

# Phylogenetic Analysis of Anaerobic Co-Digestion of Animal Manure and Corn Stover Reveals Linkages between Bacterial Communities and Digestion Performance

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## Abstract

Over 3 million tons of manures are produced annually in the United States and pose environmental and health risks if not remediated. Anaerobic digestion is an effective method in treating organic wastes to reduce environmental impacts and produce methane as an alternative energy. Previous studies suggested that optimization of feed composition, hydraulic retention time, and other operational conditions can greatly improve total solids removal and increase methane productivity. These environmental factors improve functionality by altering the microbial community structure but explicit details of how the bacterial community shifts are poorly understood. Our investigations were conducted to investigate the relationship between environmental factors, microbial community structure and bioreactor efficiency by using metagenomic analysis of the microbial communities. Our results indicated that the bioreactor with the greatest methane production, digestion efficiency and reduced levels of *E. coli*/*Shigella* had a distinctive community structure at the genus level with unique and abundant uncultivated strains of Bacteroidetes. Moreover the same bioreactor was enriched in *Aminomonas paucivorans* and Clostridia populations that can utilize secondary metabolites produced during cellulose/hemicellulose degradation to generate hydrogen and acetate. Hence specific digestion conditions that enrich for these populations may provide a route to the optimization of co-digestion systems and control the variability in reactor performance.

## Keywords

Anaerobic Co-Digestion, Bacteroidetes, Cellulose Degrading Bacterial Community

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## 1. Introduction

Anaerobic digestion can effectively degrade farm wastes and concomitantly produce methane, thereby reducing the health and environmental risks while producing energy [1] [2]. Methane is produced in anaerobic digestion when organic matter is in high concentration [3]. It is a highly cooperative process carried out by different groups of Bacteria and Archaea with distinct responsibilities [3] [4]. Schink and Stams [3] concluded that methanogenic degradation of organic matter was executed primarily by three groups of microorganisms. During anaerobic digestion of farm waste, cellulosic material is degraded by several bacterial groups providing smaller organic substrates that are further degraded by fermentative bacteria. The fermentation by-product hydrogen is used by hydrogen-oxidizing methanogens to produce methane, while acetate-cleaving methanogens produce methane from acetate.

A large number of studies focused on optimizing anaerobic digestions via manipulating the system's operational parameters, such as the substrate C:N ratio [5]-[10]. However, the microbial communities within the anaerobic digestion have not been comprehensively explored. As pointed out by Schink and Stams [3], these microbial communities are the primary force behind methane production and community shifts could affect overall performance. Studies have shown that several *Clostridium* species can decompose and partially hydrolyzed cellulose. Clostridia populations were also repeatedly reported in hydrogen and methane producing anaerobic bioreactors [8] [11]-[14]. The Clostridia group is well-known for their ability to produce hydrogen via [FeFe]-hydrogenase (*hydA*) [15] and the genes for this enzyme are found only in anaerobic bacteria and eukaryotes [16] [17]. The [FeFe]-hydrogenase energetically prefers to catalyze the reduction of protons to produce hydrogen [18] [19]. Therefore, we hypothesized that Clostridia populations are the most abundant cellulose degrading bacteria in the methane producing microbial community. Herein, a pilot-scale bioreactors study was carried on to describe microbial communities of anaerobic bioreactors fed with dairy manure (DM) and corn stover (CS). We report on phylogenetic diversity based on 16S rRNA and *hydA* genes of the communities detected under different operational conditions and conclude that a distinctive microbial community was associated with optimal solid degradation and methane production.

## 2. Materials and Methods

### 2.1. Anaerobic Bioreactors

Feedstock composition and the small pilot-scale anaerobic bioreactors (10 L) were set up and described in a companion study published by Yue *et al.* [20]. Briefly, six continuously stirred tank reactors (CSTR) were operated at 35°C with hydraulic retention times (HRTs) of 30 days, 40 days, and 50 days. Biogas production was used to evaluate the stability of the bioreactors. Three HRTs were run for each reactors. The daily gas production became constant for all six reactors during the 2<sup>nd</sup> HRT. Due to the scope and scale of the pilot operation, replication of bioreactors was impossible. The feed for dairy manure (DM) bioreactors (reactor 1, reactor 3, and reactor 5) was 5% dry matter

and 95% water. The feed for manure and corn stover (DM + CS) bioreactors (reactor 2, reactor 4, and reactor 6) was a mixture of dairy manure and corn stover in a ratio of 4:1 (wt:wt) and diluted to 5% DM using water. Detailed bioreactor performance and analytical analyses were reported in the companion study [20].

## 2.2. DNA Extraction and Targeted Gene Amplifications

Digestate samples were then taken from each bioreactor during the third HRTs. The samples were centrifuged at 10,000 RPM for 20 minutes in a Sorvall SS-34 rotor and the pellet was stored at  $-20^{\circ}\text{C}$  until extraction of community DNA. DNA was extracted by using Powersoil DNA Extraction Kit (Mo Bio Laboratory, Carlsbad, CA) with vigorous bead beating in a Biospec Mini-Beadbeater-8 (BioSpec Products, Inc., Bartlesville, OK).

Bacterial 16S rRNA gene (16S) V3-5 regions were amplified for 454 pyrosequencing according to the Human Microbiome Project protocol ([http://www.hmpdacc.org/doc/16S\\_Sequencing\\_SOP\\_4.2.2.pdf](http://www.hmpdacc.org/doc/16S_Sequencing_SOP_4.2.2.pdf)). For each polymerase chain reaction (PCR), 10 ng DNA template were used. Bacterial *hydA* were amplified according to Boyd *et al.* [17] using primer set FeFe-272F/FeFe-427R. For each *hydA* PCR, 20 ng DNA template were used.

## 2.3. Preparation for Pyrosequencing and Construction of Clone Libraries

The 16S rRNA gene PCR products were visualized on 2% Tris-Borate-EDTA (TBE) agarose gel and DNA fragments of the expected size (~569 bp) were excised and extracted from the gels by using Qiagen Gel Extraction Kit and further purified with QiaQuick PCR Product Purification Kit (Qiagen, Valencia, CA). All purified DNA products were quantitated with Qubit HS Double-stranded DNA Kit (Invitrogen, Carlsbad, CA) then composited at equal mass for a final DNA concentration of  $0.5\text{ ng}\cdot\mu\text{l}^{-1}$ . Pyrosequencing was performed at Michigan State University Research Technologies Support Facility (RTSF) by using a Roche 454 GS FLX Sequencer. Quality filtered sequences were submitted to National Center for Biotechnology Information Sequence Read Archive (BioSample accession number: SAMN04215003).

