8-2019

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Applied Microbiology and Biotechnology, Vol. 103, No. 15 (August 2019): 6353-6367. DOI.

Syntroph Diversity and Abundance in Anaerobic Digestion Revealed Through a Comparative Core Microbiome Approach

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Abstract

Anaerobic digestion is an important biotechnology treatment process for conversion of waste to energy. In this study, a **comparative core microbiome approach**, i.e., determining taxa that are shared in functioning digesters but not shared in non-functioning digesters, was used to determine microbial taxa that could play key roles for effective **anaerobic digestion**. **Anaerobic** digester functions were impaired by adding the broad-spectrum antimicrobial triclosan (TCS) or triclocarban (TCC) at different concentrations, and the **core microbiomes** in both functioning and non-functioning **anaerobic** digesters were compared. Digesters treated with high (2500 mg/kg) or medium (450 mg/kg) TCS and high (850 mg/kg) TCC concentrations lost their function, i.e., methane production decreased, effluent volatile fatty acid concentrations increased, and pH decreased. Changes in microbial community **diversity** and compositions were assessed using 16S rRNA gene amplicon sequencing. Microbial richness decreased significantly in non-functioning digesters ($p < 0.001$). Microbial community compositions in non-functioning digesters significantly differed from those in functioning digesters ($p = 0.001$, ANOSIM). Microbes identified as potentially key taxa included previously known fatty acid-degrading **syntrophs** and amino acid-degrading **syntrophs**. A diverse group of **syntrophs** detected in this study had low relative **abundance** in functioning digesters, suggesting the importance of rare microbes in **anaerobic** digester operation.

The **comparative microbiome approach** used in this study can be applied to other microbial systems where a community-driven biological phenomena can be observed directly.

Keywords:

16S rRNA; Methanogenesis; Microbial **diversity**; Rare microbial taxa; Triclosan; Triclocarban

Introduction

Demands for sustainable energy have grown with efforts to reduce dependency on fossil fuels. Water resource recovery facilities (formerly referred to as wastewater treatment plants) receive organic carbon in the influent wastewater every day, and this influent carbon is a renewable energy source that can be converted to methane via **anaerobic digestion**. **Anaerobic** digesters are operated in municipal, agricultural, and industrial treatment plants, but are susceptible to biological process upsets. Malfunction of **anaerobic** digesters can happen, for example, when they experience an organic overload (Steyer et al. [81]; Tale et al. [84]), when the microbial community is disturbed by chemical stressors (Hansen et al. [28]; Sanz et al. [69]), and when pH is lowered due to accumulation of volatile fatty acids (VFAs) or other factors (Ahring et al. [1]; Ripley et al. [64]). Knowledge about the key microbial taxa in **anaerobic** digesters and the environmental conditions necessary for their physiological activities could help to mitigate process upsets in these treatment systems (Venkiteshwaran et al. [88]), potentially by bioaugmenting digesters with these key taxa (Schauer-Gimenez et al. [70]; Tale, et al. [84]).

Important microbes in **anaerobic digestion** are those involved in hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Jimenez et al. [36]). Syntrophic reactions that are directly coupled with methanogenesis, including butyrate oxidization (Dwyer et al. [18]; Liu et al. [47]; Sieber et al. [78]),

propionate oxidization (Ariesyady et al. [2]; de Bok et al. [14]; Shigematsu et al. [77]), and acetate oxidization (Hattori et al. [30]; Ito et al. [32]; Schnürer et al. [74]; Westerholm et al. [91]), are particularly integral for proper digester function. **Syntroph** and methanogen coupling enables thermodynamically unfavorable processes to become energetically feasible by reducing product concentrations that would otherwise build up and stop reactions from continuing to occur (Schink [71]; Stams and Plugge [80]). Although microbial taxa that are capable of hydrolysis and acidogenesis of organic carbon have been characterized, only a small number of **syntrophs** in **anaerobic** digesters are well characterized, partly due to the inherent difficulty in culturing these strains (McInerney et al. [52]).

The role of microbes in digesters has been studied at community levels using biomolecular techniques. This culture-independent **approach** is particularly important since the majority of microbes that control the intermediate processes of biological waste conversion to methane are not readily culturable. Culture-independent techniques that have been used to elucidate the link between microbial taxa and function include terminal restriction fragment length profiling (T-RFLP) (Ike et al. [31]; McHugh et al. [50]), denaturing gradient gel electrophoresis (DGGE) (Cardinali-Rezende et al. [8]; Liu et al. [46]), fluorescent in situ hybridization (FISH) (Ariesyady, et al. [2]; Sekiguchi et al. [75]), and clone libraries (Chouari et al. [12]; Figuerola and Erijman [22]; Riviere et al. [66]). While TRFLP and DGGE are limited by resolution of identified taxa, FISH is limited by lack of a priori knowledge about designing probes. Clone libraries can identify taxa that have a relatively high **abundance** in a community, while key microbial processes such as syntrophic processes could be carried out by taxa that may have small relative **abundances** in the community (Narihiro et al. [56]). Next-generation sequencing (NGS) of the 16S rRNA gene provides an opportunity to identify microbial taxa, including putative **syntrophs**, that have low relative **abundance** in a digester community. However, most studies that have used NGS were observational in that the presence of microbial taxa was identified in functioning digesters (Jang et al. [35]; Kuroda et al. [40]; Lucas et al. [48]; Sun et al. [82]; Sundberg et al. [83]). While microbial taxa identified using NGS typically range from 1500 to 2000 taxa per sample at the plateau or from 5000 to 15,000 taxa per study, those taxa were rarely linked to roles in biogas conversion processes.

One of the biggest challenges in studying microbial populations and their respective roles in full-scale **anaerobic** digesters is that, while some microbial populations have adapted to the digester's microenvironment and play essential roles in digester functions, others are transiently associated with digesters. **Core microbiome approaches**, whereby microbial taxa that were shared across functioning **anaerobic** digesters were identified, were used to overcome this challenge (Lee et al. [42]; Riviere, et al. [66]; Rui et al. [68]). While this **core microbiome approach** is promising for identifying key microbial taxa in functioning digesters, it may underestimate key microbial taxa given resource and temporal heterogeneity across different **anaerobic** digesters.

In this study, a **comparative core microbiome approach** was used to link microbial taxa to their roles in the biogas conversion process during **anaerobic digestion**. In this new **approach**, microbial populations and biological processes in digesters were altered using the generic antimicrobial substances triclosan and triclocarban that target a broad range of microbial taxa (Fujimoto et al. [24]; Halden [27]). These broad range antimicrobials are used to remove multiple taxa that play roles in multiple processes in functioning **anaerobic** digesters. These antimicrobials had been used in hygiene products and toothpastes (Ying et al. [95]) and are readily found in **anaerobic** digesters due to their hydrophobic

nature (Halden [27]). Different concentrations of these two antimicrobials were added to functioning digesters, and the subsequent changes in both digester function and their microbial communities were observed. By comparing the **core microbiomes** between functioning and resultant non-functioning digester microbial communities, we identified several key microbial taxa that ostensibly play critical roles in **anaerobic digestion**.

Materials and methods

Experimental design and sampling

Lab-scale **anaerobic** digesters were operated to determine the impact of antimicrobials on digester function and microbial community. Digester setup and operation have been described previously (Carey et al. [9], [10]). Briefly, digester inoculum was collected from municipal digesters at South Shore Water Reclamation Facility (Oak Creek, WI). Fifty milliliters of the digester sludge were added to 160-mL serum bottles which served as lab-scale **anaerobic** digesters. **Anaerobic** conditions were established by injecting a N_2:CO_2 gas blend (7:3 *v*/*v*) into the digester headspace. **Anaerobic** digesters were fed daily 5 mL of synthetic primary sludge that consisted of ground dry dog food (Nutro Natural Choice, Franklin, TN) in nutrient solution (Carey et al. [9]) (compositions of the nutrient solution and dry dog food are provided in Table S1 and Table S2), and 5 mL of the digester sludge were removed daily to yield a solids retention time (SRT) of 10 days. Digesters were incubated at 35 °C (representing mesophilic **anaerobic digestion**) on a shaker table at 100 rpm.

