from alkaline hydrolysate co-digestion (Hydrolysate); and violet circles represent data from crude glycerol co-digestion (Glycerol). Shaded circles represent samples taken during the steady-state period (prior to co-digestion), while the gradient colors represent samples taken during each co-digestion period (*i.e.*, from the 1st co-digestion treatment (CT-I) to the 7th co-digestion treatment (CT-VII)). The color key is given below panel B.

Figure 2. Times-series analyses of microbiome phylogeny divergence along the first or second principle coordinate axes (*i.e.*, PC1 and PC2) based on the PCoA of the weighted UniFrac distance matrix for each co-substrate reactor microbiome compared to the control reactor microbiome. All panels show data during the co-digestion part of the study (*i.e.*, from the 1st co-digestion treatment (CT-I) to the 7th co-digestion treatment (CT-VII)). The data from the manure, hydrolysate, food waste, and glycerol reactor phylogeny is represented by red, blue, green, and purple lines, respectively. **A)** the divergence of the hydrolysate reactor microbiome and the control manure-only reactor microbiome along PC1. **B)** the divergence of the hydrolysate reactor microbiome and the control manure-only reactor microbiome along PC2. **C)** the divergence of the food waste microbiome and the control manure-only microbiome

along PC1. **D)** the divergence of the glycerol reactor microbiome and the control manure-only reactor microbiome along PC1. The 25 OTUs with the highest average relative abundance across the co-digestion period are represented as variously colored spheres. The sizes of the spheres are proportional to relative abundance of the taxon across all samples. We used differently colored spheres to highlight the OTUs that appeared to drive the changes observed between the co-digestion reactor and control (manure-only) reactor microbiomes. Green spheres represent the OTUs that were more associated with the control manure microbiome, while blue and red spheres together, indicate the OTUs associated with the co-digestion reactor microbiomes. For the hydrolysate reactor, OTUs relevant to periods when drastic microbiome shifts occurred are highlighted (*i.e.*, Blue: Days 600 – 750; Red: Days 750 – 865).

Figure 3. Reactor microbiome succession and its correlation to environmental conditions, excluding the alkaline hydrolysate reactor samples. **A)** 2D PCoA of the major principal coordinates based on the pairwise weighted UniFrac distances matrix. **B)** Constrained Distance-Based Redundancy Analysis (db-RDA) showing the environmental parameters that best explain the variation seen in microbial community structure based on weighted UniFrac distances. The color key is the same as in **Fig. 1**. Briefly, red circles represent data from control manure-only digestion (Manure); green circles represent data from food waste co-digestion (Food waste); and violet circles represent data from crude glycerol co-digestion (Glycerol).

Figure 4. 2D PCoA of the major principal coordinates based on the pairwise weighted UniFrac distances matrix for the control (MN) and glycerol (GY) reactors only. The figure key indicates which manure batch (*i.e.*, Batch 2 or Batch 3) was used for each

728 time point for both reactors.

729

730 **Table 1**

Reactor	Observed species	Chao1
Manure (control)	433 ± 63	634 ± 66
Hydrolysate	472 ± 70	718 ± 121
Food waste	412 ± 47	642 ± 102
Glycerol	457 ± 74	686 ± 111

731

Figure 1

PERIOD	CT-I	CT-II	CT-III	CT-IV	CT-V	CT-VI	CT-VII
Study Days	371 – 450	451 – 525	526 – 602	603 – 726	727 – 806	807 – 882	883 – 935
Manure							
Food Waste							
Hydrolysate*							
Glycerol**							

* Co-digestion period CT-V was extended through Day 865 in the alkaline hydrolysate reactor.

** The glycerol reactor was stopped on Day 895.

Figure 2

Figure 3

PERIOD	CT-I	CT-II	CT-III	CT-IV	CT-V	CT-VI	CT-VII
Study Days	371 – 450	451 – 525	526 – 602	603 – 726	727 – 806	807 – 882	883 – 935
Manure							
Food Waste							
Glycerol*							

* The glycerol reactor was stopped on Day 895

Figure 4