Amplified *hydA* genes were confirmed via electrophoresis (1% Tris-Acetate-EDTA agarose gel). Expected gene fragments (~500 bp) were cloned using Invitrogen Topo PCR2.1 Cloning Kit (Invitrogen, Carlsbad, CA) following the vendor's protocol. The cloned fragments were purified and sequenced at RTSF on an ABI 3730 Genetic Analyzer. Quality filtered sequences are being submitted to National Center for Biotechnology Information.

## 2.4. Sequence Processes and Community Analyses

Bacterial 16S rRNA gene sequences obtained from pyrosequencing were processed and analyzed by using the Ribosomal Database Project (RDP) Pyrosequencing Pipeline [21]. Sequences that were shorter than 250 bp, containing any N's in sequencing region, or with an average quality score (Phred score) less than 20 were discarded. Chimeras were

removed from RDP processed sequences by using USEARCH (UCHIME reference mode with Silva gold alignment database as reference) [22]. RDP Multi-classifier was used to identify each sequence to bacterial genus level (threshold 80). Bacterial 16S rRNA sequences were also aligned using RDP Aligner and then clustered by RDP Complete Linkage Clustering at 97% sequence identity. To eliminate the clustering artifacts, sequences were also trimmed to the same length (250 bp) based on the alignment profiles prior to clustering. RDP SeqMatch was used for further identification of bacterial clusters of interest.

[FeFe]-hydrogenase gene sequences were identified using Basic Local Alignment Search Tool (BLAST) against the NCBI non-redundant amino acid database (BLASTX). The closest phylogenetic affiliate was identified as the annotated match with the best Expected Value ( $E < 10^{-5}$ ).

Operational taxonomy unit (OTU), phylotype-based community analyses and clustering were performed by using statistical software R [23]. Ecological indices were estimated for each bioreactor sample based on clustered sequences by using the R package Vegan [24]. Specifically, community richness (Chao I), diversity indices (Shannon H' and Simpson's D) and community evenness (E) were calculated based on equations previously described [25]. The sampling coverage (C) of each community was calculated based on Good's method,  $C = 1 - (n_1 \cdot N^{-1})$ , where  $n_1$  represents the number of OTUs that is a singleton and N is the total number of sequences in the sample [26]. The R package Vegan was also used to perform non-metric multidimensional scaling analysis (NMDS) to correlate the dissimilarities among bioreactor samples and the shifts in phylotype abundance [24].

### 3. Results

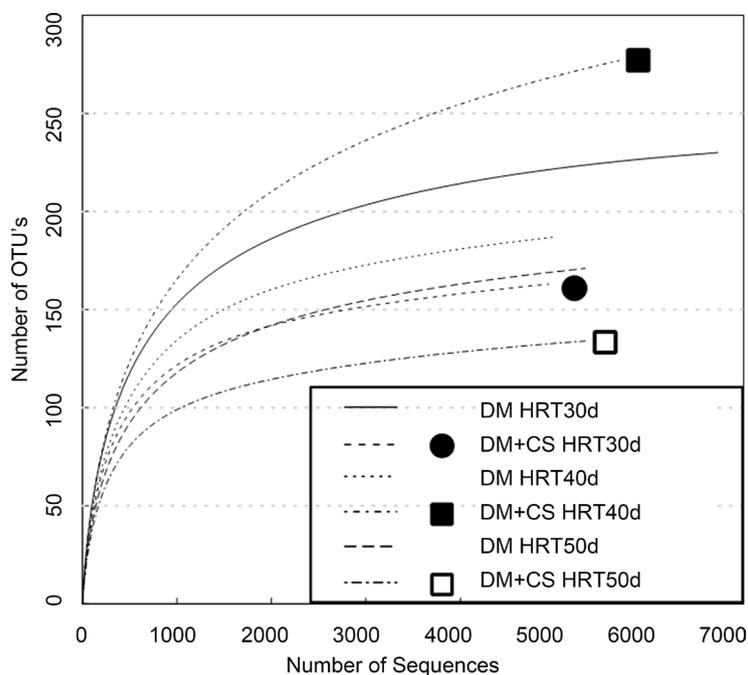
#### 3.1. Bacterial Community Comparisons

Bacterial 16S rRNA gene targeted sequencing revealed that we sampled over 98% of each anaerobic bioreactor community (Table 1 and Figure 1). The bacterial community evenness decreased as the HRT increased. The diversity indices followed the similar pattern with DM + CS bioreactor operated at HRT 40 days as the exception (Table 1).

Out of 33,006 quality-trimmed 16S rRNA gene sequences, approximately 5% were categorized as unclassified bacteria. A total of 16 bacterial phyla and 137 genera (6 groups unclassified at genus level) were identified in the anaerobic bioreactor samples. Firmicutes, Bacteroidetes, and Proteobacteria were the most abundant phyla in all six anaerobic bioreactor samples accounting for between 85% - 91% of community composition (data not shown). In the DM reactors, the abundance of Proteobacteria increased (15.4% > 34.2% > 44.5%) as the HRT increased with a concomitant decrease in Firmicutes. The noteworthy exception to this trend was in the corn stover-supplemented reactors where a precipitous decrease in Proteobacteria and *E. coli*/*Shigella* was detected at the mid range of HRT (40 days). In this reactor with the most efficient conversion to methane, the Proteobacteria dropped to 7% of the community and *E. coli*/*Shigella*

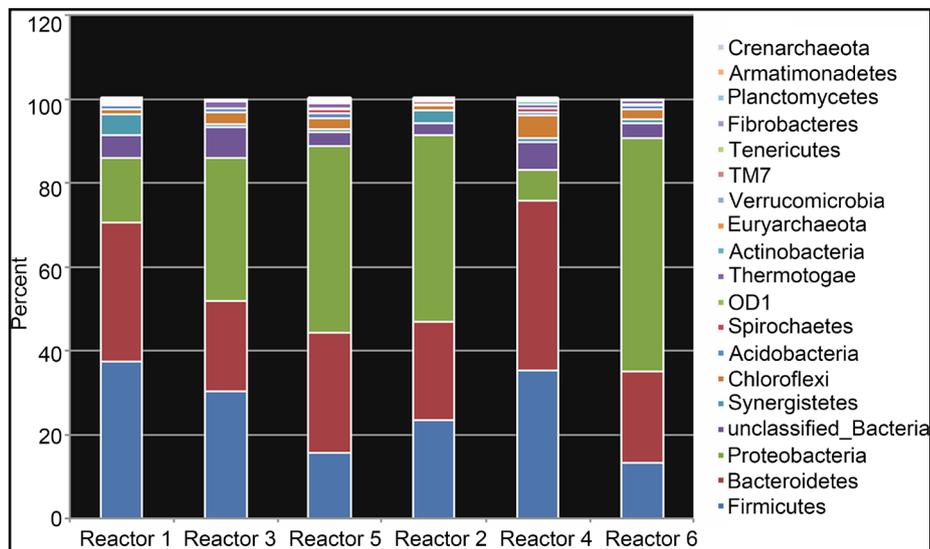
**Table 1.** Measurements of sampling size and estimation of ecological indices of bacterial community in six anaerobic bioreactors. Calculations were based on the complete linkage clustering of chimera removed and trimmed bacterial 16S rRNA gene sequence alignments at 97% similarity. N represents the total number of non-chimera sequences that passed quality trimming. Shannon index ( $H'$ ) and Simpson's index (D) were expressed in  $e^{H'}$  and 1-D, respectively to emphasize the differences. E represents the community evenness. C represents the sampling coverage via gene-targeted pyrosequencing.