Eight sets of triplicate digesters were operated. For two digester sets, background TCS or TCC concentrations were maintained by adding TCS and TCC into the digesters at 30 mg/kg, the concentrations found in the inoculum sludge taken from municipal **anaerobic** digesters (background groups) (Carey et al. [9]; [10]). For six digester sets, digesters fed low (130 mg/kg for TCC, 100 mg/kg for TCS), medium (450 mg/kg for TCC, 850 mg/kg for TCS), and high (850 mg/kg for TCC, 2500 mg/kg for TCS) concentrations of each antimicrobial were prepared. The low concentrations were selected based on the 95th percentile concentrations found in a national survey of US biosolids (USEPA [86]). The medium and high concentrations corresponded to IC_{10} and IC_{50} concentrations determined during short-term (10 day) preliminary batch testing (Carey et al. [9]). All eight digester sets were initially acclimated by feeding the synthetic primary sludge for 45 days at background TCS and TCC concentrations before antimicrobial concentrations were increased on day 46. The elevated antimicrobial concentrations were maintained for an additional 65 days (> 6 SRT values, 110 days total). Digesters were operated in triplicate and for multiple solids retention times so that reproducible changes in community structure stemming from antimicrobial exposure could be **revealed**. Biomass samples were taken from the digesters on day 0 (seed), day 45 (pre-treatment), and on day 110 (post-treatment) for microbial composition analysis.

Digester function

For digester functional analysis, gas production was measured daily using a protocol previously described (Schauer-Gimenez et al. [70]). Biogas methane composition was determined using gas chromatography with thermal conductivity (GC-TCD) using a protocol described previously (Schauer-Gimenez et al. [70]). The methane production was calculated by multiplying methane composition by gas production (mL/day). The sum of VFA concentrations was determined using a GC-FID as described

previously (Venkiteshwaran et al. [88]). The pH was determined with a pH meter (Orion 4 Star, Thermo, Waltham, MA). Our previous research **revealed** that TCC degradation was negligible as indicated by observing target TCC concentrations in the effluent (Carey et al. [9]). It is possible that biodegradation slightly altered TCS concentrations in the effluent with approximately 25% removal in low digesters and 10% removal in medium digesters (Carey et al. [10]).

DNA extraction and Illumina sequencing

DNA was extracted from 1.8 mL of digester contents using MP FastDNA SPIN kits (Solon, OH) according to manufacturer's protocol with minor adjustments as described previously (McNamara et al. [54]). The extracted DNA samples were sent to a commercial sequencing facility (MRDNA, Shallowater, TX). The V4 region of 16S rRNA gene was amplified using 515F (with barcode on forward primer) and 806R primers which are known to prime both bacterial and archaeal taxa (Caporaso et al. [7]; Walters et al. [90]). PCR amplicons were purified using AmPure XP beads (Beckman Coulter, Inc., Brea, CA). Libraries were prepared following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed using Illumina MiSeq platform with v3 300 base single read protocol. The raw sequence reads were deposited in the Short Reads Archive (SRA) and are publicly available with the accession number from SRR5244210 **through** SRR5244263.

16S rRNA sequence data analysis

Raw output data were processed using a commercial sequencing facility pipeline (Dowd et al. [17]) (MRDNA Shallowater, TX). In short, raw reads with ambiguous base or low average quality (<Q25) or short length (< 200 base) were removed. Chimera sequences were identified using UCHIME (Edgar et al. [20]) and removed. The processed reads were denoised using protocol described previously (Reeder and Knight [63]). Quality reads were clustered using UCLUST (Edgar [19]) based on the sequence similarity and OTUs were assigned at the 97% sequence similarity cutoff level. The representative sequence read from each OTU with 97% sequence similarity cutoff was classified for its identity using BLASTn with best-hit filtering algorithm against a curated database derived from GreenGenes, RDPII, and NCBI. For the subsequent alpha and beta **diversity** analyses, the number of reads per sample was synchronized by rarefying reads at 17,925, the lowest reads of all samples. Richness of each sample was determined as the number of observed OTUs at 17,925 reads.

The effect of antimicrobials on richness was assessed using Wilcoxon rank sum test. The effect of antimicrobials on microbial community composition was visualized using principal coordinate analysis (PCoA) with Bray-Curtis distance using vegan and labdsv packages in R. The relative **abundance** bar graphs were generated at phylum, class, family, and genus level resolution using phyloseq with tax_glom function in R to assess the effect of antimicrobials on microbial **abundance**.
Relative **abundance** was used instead of actual quantity in this study, partly because there was no significant difference in 16S rRNA gene copies between functioning and non-functioning digesters (Carey et al. [9], [10]). DNA internal standards were not added to assess DNA extraction efficiency because all digesters were operated with the same inoculum source community and because one internal standard might not represent extraction efficiency of all taxa. Microbial richness plots, PCoA plots, and relative **abundance** bar plots at different taxonomic levels were synthesized using ggplot in R. The effect of antimicrobials on the relative **abundance** of microbial taxa at different taxonomic level was performed using STAMP (Parks et al. [57]) with White's non-parametric test (White et al.

[92]). **Core microbiome** analysis was performed by converting the relative **abundance** OTU table into binomial ("1" for presence and "0" for absence) using "IF" function in excel. Venn diagrams were calculated using vennCounts and vennDiagram functions in the limma package in R. The calculated Venn diagram was visualized using draw.triple.venn function in VennDiagram package in R. R version 3.1.1 was used throughout the study (R_Core_Team [62]).

Results

The use of antimicrobials to create functioning and non-functioning digesters

Before antimicrobial concentrations were increased on day 46 of operation, all digesters demonstrated stable methane production (67.4 ± 4.8 mL/day), near-neutral pH (7.21 ± 0.03), and low effluent VFA concentrations (231 ± 140 mg/L) (Table S3). At day 110 (over 6 solid retention time values following introduction of the antimicrobial), when the digesters had reached quasi steady-state under their respective antimicrobial loadings, loss of digester function was observed in three sets of digesters: medium-TCC digesters, high-TCC digesters, and high-TCS digesters. These three digester sets are referred to as the non-functioning digesters. In these three sets, the average concentration of VFAs was 15,400 mg/L as compared to 31 mg/L in the background digester sets. Similarly, pH and methane production decreased substantially in non-function digester (Table S3, Fig. 1). In contrast, the low and medium TCS digesters and the low TCC digesters continued to maintain function and demonstrated similar methane production, VFA concentrations, and pH as the background control digesters; these digesters including background control digesters are referred to as the functioning digesters (Table S3, Fig. 1). As expected, methane production and VFA concentrations were negatively correlated, whereas methane production and pH were positively correlated (Fig. 1).

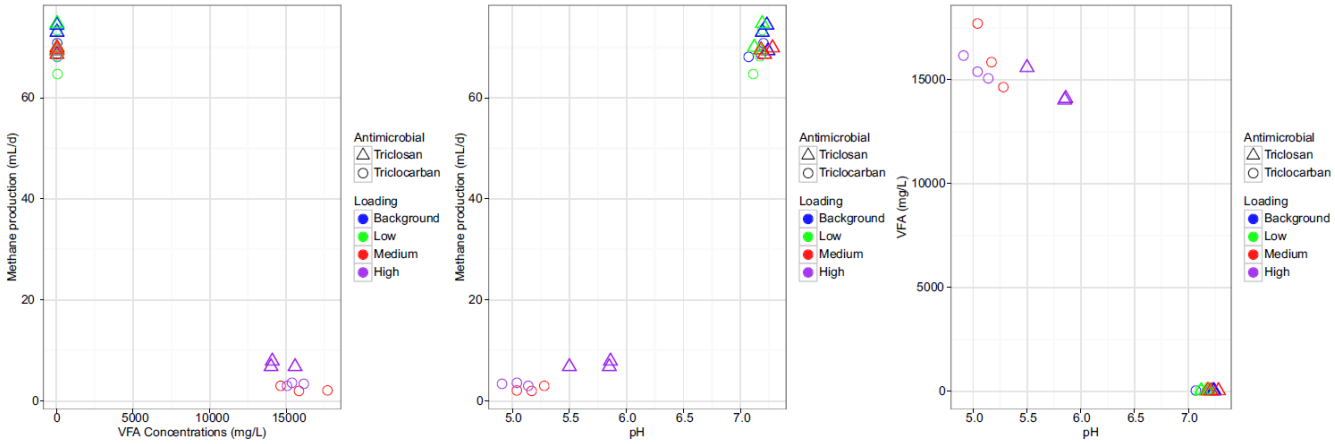

Fig. 1Changes in digester function after antimicrobial treatments with TCC and TCS at day 110. The relationship between pH, methane gas production, and VFA concentrations was depicted. A positive relationship was observed between pH and methane gas production, while negative relationships were observed between pH and VFAs and VFAs and methane gas production. Non-functioning digesters had low methane gas production, high VFA concentrations, and lower pH

Diversity in functioning and non-functioning digesters

Microbial richness (i.e., alpha **diversity**) determined by the number of observed OTUs was between approximately 2000 and 2500 OTUs in background digesters throughout the duration of experiment

(Fig. 2). The average number of observed OTUs decreased from 2050 OTUs in functioning digesters to 831 OTUs in non-functioning digesters at day 110 following the addition of the antimicrobials. The richness strongly correlated to the functionality of the digesters; non-functioning digesters had significantly lower numbers of observed OTUs than functioning digesters at day 110 ($p < 0.001$, Wilcoxon test).

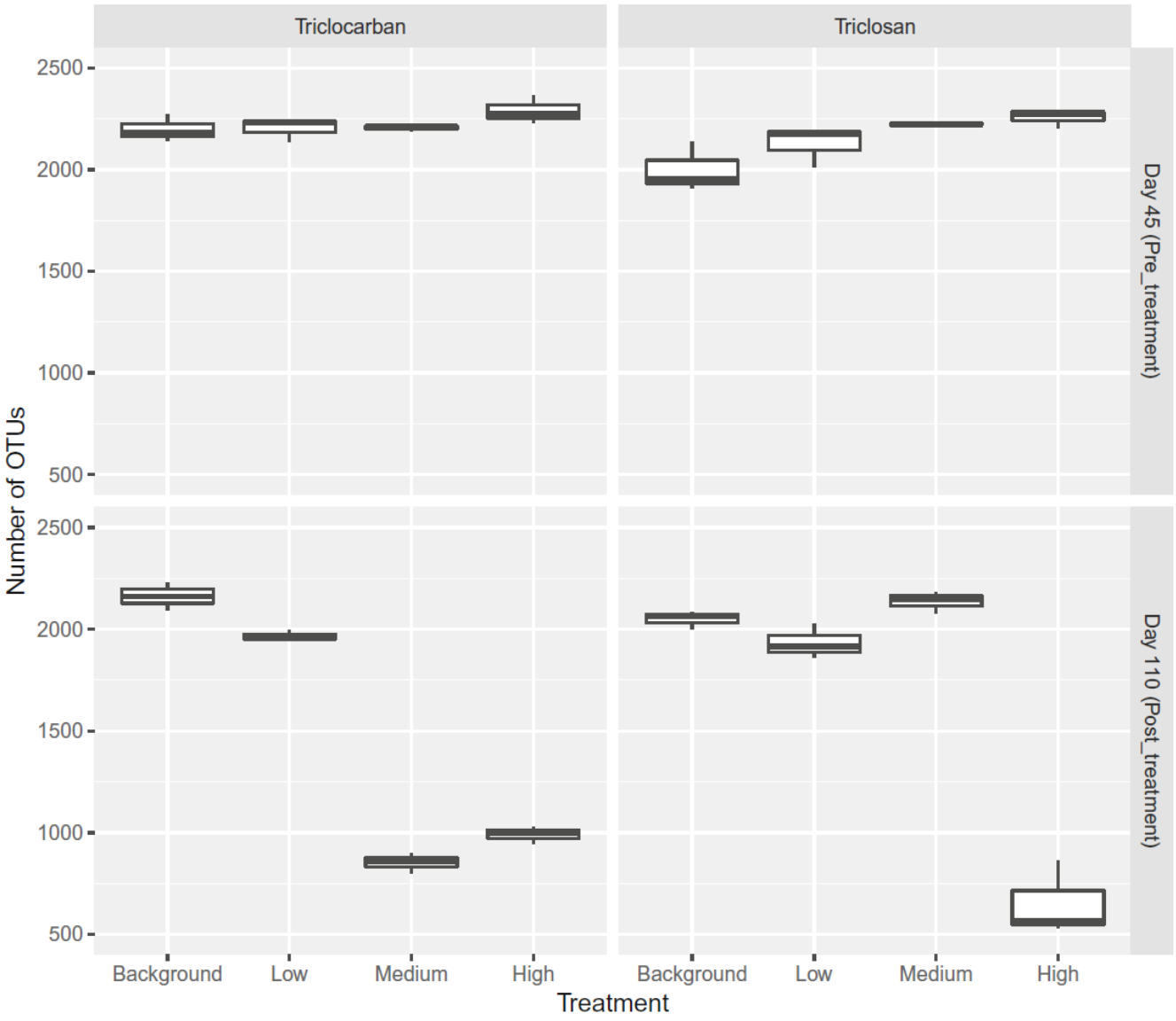

Fig. 2 The effect of TCC and TCS treatments on microbial richness represented by the number of observed OTUs. The effect of treatment levels (background, low, medium, and high) on richness was depicted before (day 45) and after (day 110) the addition of antimicrobials (n = 3)

The microbial community compositions in non-functioning digesters were also substantially altered compared to the background controls. Microbial communities at day 45 (i.e., pre-treatment) were similar to each other, but at day 110 microbial communities in non-functioning digesters were distinct from microbial communities in functioning digesters as a result of the antimicrobials added (Fig. 3: PCO1 axis). The non-functioning microbial communities were clustered together and were significantly different from the functioning communities at day 110 ($p = 0.001$: ANOSIM). With respect to the effect

of different antimicrobials, microbial community compositions in high TCS digesters were also different from those in medium and high TCC digesters (p = 0.011: ANOSIM), though the difference was much less relative to the differences between the background digesters and these non-functioning digesters (Fig. 3). It is noted that, in the background digesters that were fed with synthetic primary sludge, the microbial communities changed drastically over time from day 0 to day 45 as the communities acclimated to the feed. The change in microbial communities over time was captured by the second axis of PCoA plot (Fig. 3: PCO2 axis). The change from day 45 to day 110 in the background digesters was relatively minor (based on distance the communities moved on the second axis) compared to the change over the first 45 days.