Influent	100% DM			80% DM + 20% CS		
HRT (days)	30	40	50	30	40	50
N	6729	5003	5328	4939	5684	5323
OTU	230	187	171	163	277	134
ChaoI	240.62	208.94	190.50	193.67	332.80	146.21
$e^{H'}$	70.62	49.30	36.10	40.64	73.62	27.30
E	0.78	0.75	0.70	0.73	0.76	0.68
1-D	0.97	0.94	0.91	0.91	0.97	0.87
C	99.64	99.46	99.49	99.51	98.89	99.64



**Figure 1.** Estimation of community coverage by rarefaction analysis. Rarefaction curves of anaerobic bioreactor 16S rRNA gene sequences were constructed based on the estimation of OTUs and ChaoI indices at 97% similarity.

were reduced to ~2%. The relative abundance of phylum Bacteroidetes ranged from 21% - 40% with no apparent relationship to the HRT. Clostridia, unclassified Bacteroidetes, and *E. coli/Shigella* were the major groups observed within the Firmicutes, Bacteroidetes and Proteobacteria, respectively. At the phylum level a general similarity at the level of presence/absence of phyla across reactors was seen (Figure 2). However reactor 4 (corn stover supplemented at 40 d HRT) was clearly distinct in terms of the relative abundances of phyla. This difference is seen more dramatically at the genus



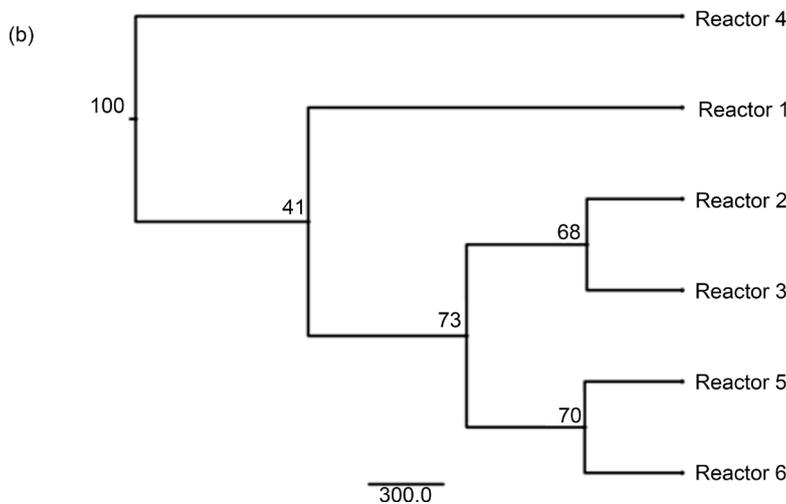
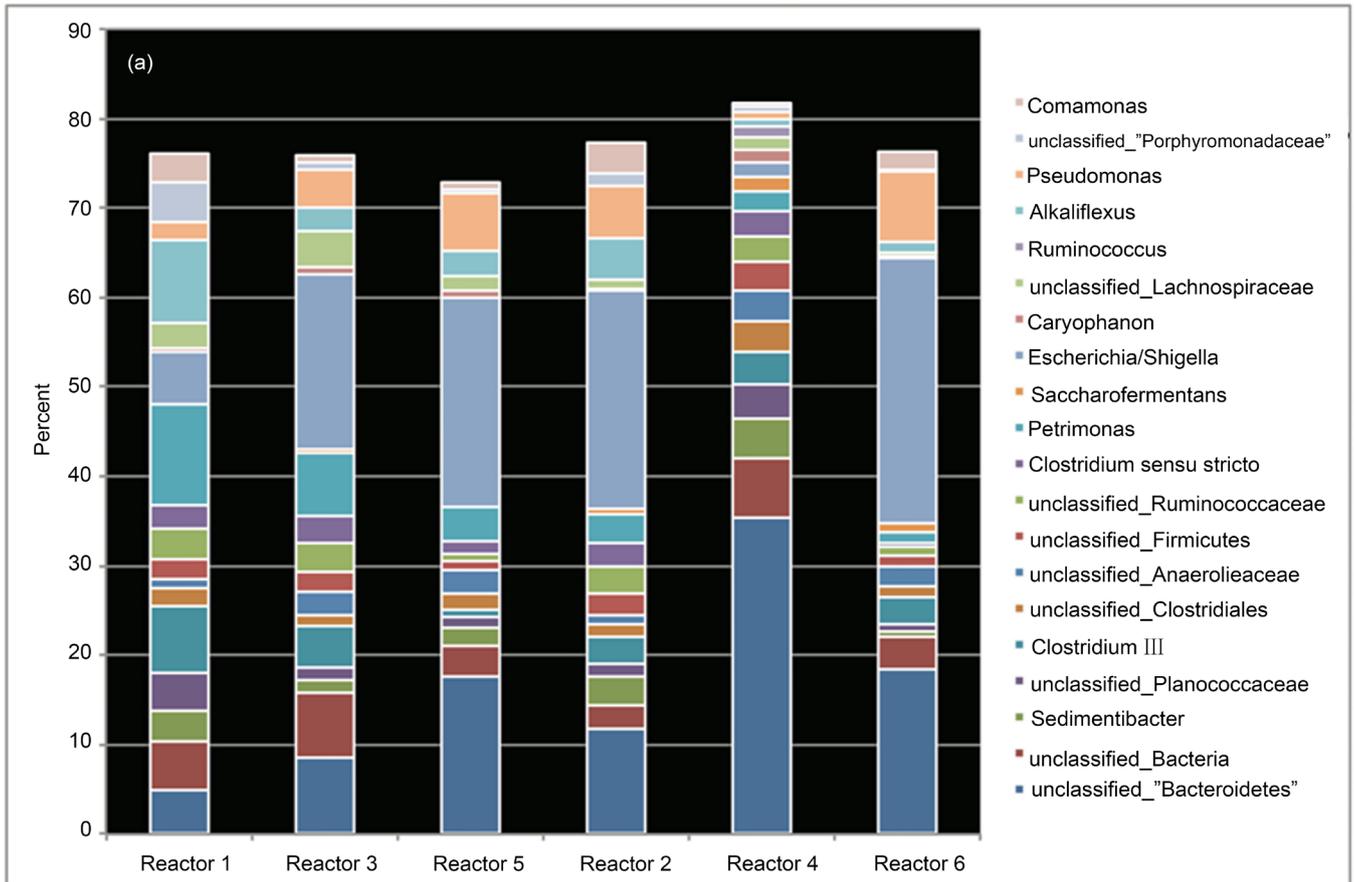
**Figure 2.** The phylum distribution of 16S rRNA DNA sequences in anaerobic digestors.