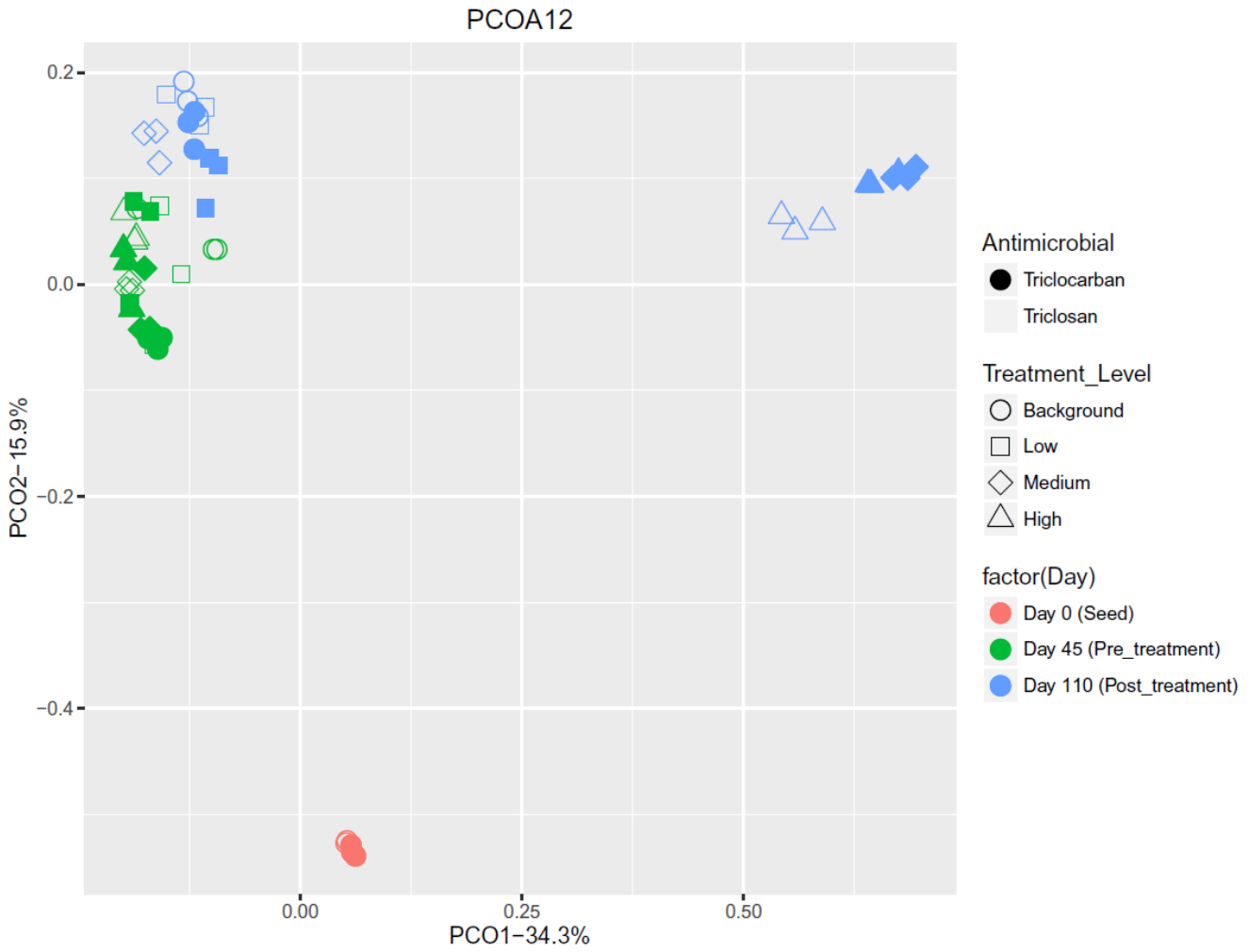

Fig. 3The effect of TCC and TCS treatments on microbial community compositions. The PCoA plot was generated using Bray-Curtis dissimilarity distance matrix derived from rarefied OTUs table with 97% similarity cutoff. Temporal effect and treatment effect on microbial community compositions were depicted along PCO1 and PCO2 axis, respectively. The closed symbols are TCC and the open symbols are TCS

Taxa analysis at phylum and class levels

Feeding digesters with synthetic primary sludge for 45 days enriched both *Firmicutes* and *Bacteroidetes* in **anaerobic** digesters from less than 10% to nearly 30% of the relative **abundance**, respectively (Fig. S1). At day 45, microbial communities of all digesters were

similar to each other at the phylum level (Fig. S1). At day 110, the background digesters were dominated by the phyla *Firmicutes* (35.1%), *Bacteroidetes* (24.5%), *Spirochaetes* (16.1%), *Proteobacteria* (10.4%), *Euryarchaeota* (3.8%), and *Chloroflexi* (2.1%) (Fig. S1). Within phylum *Proteobacteria*, the class *Betaproteobacteria* and *Deltaproteobacteria* accounted for a majority of taxa, whereas *Gammaproteobacteria* represented lower relative **abundance** in the background (Fig. S2).

The antimicrobial concentrations that resulted in non-functioning digesters changed the microbial communities at phylum and class levels as seen on day 110. The **abundance** of the class *Betaproteobacteria* and *Deltaproteobacteria* decreased in all non-functioning digesters (Fig. S2, $p < 0.01$ and $p < 0.01$, respectively). The **abundance** of *Gammaproteobacteria* also substantially diminished in TCC-treated non-functioning digesters, but were dominant in TCS-treated non-functioning digesters in which they accounted for nearly 60% of the relative **abundance** in the community (Fig. S2). The relative **abundance** of phylum *Spirochaetes* was substantially diminished in non-functioning digesters ($p < 0.01$, Fig. S1). The relative **abundance** of the phylum *Chloroflexi* was also reduced ($p < 0.01$), although they only accounted for a minor fraction in background digesters at day 110. TCS and TCC treatments did not change the relative **abundance** of *Firmicutes* ($p = 0.91$). The relative **abundance** of *Bacteroidetes* was also unaffected in TCC-treated non-functioning digesters but were diminished in TCS-treated non-functioning digesters. The **abundance** of the Archaea phylum *Euryarchaeota* remained unaffected before and after treatment with TCS and TCC when relative **abundance** was determined at phylum level ($p = 0.12$).

Taxa analysis at family and genus levels

Although no changes in archaeal relative **abundance** were observed after TCS and TCC treatments at the phylum level, the TCS and TCC treatments impacted archaeal communities at the family level (Fig. 4). In the non-functioning digesters, antimicrobials reduced the **abundance** of two families, *Methanosaetaceae* and *Methanomicrobiaceae*, relative to the background digesters ($p < 0.01$ and $p < 0.01$, respectively), while the relative **abundance** of the family *Methanobacteriaceae* substantially increased in non-functioning digesters relative to the background digesters ($p < 0.01$).

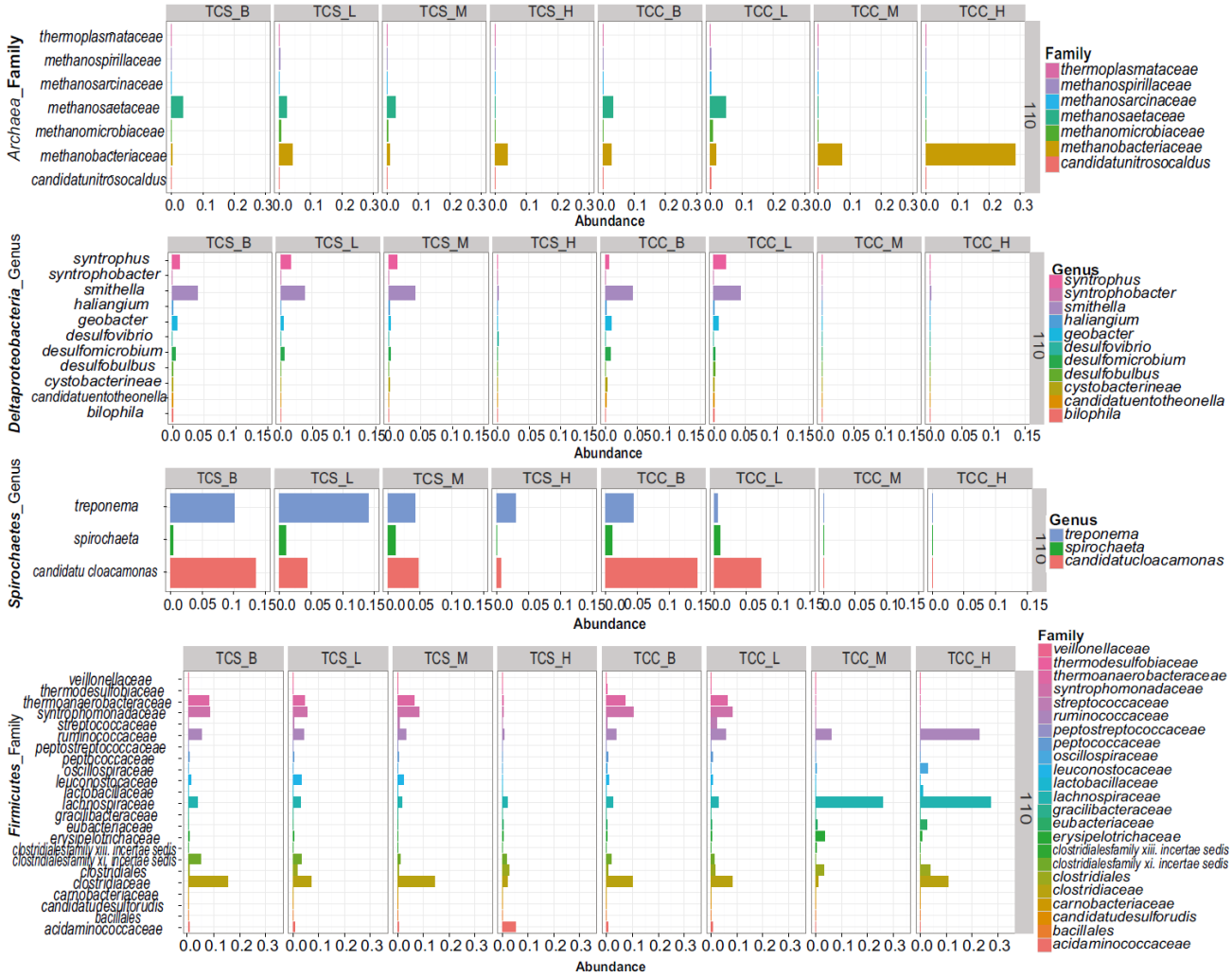

Fig. 4Relative **abundance** of microbial taxa determined at family or genus level across treatment groups at day 110. The relative **abundance** for each microbial taxon was plotted on X -axis, while the name of each taxon either at genus or family level was presented on Y -axis. Letters B, L, M, and H represent background, low, medium, and high, respectively

The *Betaproteobacteria* and *Deltaproteobacteria* that accounted for a major proportion within *Proteobacteria* in background digesters were diminished in non-functioning digesters. Within the *Betaproteobacteria*, the relative **abundance** of the genera *Brachymonas* and *Azospira* decreased in non-functioning digesters (Fig. S3, $p < 0.01$ and $p < 0.01$, respectively). Within the *Deltaproteobacteria*, the relative **abundance** of the genera *Syntrophus* and *Smithella* decreased in non-functioning digesters (Fig. 4, $p < 0.01$ and $p < 0.01$, respectively). *Gammaproteobacteria* genus *Succinivibrio* became dominant in high TCS-treated non-functioning digesters accounting for between 30 and 60% of the entire microbial communities in the digester, while they represented less than 2% in medium and high TCC-treated non-functioning digesters (data not shown).

Within phylum *Spirochaetes*, the relative **abundance** of genera *Spitochetea* and "*Candidatus* Cloacamonas" was substantially reduced in non-functioning digesters relative to those in functioning digesters (Fig. 4, $p < 0.01$ and $p < 0.01$, respectively). The **abundance** of genus *Treponema* was also low in digesters treated with the medium and high TCC relative to

background, but the high TCS-treated non-functioning digesters had an **abundance** of this genus similar to the **abundance** seen in background digesters.

While TCC and TCS treatments did not substantially change the total relative **abundance** of *Firmicutes* at the phylum level, the antimicrobials affected *Firmicutes* populations differently at family levels. When digesters were treated with high TCS and medium and high TCC concentrations, the relative **abundance** of families *Thermoanaerobacteraceae* and *Syntrophomonadaceae* decreased substantially (Fig. 4, $p < 0.01$ and $p < 0.01$, respectively). Other families were less affected by TCS and TCC amendments. Likewise, the treatments with high TCS and medium and high TCC affected *Bacteroidetes* populations differently at the family level. The **abundance** of families *Porphyromonadaceae*, *Bacteroidales*, and *Bacteroidaceae* decreased in all non-functioning digesters (Fig. S3, $p < 0.01$ for each of the three families). In contrast, the family *Prevotellaceae* increased its **abundance** in medium and high TCC affected digesters, while its **abundance** was slightly diminished in high TCS-treated communities.

Changes in microbial relative **abundance** before and after antimicrobial treatments were also analyzed at OTU level (with 97% sequence similarity cutoff) by comparing microbial communities between functioning and non-functioning digesters at day 110. For this analysis, only OTUs with average relative **abundances** greater than 0.05% (9 reads out of 17,925 rarefied reads) in functional digesters were considered. The analysis identified 252 OTUs that were significantly lower in non-functioning digesters relative to functioning communities ($p < 0.01$, White's non-parametric test). Among the 252 taxa, 92 taxa had a relative **abundance** between 0.05 and 0.1% in functioning digesters, 123 taxa had a relative **abundance** between 0.1 and 0.5% in functioning digesters, while 37 taxa had a relative **abundance** greater than 0.5% in functioning digesters (Fig. S4). These 252 potentially key microbial taxa included known methanogens such as genera *Methanosaeta*, *Methanobrevibacter*, and *Methanoculleus*. Sixty-seven previously described syntrophic bacterial OTUs (out of 252 OTUs), which were represented by genera *Clostridium* (Firmicutes), *Syntrophomonas* (Firmicutes), *Smithella* (Deltaproteobacteria), *Syntro phus* (Deltaproteobacteria), *Longilinea* (Chloroflexi), and *Gelria* (Firmicutes), were also identified as potentially key microbial taxa. The numerically dominant syntrophic taxa were *Clostridium* (19 OTUs), *Syntrophomonas* (16 OTUs), and *Smithella* (15 OTUs) in that order. Many previously characterized **syntrophs** (56 out of the 67 OTUs) were found in minor fractions that had relative **abundance** between 0.05 and 0.5% in functioning digesters (Table 1).

Table 1. Key microbial taxa identified to be significantly more abundant in functioning digesters

Relative abundance (# of OTUs)	**Phylum/class**	**(# of OTUs)**	**Genus**	**(# of OTUs)**
3.0% > taxa > 0.5% (n = 37)	*Firmicutes*	12	*Gelria*	5
			Clostridium	2
			Syntrophomonas	2
			Others	3
	Bacteroidetes	11	*Proteiniphilum*	3
			Bacteroides	2

			Paludibacter	2
			Others	4
	Spirochaete	7	*Candidatus_Cloacamonas*	5
			Others	2
	Betaproteobacteria	3	Azospira	3
	Euryarchaeota	2	*Methanosaeta*	1
			Methanobrevibacter	1
	Chloroflexi	1	*Longilinea*	1
	Deltaproteobacteria	1	*Smithella*	1
0.5% > taxa > 0.1% (n = 123)	*Firmicutes*	38	*Clostridium*	8
			Syntrophomonas	8
			Tissierella	4
			Gelria	3
			Others	15
	Bacteroidetes	31	*Proteiniphilum*	12
			Paludibacter	6
			Others	13
	Deltaproteobacteria	16	*Smithella*	10
			Syntrophus	3
			Others	3
	Spirochaetes	9	*Treponema*	5
			Candidatus_Cloacamonas	3
			Spirochaeta	1
	Betaproteobacteria	7	*Azospira*	3
			Brachymonas	3
			Azoarcus	1
	Euryarchaeota	4	*Methanosaeta*	3
			Methanoculleus	1
	Synergistetes	4	*Thermovirga*	3
			Candidatus_tammella	1
	Other phyla	14	Others	14
0.1% > taxa > 0.05% (n = 92)	*Firmicutes*	35	*Clostridium*	9
			Syntrophomonas	6
			Gelria	5
			Tissierella	4
			Others	11
	Bacteroidetes	29	*Proteiniphilum*	11
			Paludibacter	6
			Prevotella	3
			Others	9
	Euryarchaeota	5	*Methanosaeta*	4
			Methanobacterium	1
	Betaproteobacteria	5	*Azospira*	5