level (**Figure 3(a)**) where similarities can be easily detected between reactors 2, 3, 5 & 6 and reactor 4 is seen as distinctly different. Comparative analysis using Wards clustering of Euclidian distances (bootstrapped to 500 replicates) revealed strong similarities between reactors 2, 3, 5 & 6 (**Figure 3(b)**) with reactor 1 intermediate between 4 and the remainder. Clustering with Bray-Curtis dissimilarity gave similar results (data not shown). The unclassified Bacteroidetes increase from a 12% average in the other 5 reactors to 35% in reactor 4. This and small increases in other anaerobe phylotypes was accommodated by substantial reductions in the gamma-Proteobacteria populations. The combined *Pseudomonas-Escherichia-Shigella* percentages summed to 2.2% in reactor 4 compared to 25.8% average sum in the other 5 reactors. Reactors 5 & 6 were at the greatest HRT while reactors 2 & 3 were at different HR times. The community differences between the reactors with 30 d HRT presumably reflects the differences in the inoculum with reactor 2 supplemented with corn stover.

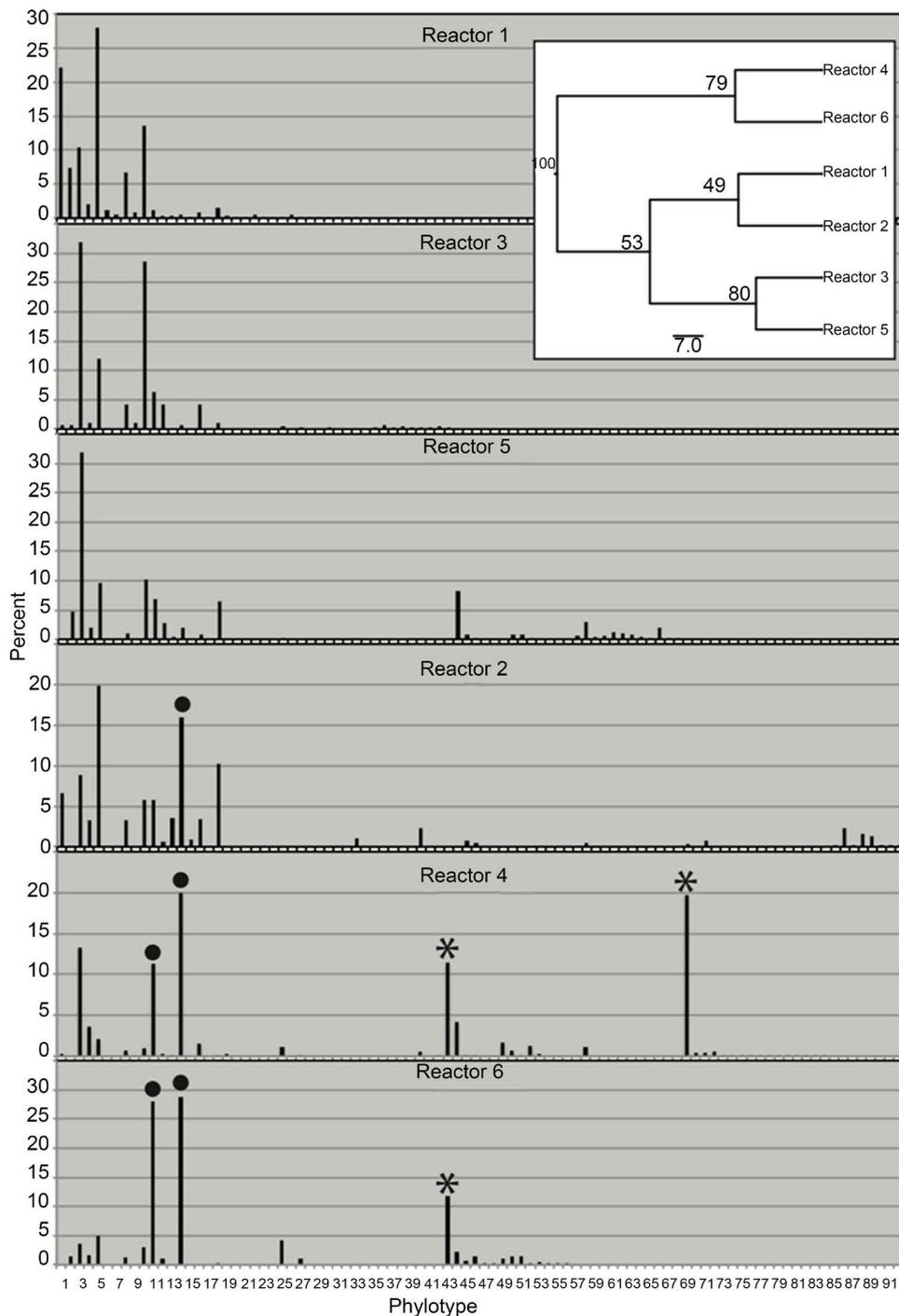
### 3.2. Shifts within Phylum Bacteroidetes

Based on a 97% cutoff, 91 different Bacteroidetes phylotypes could be detected across six reactors, many of which were singletons. These are shown in **Figure 4** where each bar represents the percentage of a Bacteroidetes phylotype in the reactor. Reactors 1, 3 & 5 were dominated by phylotypes #3, #5 & #10, accounting for up to 70% of the Bacteroidetes. In sharp contrast, four unclassified Bacteroidetes phylotypes (#11, #14, #43, and #69) comprised 62% of the phylum in bioreactor 4 (DM + CS HRT40d). Phylotypes #11 & #14 were present at low levels in reactors 1, 3 & 5 and substantially more abundant in the corn stover supplemented reactors. Phylotype #43 was unique to bioreactors 4 & 6 (over 11%) while phylotype #69 was uniquely abundant in reactor 4 (DM + CS HRT40d). Over 11% and approximately 20% of the Bacteroidetes abundance was contributed by clusters #43 and #69 respectively. The insert in **Figure 4** shows the

Wards clustering (Euclidian distance) of the reactors based solely on the Bacteroidetes phylotypes. This analysis indicated that the Bacteroidetes populations in reactors 4 & 6 were more similar then to the remaining four reactors (bootstrap analysis highly



**Figure 3.** Panel (a). The top 20 most abundant genera detected in the bioreactors. Panel (b). Wards clustering (Euclidian distance) of the reactor communities. Confidence levels are indicated at the nodes and are based on bootstrapping at 500 replicates.