	Deltaproteobacteria	4	*Smithella*	4
	Other phyla	14	Others	14

Core microbiome analysis

Microbial taxa that are present and prevalent in functioning digesters, but not present and prevalent in non-functioning digesters were examined using **core microbiome** analysis. Treatments with different concentrations and types of antimicrobials created functioning digesters that had different microbial community compositions and structures. The **core microbiome approach** helped to identify shared microbial taxa from those variable community compositions within functioning digesters that were not present in non-functional communities. Multiple cutoff levels were used for presence/absence criteria. When the lower relative **abundance** cutoff level (0.01% or greater = 2 reads out of 17,925 reads) was used for presence/absence criteria, the number of OTUs identified as part of the **core microbiome** was high and overlaps between functioning and non-functioning groups were detected (Fig. 5). When the cutoff level for presence/absence was increased to 0.05% or greater (9 reads out of 17,925 reads), only 186 OTUs were identified to be **core** OTUs in functioning digesters, and they were not shared with any non-functioning digesters (Fig. 5). These 186 OTUs are candidates as potentially key microbial taxa in functioning digesters because they were present as **core** members in the functioning digester sets, but were not present in the non-functioning digester sets. Their identities and functions obtained from literature reviews were summarized in Table 2. Several previously described **syntrophs** were identified as members of the functioning **core microbiome**, which includes *Clostridium*, *Syntrophomonas*, *Smithella*, *Longilinea*, and *Gelria*.

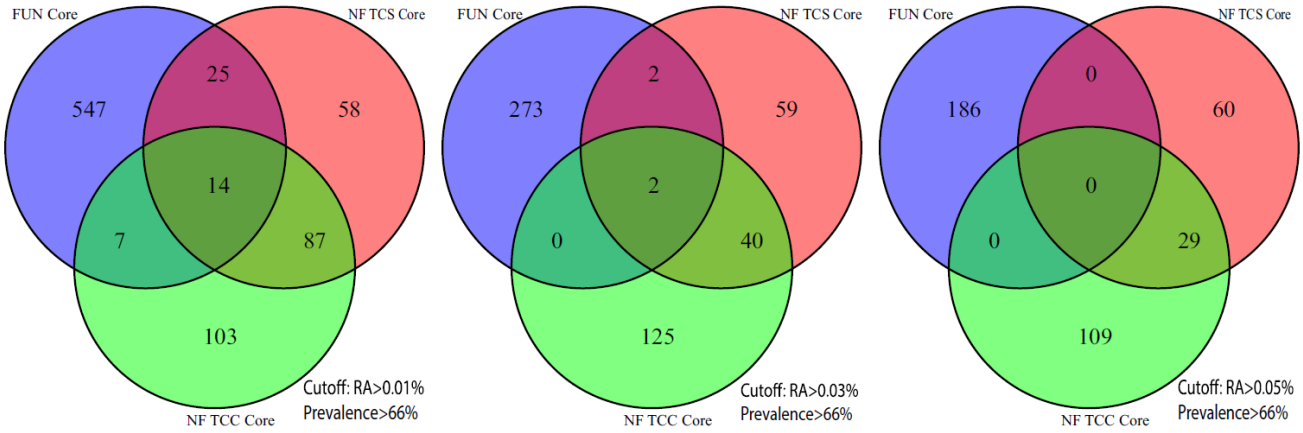

Fig. 5 VENN diagram that depicts **core** microbial taxa associated with functioning and non-functioning digesters. FUN and NF denote functioning and non-functioning digesters, respectively. The numbers in each category of VENN diagram represent the number of **core** taxa found in each category. The criteria for "presence" were altered by adjusting cutoff level of relative **abundance** (RA)

Table 2 Key microbial taxa that are identified to be present in only healthy functioning **anaerobic** digesters using **core microbiome approach**

Taxa_ID_Genus (phylum/class)	**Number of OTUs in**	**Functions**	**References**

	healthy core (n = 186)		
Proteiniphilum (Bacteroidetes)	16	Proteolytic acidogenesis	(Chen and Dong 2005)
Smithella (Deltaproteobacteria)	13	Propionate oxidation**	(Liu et al. 1999)
Clostridium (Firmicutes)	12	Acetate oxidation**	(Schnürer et al. 1996)
Paludibacter (Bacteroidetes)	11	Acidogenesis	(Ueki et al. 2006)
Syntrophomonas (Firmicutes)	11	Fatty acid degradation*	(McInerney et al. 1981)
Gelria (Firmicutes)	10	Glutamate degradation**	(Plugge et al. 2002)
Azospira (Betaproteobacteria)	8	Chlorate-reducing bacteria	(Byrne-Bailey and Coates 2012)
Candidatus cloacamonas (Spirochaetes)	8	Amino acid degradation***	(Pelletier et al. 2008)
Methanosaeta (Euryarchaeota)	7	Acetoclastic methanogenesis	(Patel and Sprott 1990)
Prevotella (Bacteroidetes)	6	Glucose fermentation	(Shah and Gharbia 1992)
Tissierella (Firmicutes)	6	Creatine degradation	(Harms et al. 1998)
Thermovirga (Synergistetes)	4	Amino acid degradation	(Göker et al. 2012)
Bacteroides (Bacteroidetes)	4	Hydrolysis of polysaccharide	(Xu et al. 2003)
Alkaliflexus (Bacteroidetes)	3	Hydrolysis/acidogenesis	(Zhilina et al. 2004)
Blautia (Firmicutes)	3	Acetogenesis from H2/CO2	(Bernalier et al. 1996)
Brachymonas (Betaproteobacteria)	3	Cyclohexane degradation	(Rouvière and Chen 2003)
Dethiobacter (Firmicutes)	3	Thiosulfate reduction with H2	(Sorokin et al. 2008)
Longilinea (Chloroflexi)	3	Sucrose/yeast extract utilization**	(Yamada et al. 2007)
Others	55	Miscellaneous	

*Confirmed syntrophic reactions in co-culture **Confirmed syntrophic reactions in co-culture, also grow in pure culture ***Predicted syntrophic reactions

Discussion

The aim of this study was to identify important microbial taxa that could be responsible for stable gas production by comparing **core microbiomes** in functioning digesters to those in non-functioning digesters. This study was setup to test this **comparative core microbiome approach** as a tool to narrow down the microbial taxa that play roles in gas production from nearly 2000 OTUs

per **anaerobic** digester sample typically identified using NGS. The two antimicrobials used in this study, TCS and TCC, altered microbial populations differently. TCS was effective in altering digester function at the high concentration tested, whereas both medium and high concentrations of TCC altered digester function. The use of broad-spectrum antimicrobials was effective because (1) the antimicrobial concentrations used in this study did not remove the entire bacterial populations, rather eliminated or diminished a fraction of the microbial population that was present in functioning digesters, and (2) both antimicrobials successfully impaired digester function, i.e., methane gas production, VFA removal, and near-neutral pH maintenance of the digesters. The resulting non-functioning microbial communities were slightly different from each other and were substantially different from functioning communities based on microbial richness and microbial compositions.

We postulated that the taxa that were removed in non-functioning digesters, but remained present in functioning digesters, potentially played essential roles in digester function. We focused on the bacterial taxa that were diminished in non-functioning digesters regardless of the antimicrobial type and concentration used. In other words, comparisons were made between all functioning digesters including background control digesters and all non-functioning digesters at day 110 (post-treatment communities). Nearly 250 microbial taxa that could potentially play key roles in functioning digesters were identified by assessing change in relative **abundance**, while presence/absence based **comparative core microbiome approach** identified 186 potentially key taxa. These taxa were conservative estimates because the number of reads that were sequenced were not sufficient enough to cover all **diversity** that were present in functioning digesters based on rarefaction curves (Fig. S5).

These potentially key microbial taxa included previously described fatty acid-degrading **syntrophs** and several known or putative amino acid-degrading **syntrophs**. **Syntrophs** are important populations in gas-producing **anaerobic** digesters, yet they have not been fully characterized due to difficulties in isolating them. In this study, potential **syntrophs** were determined based on published information in previous literatures. The metabolic processes of these **syntrophs** described in previous studies include acetate oxidization by *Clostridium* (Schnürer et al. [73]), propionate oxidization by *Smithella* (de Bok, et al. [14]; Liu et al. [45]), butyrate oxidization by *Syntrophus* (Jackson et al. [34]), and other fatty acid catabolisms by *Syntrophomona*s (McInerney et al. [51]). In contrast to fatty acid-degrading **syntrophs**, less information is available for **syntrophs** that degrade amino acids (Morris et al. [55]). *Gelria*, a previously described **syntroph** that degrade amino acids when coupled with H_2-consuming methanogen (Plugge et al. [60]), was also identified as a potentially key microbial taxon in this study. A genus, "*Candidatu* Cloacamonas" within phylum *Spirochaetes* or a new candidate division WWE1, that was identified as a potentially key microbial taxon, was also suggested to perform syntrophic amino acids degradation, although the syntrophy has not been confirmed using co-culture experiments (Pelletier et al. [59]).

Syntrophs were present with low relative **abundance** in functioning digesters. Fifty-six out of 67 previously described **syntrophs** were present with relative **abundance** ranging from 0.05 to 0.5% in functioning digester communities. This result suggests that these **syntrophs** play key roles in digester environments even though they are present in relatively low **abundances**. The low relative **abundance** of syntrophic taxa was expected by the low energy gained via syntrophic reactions (Jackson and McInerney [33]; Kato and Watanabe [37]; Leng et al. [44]; Schink [72]), but has not been

studied extensively using functioning **anaerobic** digesters. **Syntroph abundance** could also be limited by the **abundance** of their partner methanogens, which have direct physical association with **syntrophs** (Kato and Watanabe [37]; Kouzuma et al. [39]; Walker et al. [89]) and are also present with low relative **abundance** in functioning digesters (Blume et al. [4]). This finding could also mean that the low **abundance** of **syntrophs** is a limiting factor in **anaerobic digestion** and could be the cause of upset when excess amount of organics are loaded in a short time period. A previous study reviewed ecological roles of rare microbial populations and the importance of microbial taxa that are present with low **abundance** in a community (Lynch and Neufeld [49]). Although microbial taxa with low relative **abundances** in a community play essential roles in mixed communities, taxa that had **abundances** lower than 0.1% of a community were often ignored (Lynch and Neufeld [49]). The findings from our study suggest that taxa with low relative **abundance** should not be ignored, particularly in a complex system where multiple reactions happen in a series involving various intermediates. Furthermore, these taxa with low relative **abundance** were difficult to detect using clone libraries and other previously available molecular techniques, which validate the utilization of deep sequencing to elucidate microbial processes in **anaerobic** digesters (Rittmann et al. [65]). Although only previously characterized syntrophic taxa were included in this analysis, it is also possible that other **syntrophs** were present that have not yet been characterized.

The **diversity** of **syntrophs** found in potentially key microbial taxa was higher than presumed previously. Unlike the low OTU **diversity** observed with the methanogens (11 OTUs out of 252 OTUs), the number of OTUs detected as known **syntrophs** was high (67 OTUs out of 252 OTUs). This in turn suggests that multiple syntrophic reactions with different substrates could happen with shared hydrogen scavengers in digesters, which cannot be inferred from observations in co-culture alone. Most of the **syntrophs** previously identified have been isolated and studied using co-culture in the laboratory. While a co-culture visualizes one to one interaction between a methanogen and its syntrophic reaction partner, these results suggest that one methanogen taxon with multiple syntrophic partners could occur in mixed culture. Furthermore, several **syntroph** OTUs that belong to the same genus were found in this study (i.e., 14 OTUs belong to genus *Smithella* in 0.05 to 0.5% fraction). This finding suggests that closely related **syntrophs** co-existed in the digesters. We do not know if these OTUs within the same **syntroph** genus metabolize the same compounds (functionally redundant) or different compounds (each OTU is adapted to utilize slightly different resources). It is possible that complex resources in a digester (i.e., synthetic primary sludge plus nutrient medium) allowed co-existence of a diverse group of **syntrophs**. It is also important to note that the genus *Clostridium*, one of the **syntrophs** identified as a key taxa in this study, contains several species that possess broad metabolic functions which are not limited to syntrophic reactions. Unlike the genus *Clostridium*, most of the syntrophic taxa described here do not possess diverse metabolic functions.
Genera *Syntrophomonas* (McInerney et al. [51]) and *Smithella* (Liu et al. [45]) and *Gelria* (Plugge et al. [60]) are considered as obligate **syntrophs**, while genus *Longilinea* is a facultative **syntroph** (Yamada et al. [94]).

Previously, functional redundancy of bacterial taxa was observed specifically for taxa that are responsible for hydrolysis and acidogenesis, while methanogens and **syntrophs** were believed to be less diverse and less redundant due to inflexibility in function (Briones and Raskin [5]). If syntrophic taxa within the same genus are functionally redundant rather than utilizing different substrates, then

the high **syntroph diversity** would potentially lead to more resilience in **anaerobic** digesters. A pool of functionally redundant taxa with different sensitivities to different stresses that wastewater treatment plants receive is necessary in order for wastewater treatment plants to properly operate upon exposure to stresses. Although a majority of syntrophic taxa were lost in non-functioning digesters in our experiments, we are not certain if the high **diversity** of **syntrophs** in functioning digesters relative to non-functioning was a random coincidence or if there was a reasonable mechanism behind this observation; for instance, **syntrophs** could be more susceptible to the antimicrobials used in this study since they lack energy to pump out the antimicrobials via efflux pumps, a common resistance mechanism against these generic antimicrobials (Chuanchuen et al. [13]; Pycke et al. [61]).

Methanogens are essential microbial taxa for methane production in **anaerobic** digesters. In this study, three methanogen families were identified in the functioning background microbial communities, and these three taxa responded to antimicrobials differently. Euryarchaeota taxa that belong to families *Methanosaetaceae* and *Methanomicrobiaceae* decreased in non-functioning digesters, while the **abundance** of the family *Methanobacteriaceae* increased in the non-functioning digesters. Family *Methanosaetaceae* includes the genus *Methanosaeta*, one of the only two genera known to convert acetate into methane via the acetoclastic pathway, while both *Methanomicrobiaceae* and *Methanobacteriaceae* reduce carbon dioxide with hydrogen to synthesize methane via the hydrogenotrophic pathway (Demirel and Scherer [16]). Some Euryarcheota taxa that belong to the family *Methanobacteriaceae* became dominant in non-functioning digesters that had lower pH, most likely because they were resistant to the acidic conditions that are also found in acidic peatlands (Kotsyurbenko et al. [38]). Even though hydrogenotrophic methanogens were present in non-functioning digesters, methane was not steadily produced. This lack of methane production is likely because *Methanobacteriaceae* produce methane poorly (Kotsyurbenko et al. [38]) and/or some metabolic pathways carried out by **syntrophs** that co-occur with hydrogenotrophic methanogen were cutoff by these antimicrobials or low pH environments.