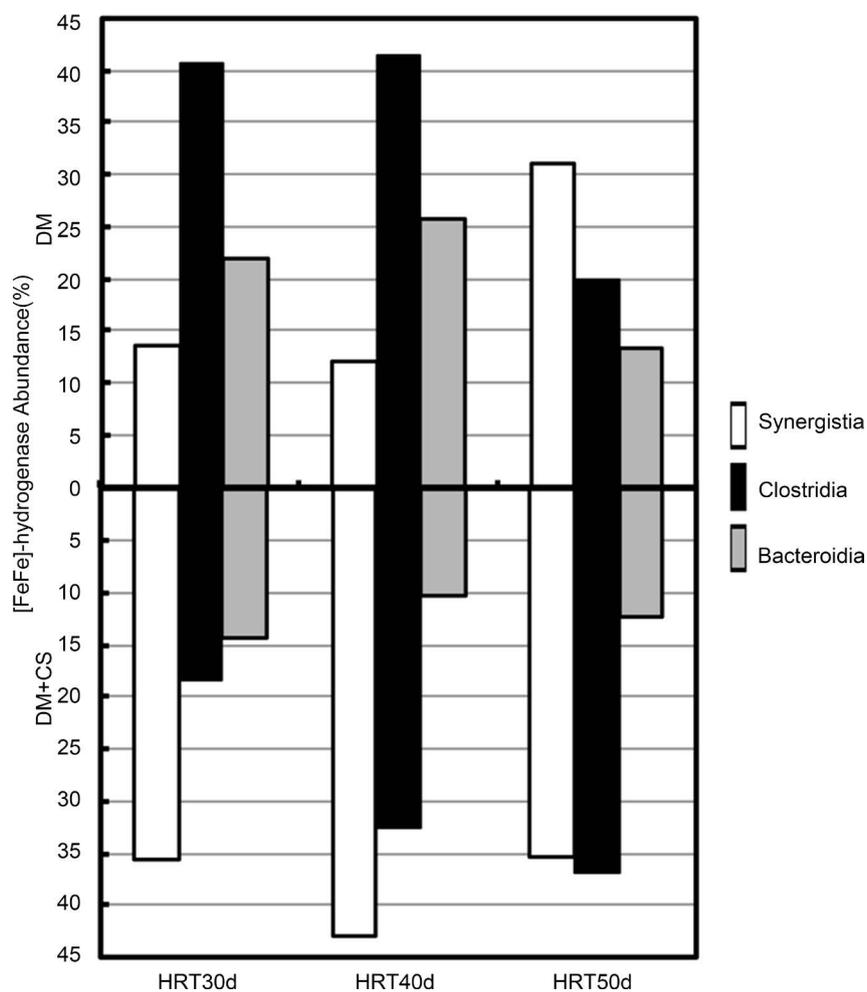
**Figure 4.** Bacteroidetes diversity and distribution in reactors. Clades within the Bacteroidetes at 97% similarity were determined through the RDP Pipeline. The insert shows Wards clustering (Euclidian distance) of the bioreactors based solely on the Bacteroidetes populations. (97% sequence similarity) between the DM + CS bioreactor with a HRT of 40 days and other bioreactor samples. Labels HRT30d, HRT40d and HRT50d represent bioreactors with a HRT of 30, 40, and 50 days, respectively. Symbol • denote populations (#11 and #14) of unclassified Bacteroidetes that are highly abundant in all DM + CS bioreactors, Symbol \* denote populations in DM + CS bioreactors at long HRTs (#43) or only in reactor 4 (#69).

supported the separation).

### 3.3. Hydrogen Producing Population Profiles

We obtained 45 to 76 *hydA* gene clones from each reactor sample (352 *hydA* gene clones from 6 bioreactors). The coverage based on Good's method ranged from 72% - 94% with a median of 87%. A total of 49 hydrogenase OTUs were detected of which 28 were affiliated with class Clostridia. Three major taxonomic affiliations (class Synergistia, Clostridia and Bacteroidia) were identified, accounting for 64% - 86% of the detected hydrogenases sequences. The remaining [FeFe]-hydrogenase OTUs, from more abundant to less abundant, belonged to Spirochaetales, Deltaproteobacteria, Thermotogales, Negativicutes, Candidatus Cloacamonas, Victivallales, and Gammaproteobacteria.

The relative abundance of Synergistia-like *hydA* gene was more prevalent in DM + CS bioreactors than DM bioreactors (Figure 5). In contrast, Bacteroidia-like *hydA*



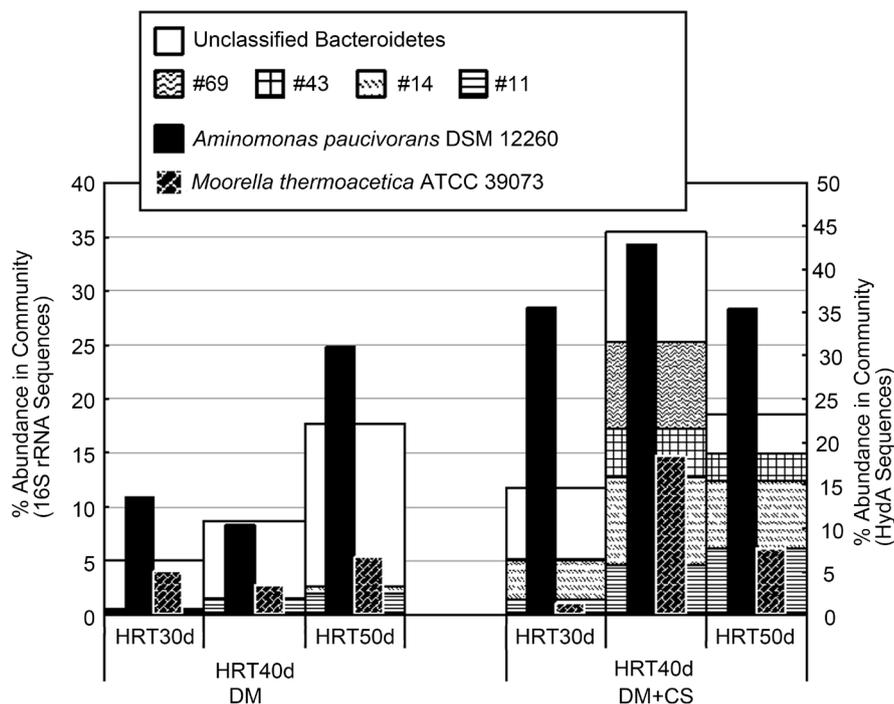
**Figure 5.** The relative abundance changes of the three most abundant [FeFe]-hydrogenase containing bacterial groups in anaerobic bioreactors fed with DM and DM + CS. Labels HRT30d, HRT40d and HRT50d represent bioreactors with a HRT of 30, 40, and 50 days, respectively.

genes were more abundant in the DM bioreactors. The relative abundance of Bacteroidia-like *hydA* gene was the least abundant in DM + CS HRT40d (reactor 4) sample. The relative abundance of Clostridia-like *hydA* gene decreased with increasing HRT in the DM bioreactors and increased in the DM + CS bioreactors.

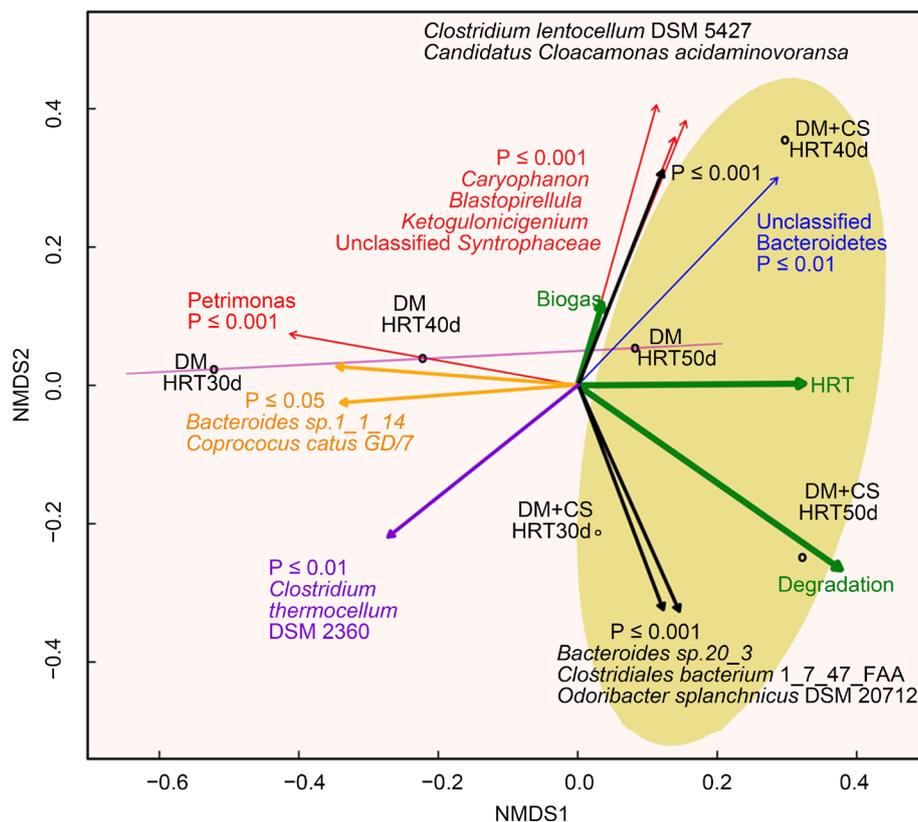
At the species level, *hydA* gene sequences similar to those found in *Aminomonas paucivorans*, which comprised of 98% of *Synergistetes*-like *hydA* genes, was the most abundant. [FeFe]-hydrogenase gene sequences similar to those of *Moorella thermoacetica*, a member of class Clostridia, were also abundant in the DM + CS bioreactors. These two *hydA* gene relative abundance profiles in bioreactors were similar to the relative abundance profile for unclassified Bacteroidetes 16S rRNA genes (Figure 6).

### 3.4. Non-Metric Multidimensional Scaling Analysis

Non-metric multidimensional scaling (NMDS) revealed that the anaerobic bioreactor community shifts correlated strongly with changes in HRT, especially in the DM bioreactors (Figure 7). Fitting the detected bacterial genera to the bioreactor community distances revealed that the abundance of *Petrimonas* decreased significantly as the HRT increased and when corn stover was added. The abundance of several genera, including *Caryophanon*, *Blastopirellula*, *Ketogulonicigenium*, unclassified *Syntrophaceae* and unclassified Bacteroidetes significantly more abundant in DM + CS 40d (Figure 7 red



**Figure 6.** The abundance of [FeFe]-hydrogenase containing bacteria in anaerobic bioreactor samples compared to the abundance pattern of unclassified Bacteroidetes (background thick columns) determined by 16S rRNA gene sequencing. The distribution of clusters #11, #14, #43 and #69 were also plotted as part of unclassified Bacteroidetes within each sample. Labels HRT30d, HRT40d and HRT50d represent bioreactors with a HRT of 30, 40, and 50 days, respectively.



**Figure 7.** NMDS analysis of the bacterial community in anaerobic bioreactors (based on 16S rRNA gene sequences at 97% similarity cutoff). The green arrows indicate the increase of biogas production (Biogas), HRT (HRT) and TS removal rate (Degradation). The red and blue arrows indicate bacterial genera that shifted significantly, where  $P \leq 0.001$  and  $P \leq 0.01$ , respectively. The black, yellow and purple arrows represent the [FeFe]-hydrogenase phylogenetic affiliations that shifted significantly ( $P \leq 0.001$ ,  $P \leq 0.05$ , and  $P \leq 0.01$ , respectively). All arrows point towards the abundant range of the gradients. Labels HRT30d, HRT40d and HRT50d represent bioreactors with a HRT of 30, 40, and 50 days, respectively.

and blue arrows). Fitting of the bacterial community based *hydA* gene identification showed that the abundance of those most closely affiliated with *Clostridium lentocellum*, and *Candidatus Cloacamonas acidaminovoransa* were found significantly associated with the DM + CS HRT40d (reactor 4). The abundance of several other *hydA* genes, such as *Clostridium thermoCELLUM*, and *Bacteroides* sp. 20\_3, shifted significantly along the HRT gradient and upon the addition of corn stover (Figure 7).