In this study, 19 OTUs that belong to the phylum *Spirochaetes* were identified as one of the potentially key microbial populations that are responsible for healthy digester functioning. Several previous studies have identified *Spirochaetes* in **anaerobic** digesters as a key microbial phylum (Ariesyady et al. [2]; Fernandez et al. [21]; Lee et al. [42], [43]). Though their putative functions in **anaerobic** digester environments have been discussed (Ariesyady et al. [2]; Fernandez et al. [21]; Lee et al. [43]), their actual metabolic roles in digesters remain unknown as the cultivation and isolation of *Spirochaetes* strains is challenging.

Two Betaproteobacteria genera, *Brachymonas* and *Aazospira,* were identified as potentially key microbial populations for functions of **anaerobic** digesters, though their exact roles in functioning digesters remain unknown. Genus *Brachymonas* was previously identified as a member of the **core microbiome** in **anaerobic** digesters (Riviere et al. [66]). In another study, *Brachymonas* was identified to be capable of degrading chlorohexane anaerobically (Rouvière and Chen [67]). Genus *Aazospira* was also reported to have affiliation to **anaerobic** condition where it was found to degrade chlorate anaerobically (Byrne-Bailey and Coates [6]). Although the association of these two Betaproteobacteria taxa with **anaerobic** conditions have been reported, the role of these two taxa in digester functions remains unknown.

Some *Bacteroidetes* populations were identified as potentially key microbial populations in this study. One such taxon was the genus *Proteiniphilum* that was previously described as facilitator that accelerates the rate of propionate oxidizing syntrophic reactions when coupled with methanogens in triculture (Chen and Dong [11]). A proposed function of this genus was described as a fermenter that converts polypeptides into propionic and acetic acids (Chen and Dong [11]; Hahnke et al. [26]).

The potentially key microbial populations for digester functions identified in this study may not be generalizable for other **anaerobic digestion** systems. In this study, microbial populations were altered using antimicrobials, while all other operational parameters were kept the same, including the seed populations and organic feed. This setup naturally led to differences from full-scale **anaerobic** digester systems. For instance, we used synthetic primary sludge in place of actual sludge because we wanted to create a steady-state microbial community over time. The enrichment using synthetic primary sludge could select for different steady-state microbial communities from ones typically seen in **anaerobic** digesters in water resource recovery facilities. Furthermore, the seed we used for this study came from a single water resource recovery facility, so the **diversity** of microbial taxa we started with was limited to what was present in the seed biomass. Therefore, the results from this experiment are not necessarily reflective of microbial communities in all digesters. Indeed, different **anaerobic** digesters receive different wastes at different time points; thus, the compositions of wastes and the role of microbes vary throughout time within a single **anaerobic** digester (Lee et al. [42]). The findings in this study highlight important taxa in the digesters studied.

Another limitation to this study is that, although nearly 250 microbial taxa were identified to be potentially important in **anaerobic** digester function, none of the taxa including potentially key **syntrophs** described here were experimentally proven to be directly linked to the change in digester function. Though 16S rRNA gene amplicon sequencing has been used to infer key microbial taxa in **anaerobic** digesters, this sequencing technique is not sufficient to identify actual metabolic processes performed by microbial taxa in the digesters. These 250 microbial taxa serve as a starting point on which taxa studies can be focused. Furthermore, there is a possibility that some key taxa for biogas production were not removed from the non-functioning digesters by antimicrobials. Our comparison **approach** attempted to identify **core** microbial taxa that are present in functioning digesters but lost in non-functioning digesters. Taxa that are present in both functioning and non-functioning digesters might play a key role in digester functions, but our **comparative core microbiome** analysis did not identify taxa that were abundant and shared between functioning and non-functioning digesters. When the **comparative core** microbime **approach** is applied to other systems, such taxa should be considered as potentially important. If present, these taxa could be explored further to be conservative. Another limitation of this study is that changes in digester function and microbial communities were measured at day 110, which was approximately six SRT values after antimicrobials were fed and maintained. As noted in the methods, both antimicrobials were still present in the digesters at anticipated concentrations, and the microbial taxa enriched in the non-functioning digesters could have been due to direct impacts from antimicrobials or from the altered digester environmental conditions such as lowered pH that were indirect impacts of adding antimicrobials. Future work should investigate finer temporal scales to track changes in bacterial compositions and digester functions in real time to elucidate the association between the microbial taxa and changes in digester functions.

Although we focused on microbial taxa that were diminished in both TCS- and TCC-treated non-functioning digesters, there was a difference in microbial community compositions between the two antimicrobials. One notable difference was that the class *Gammaproteobacteria* accounted for nearly 60% of the relative **abundance** in non-functioning digesters that were treated with high level of TCS, while *Gammaproteobacteria* taxa accounted for less than 10% in TCC-treated non-functioning digesters. *Gammaproteobacteria* are known to have resistance mechanisms against TCS including mexB (efflux pump: (Chuanchuen et al. [13])) and fabV (isoenzyme of fabI: (Zhu et al. [97])); both found in *Pseudomonas*. Therefore, it is reasonable to assume triclosan could eliminate *Gammaproteobacteria* less efficiently than triclocarban did. Degradation of TCS by *Gammaproteobacteria* is not known, while degradation of TCS can occur in other taxa including phylum *Chloroflexi* (McNamara and Krzmarzick [53]) and genus *Sphingopyxis* (in class *alphaproteobacteria*) (Lee et al. [41]). The dominance of *Gammaproteobacteria* could also be due to the tolerance of some *Gammaproteobacteria* to acidic pH conditions (Foster [23]). There was a difference in VFA compositions between TCS- and TCC-treated non-functioning digesters (Fig. S6). High propionate concentration in TCS-treated non-functioning digesters suggest that propionate oxidizing bacteria were inactivated in those digesters.

This study demonstrated that the **comparative core microbiome approach** is a useful screening method to identify potentially important microbial populations for digester function. The **approach** taken could apply to other systems where the microbial **diversity** is high and community level function can be observed directly. While broad-spectrum antimicrobials were added in this study, targeted inhibitors could be used in a similar way specifically when a priori knowledge about targets and their inhibitors are available. This **approach** is not mechanistic, but was used to narrow down the potentially key microbial taxa from a massive number of OTUs that are resultant of NGS sequencing. This study also **revealed** a diverse group of **syntrophs** that are present with low **abundance** are potentially important for proper digester function. While nearly 250 taxa that could potentially play key roles in digester function were identified, these microbial taxa need to be explored further using isolation techniques, microautoradiography (MAR) with genus specific probes, and/or collecting samples within shorter time frames to give resolution for mechanistically linking changes in microbial community to functional changes. While there were no significant differences in microbial quantity based on qPCR of 16S rRNA copies (Carey et al. [9], [10]), the microbial activities could have been different across treatments. Activity measurements using ATP quantification assays across treatment groups could have helped to identify if such difference existed. RNA sequencing of both ribosomal RNA and expressed functional genes may also be used to examine activities and detailed microbial functions in active **anaerobic** digesters (De Vrieze et al. [15]; Vanwonterghem et al. [87]), while other techniques including qPCR could also be used to target specific functional genes. Upon definitive identification of key microbial taxa for digester functions, sustainable management and operation of **anaerobic** digester could be enhanced by removing the stresses to the key microbes in digesters or by bioaugmenting with the loss of key microbial populations.

E-supplementary data of this work can be found in an online version of the paper.

Funding information

M.F. was funded by a Binsfeld Fellowship and by the Marquette University Water Quality Center. D.E.C. was funded by the Marquette University Water Quality Center, the Lafferty Family Foundation, and a Binsfeld Fellowship.

Compliance with ethical standards

Ethical statement

No animals or human subjects were used to generate data in this research article.

Conflict of interest

The authors declare that they have no conflict of interest.

Electronic supplementary material

Graph: (PDF 1135 kb)

Publisher's note

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