#### 4. Discussion

Anaerobic digestion is an efficient method of converting agricultural wastes into methane. The accompanied study by Yue *et al.* [20] demonstrated that the addition of corn stover in anaerobic digestion maximized biogas production and enhanced total solids reduction. The reported observation concluded that cellulose and hemicellulose degrading bacteria and associates contributed to the generation of hydrogen and acetate, which in turn supported the growth of methanogens.

#### 4.1. The Significance of Clostridia and Bacteroidetes

We observe a large number of Clostridia by 16S rRNA gene profiling in this pilot scale reactor study and we anticipated greater abundance and diversity of Clostridia in the reactors supplemented with corn stover because of their cellulolytic abilities. However, Bacteroidetes were equally abundant as the Clostridia in all bioreactors. Moreover, in Bioreactor 4 where methane production and total solids reduction were greatest, unique populations of uncultivated Bacteroidetes were abundant. The shifts in the Clostridia-like *hydA* gene abundances indicated that unique Clostridia populations became more prominent with the addition of corn stover and the increase of HRT (Figure 5). The NMDS analysis (Figure 7) also showed that three Clostridia populations associated with the DM + CS bioreactors increased significantly (*Clostridium lentocellum*, *Candidatus Cloacamonas acidaminovorans*, and *Clostridiales bacterium 1\_7\_47\_FAA*). These observations suggested that although cellulolytic Clostridia were important, the role of specific populations of Bacteroidetes in anaerobic cellulose degradation should not be ignored. Moreover, populations of Clostridia may be crucial in both hydrogen production and cellulose degradation in our bioreactors.

Like class Clostridia, Bacteroidetes populations were also commonly reported as cellulose/hemicellulose degraders [27]-[29] and observed in anaerobic digestion at low abundance (average 17%) [30]-[34]. There were 21% - 40% of Bacteroidetes populations in our pilot bioreactors and we observed a shift from the dominance of identifiable Bacteroidetes to unclassified Bacteroidetes, which correlated with the overall improved system performance reported in the companion study [20]. The four unclassified Bacteroidetes clusters that were uniquely abundant in corn stover supplemented bioreactors were highly similar to bacterial 16S rRNA gene clones found in methanogenic environments, specifically, domestic wastewater enriched microbial fuel cells (#43, SeqMatch score = 0.998), biogas slurry (#11, SeqMatch score = 0.966), Kinneret lake sediments (#14, SeqMatch score = 0.983), methanogenic zone of a hydrocarbon- and chlorinated-solvent contaminated aquifer (#69, SeqMatch score = 0.988) and an anaerobic bioreactor fed with butyrate and sulfate enriched paper mill wastewater (#69, SeqMatch score = 0.988, unpublished work) [35]-[38]. Further, a polysaccharide utilization locus in uncultured Bacteroidetes phylotype (SRM-1) was recently identified, which allows SRM-1 to target different major components of plant biomass [39]. Thus, unclassified Bacteroidetes may be more crucial in anaerobic degradation of recalcitrant carbons than we have previously perceived. While the 16S rRNA gene based survey suggested that members of Bacteroidetes played an important role in the anaerobic digestion, the *hydA* clone library profile revealed that Bacteroidetes-like *hydA* genes were not as abundant as would be expected if they were linked to these important Bacteroidetes populations. Bacteroidetes-like *hydA* genes were also less abundant in the DM + CS than the DM bioreactors (Figure 5). The 16S rRNA and *hydA* gene profiling together suggests that unclassified Bacteroidetes might play a substantial role in cellulose/hemicellulose degradation and total solid removal but not as direct providers of substrates for methanogens in biogas production.

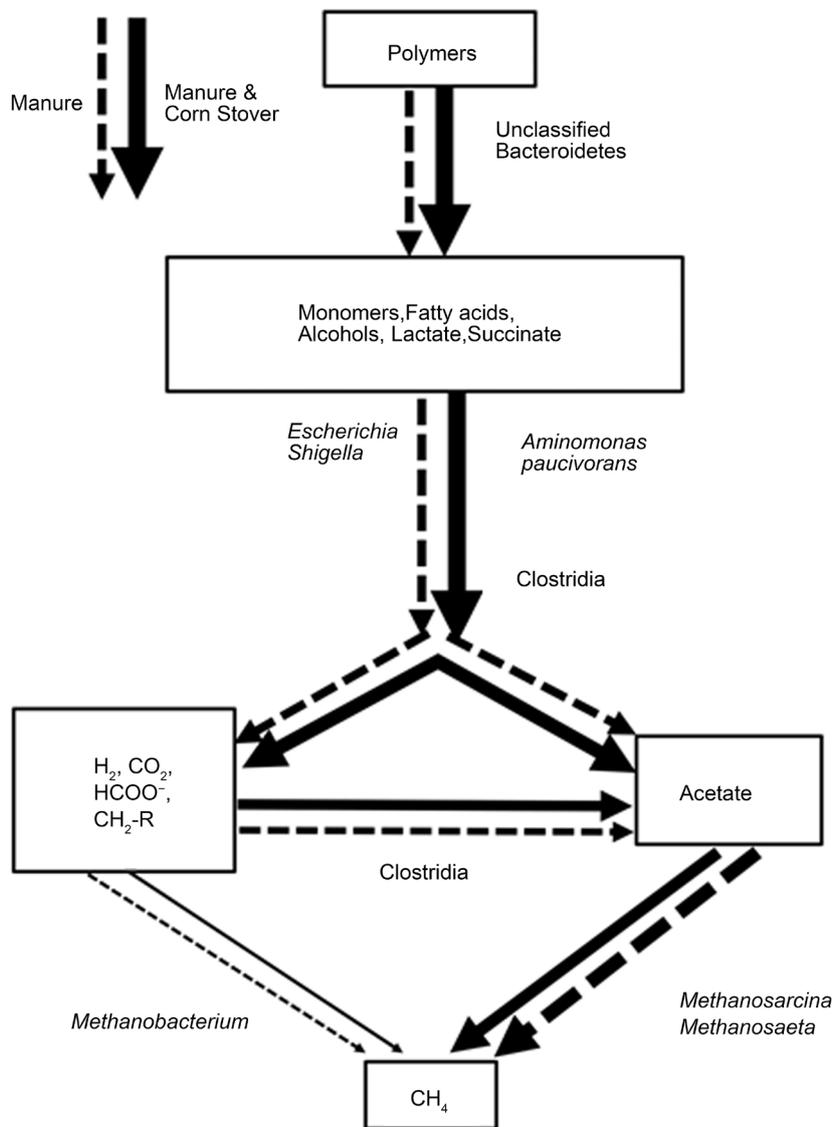
## 4.2. The Hydrogen Producing Populations

Besides Clostridia-and Bacteroidetes-like *hydA* genes, we observed a high abundance of Synergistia-like hydrogenase genes (at least 35%) in all of the DM + CS bioreactors. This observation suggested that Synergistia were active in hydrogen production in cellulose/hemicellulose rich environments. At species level, Synergistia was solely contributed by *Aminomonas paucivorans*, an anaerobic bacterium that was previously identified as an amino-acid-degrader. Baena *et al.* [40] described an *A. paucivorans* isolate that was able to grow on arginine, histidine and glutamate when cultivated syntrophically with methanogen *Methanobacterium formicum*. The end products of the co-culture were propionate, acetate, CO<sub>2</sub> and methane [40]. There are no previous studies indicating that *Aminomonas paucivorans* is capable of cellulose degradation. Hence, concluding from the available information and our observations, *A. paucivorans* might be contributing largely to hydrogen production by utilizing the secondary fermentation products of degraded dairy manure [40].

## 4.3. The Anaerobic Co-Digesting Ecosystem

Anaerobic bioreactor is a complex ecosystem and frequently referred to as a black box. This pilot study was designed to correlate the microbial community structure with operating conditions. From our results, we postulate that a unique community structure including specific uncultivated Bacteroidetes populations and syntrophic populations in anaerobic bioreactors fed with agricultural wastes led to optimal methane production, total solids reduction, and reduced *E. coli*/*Shigella* abundance (Figure 8).

NMDS analysis revealed a complex network among bacterial communities in the methane producing anaerobic bioreactors. Correlating 16S rRNA gene with *hydA* gene diversities, we observed that the high abundance of unclassified Bacteroidetes was significantly associated with the DM + CS bioreactor with a HRT of 40 days that produced the most methane. Besides unclassified Bacteroidetes, the abundance of *Caryophanon*, *Blastopirellula*, *Ketogulonicigenium* and unclassified *Syntrophaceae* increased significantly in the DM + CS bioreactor with a HRT of 40 days. Little has been discovered regarding the metabolic capabilities of *Caryophanon* and *Blastopirellula*. Members of *Ketogulonicigenium* and *Syntrophaceae* were previously identified as fermenters with versatile metabolisms [41]-[44]. Further, the syntrophic importance of members of *Syntrophaceae* was linked to methane production systems using hydrocarbons as substrate [45]-[47]. It is important to note that hydrocarbons are secondary metabolites from plant material degradation. Hence, unclassified Bacteroidetes might play a substantial role in primary cellulose/hemicellulose degradation and that genera *Caryophanon*, *Blastopirellula*, *Ketogulonicigenium* and unclassified *Syntrophaceae* could potentially participate in secondary fermentation (Figure 8). NMDS analysis also showed that different members of Clostridia and Bacteroidetes participated in different activities among all six anaerobic bioreactors. For example, *Bacteroides* sp. 1\_1\_14 appeared to be affiliated with hydrogen production during dairy manure digestion at a low HRT, while *Bacteroides* sp. 20\_3 was found significantly associated with DM + CS digestion



**Figure 8.** The potential biogas production model in anaerobic bioreactors. Thick lines indicate potential important processes (figure adapted from Schink & Stams [3]).

at an elevated HRT. Similarly, *C. thermocellum*-like *hydA* genes were found to be significantly correlated with the low HRT bioreactors and *Clostridiales* bacterium and *Odoribacter splanchnicus* might play a significant role during methane production in DM + CS digestion at elevated HRT. Although *A. paucivorans* (a member of family *Synergistaceae*) and *M. thermoacetica* (a member of class *Clostridia*) like *hydA* genes were highly abundant and varied in anaerobic bioreactors, NMDS analysis indicated that the shifts of their abundance were not significant across all bioreactors (Figure 7). However, we observed that the abundance of these two groups of *hydA* genes followed the abundance pattern of unclassified Bacteroidetes determined based on 16S rRNA gene sequences (Figure 6). From these observations, we hypothesized that hydrogen-producing population *A. paucivorans* responded to the growth of unclassified Bacteroidetes. This is

consistent with our prediction that unclassified Bacteroidetes were responsible for cellulose/hemicellulose degradation but not for hydrogen production. Subsequently, cellulose/hemicellulose degradation products, such as xylose, glucose and cellulobiose, could be consumed by hydrogen producers (*A. paucivorans* and *M. thermoacetica*).

The companion study [20] described the archaeal community structures in these anaerobic bioreactors, where they observed that the communities were dominated by *Methanobacterium*, *Methanosarcina* and *Methanosaeta* populations (Figure 8). Members of *Methanobacterium* are known for producing methane via hydrogen oxidation. The presence of this group of methanogens may be metabolically link to hydrogen produced by *A. paucivorans*, Clostridia and Bacteroidetes. The literature indicates that many members of *hydA* containing bacteria generate acetate as one of the final products [17]. The presence of a large amount of *Methanosarcina* and *Methanosaeta* suggests that acetate was a substantial source for methane generation. Yue *et al.* [20] also concluded that the community shifts from *Methanosarcina* dominant to *Methanosaeta* dominant was due to the decrease in acetate concentration in the bioreactors as HRT increased. This also explains the decreasing trend in *Methanobacterium* abundance in both the DM and DM + CS bioreactors as the HRT increased from 30 to 50 days. These observations are consistent with the observation that the biogas/methane production rate approached plateau when the operational HRT was more than 40 days.

## 5. Conclusion

Our results, while descriptive, suggest that specific populations of unclassified Bacteroidetes, Clostridia and supporting metabolic guilds are required to establish optimal conditions for methane generating farm waste digesters. Establishing optimal physical and chemical conditions in an anaerobic digester may select for the optimal microbial community only if the members are present. However, knowing with certainty the structure of this microbial community provides the option of preseeded reactors with critical populations so that digestion rapidly reaches and maintains efficient operation of what is a complex community metabolism. Information regarding key populations involved in co-digestion might lead to rational selective strategies for initiating and maintaining optimal community structure. While it is not surprising that close attention to operating conditions will optimize waste processing, it might be greatly enhanced if an optimal community structure could be seeded into reactors and nurtured rather than select and hope for optimality.

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