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Metagenomic Insights Into The Full-Scale Anaerobic Digestion of Cow Manure and Pre-Treated Food Waste

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METAGENOMIC INSIGHTS INTO THE FULL-SCALE ANAEROBIC DIGESTION OF COW MANURE AND PRE-TREATED FOOD WASTE

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ABSTRACT

Anaerobic digestion provides many benefits, such as diverting organic waste from landfills, promoting nutrient recovery, reducing greenhouse gas emissions, and producing renewable energy. A complex community of microorganisms is responsible for the anaerobic digestion process that converts organic matter into biogas, a renewable energy source. The start-up phase of an anaerobic digester is considered a crucial point in solidifying the core microbial community that will ensure a stable anaerobic digester. A full-scale anaerobic digester that co-digests cow manure and pre-treated food waste from a hydrolysis tank was studied from start-up through stable performance in order to better understand the dynamics of the core microbial community, assess gene abundance, and relate population dynamics to operational parameters.

The microbial community in the hydrolysis tank, which was operated at a low pH of 3.5 to 4.4, consisted of organisms known to ferment carbohydrates to acetate and lactate, such as those in the genera *Lactobacillus*, *Prevotella*, and *Bifidobacterium*. Genes for the Bifid shunt were abundant while genes for hydrogen production and ferredoxin production and consumption were absent, suggesting that fermentation likely did not result in H_2 production.

The methanogenic archaeal community in the anaerobic digester had a high relative abundance up to 25%. The inoculation of the digester had little impact on abundant organisms, as *Methanoculleus* and an *unclassified Methanomicrobiales* genera were highly abundant before and after inoculation, though the abundance of total methanogenic archaea decreased after inoculation. While some genes related to acetoclastic methanogenesis were present, two diagnostic genes of this pathway (ack and pta) were absent, suggesting that hydrogenotrophic methanogenesis was the primary route. The high abundance of genes involved in ferredoxin cycling and H_2 generation suggest that bacteria in the digester were likely producing H_2.

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CHAPTER 1

INTRODUCTION

Anaerobic digestion is the process of converting organic waste into renewable energy using microorganisms in an engineered environment. This process allows organic waste streams such as food and agricultural waste to be diverted from conventional disposal methods and transformed into biogas and other valuable byproducts. Biogas can be combusted to create electricity or upgraded into pipe-line quality renewable natural gas.

Food waste is a persistent problem, with one third of all food produced in the United States being disposed of in landfills or by incineration (Jaglo et al., 2021). Landfilling food waste results in methane production from the anaerobic breakdown of organic material while incinerating food waste releases carbon dioxide during combustion. A more sustainable alternative to these food waste disposal methods is composting. Composting consists of the aerobic decomposition of food waste, creating a valuable product that can be used as fertilizer. When composting is done correctly, a small amount of carbon dioxide is released (Fan et al., 2021). However, if compost piles are not well maintained, anaerobic conditions occur which release methane, a more harmful greenhouse gas than carbon dioxide (Fan et al., 2021). Anaerobic digestion provides a safe alternative by capturing biogas in a controlled environment to use as renewable energy.

Agricultural waste primarily consists of manure. Manure is often stored in lagoons and applied to fields as a fertilizer. Storage ponds are stagnant and open,

allowing manure to release methane directly to the atmosphere (Baral et al., 2018). While the nutrients in manure help crops grow, excess nutrients such as nitrogen and phosphorus can be transported by stormwater runoff into nearby waterways. This nutrient overload can cause algal blooms and eutrophication which harm watershed health. Anaerobic digestion can alleviate agricultural pollution by directing carbon in manure to beneficial products and by directing nutrients in manure to a central processing location prior to field application.

The microbial community in an anaerobic digester is responsible for converting these complex waste streams into biogas. The abundance and classification of microbial species present in an anaerobic digester is impacted by process parameters and can determine process performance (Chen et al., 2016; Wang et al., 2018). However, little is known about how anaerobic digester microbial communities are enriched during the start-up phase. This work will investigate elucidate changes in the abundance and taxonomy of genes and microbial organisms during the start-up phase of a full-scale anaerobic digester using time-series metagenomics. Understanding how factors such as inoculation, the beginning of co-digestion with food waste, and operational parameters impact the microbial community can lead to more efficient start-up of anaerobic digesters and provide fundamental insights into the assembly and stability of anaerobic digestion microbial communities.

CHAPTER 2

LITERATURE REVIEW

OVERVIEW

Anaerobic digestion consists of four different phases (Figure 1.1): hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Dalke et al., 2021). Hydrolysis breaks down organic chemical polymers (e.g., complex carbohydrates, proteins, nucleic acids, and lipids) into monomers (e.g., sugars, amino acids, nucleotides, and fatty acids. Hydrolysis depends on microorganisms that produce extracellular and intracellular enzymes that cleave the chemical bonds between monomeric units of a polymer, and hydrolysis is often the rate limiting step of anaerobic digestion (Mata-Alvarez et al., 2014; Xing et al., 2020). After hydrolysis, monomers are fermented into a variety of carbonaceous intermediates that include carboxylic acids, alcohols, and aldehydes. Short-chain volatile fatty acids (e.g., acetate, propionate, and butyrate) are typical carbonaceous intermediates. In addition, fermentation produces hydrogen gas and carbon dioxide. Acetogenesis directs carbon to acetate and includes homoacetogenesis, which converts hydrogen gas and carbon dioxide to acetate. Bacterial populations are involved in hydrolysis, acidogenesis, and acetogenesis; however, only archaeal populations are involved in methanogenesis (Chen et al., 2016).

Methanogenic archaea perform the last step of methanogenesis. There are two different types of methanogens: acetoclastic and hydrogenotrophic. Acetoclastic methanogens convert acetate into methane while hydrogenotrophic methanogens convert

CO_2 and H_2 into methane. Methanogens have a very slow growth rate and are sensitive to environmental factors (Vesilind and Worrell, 2012).

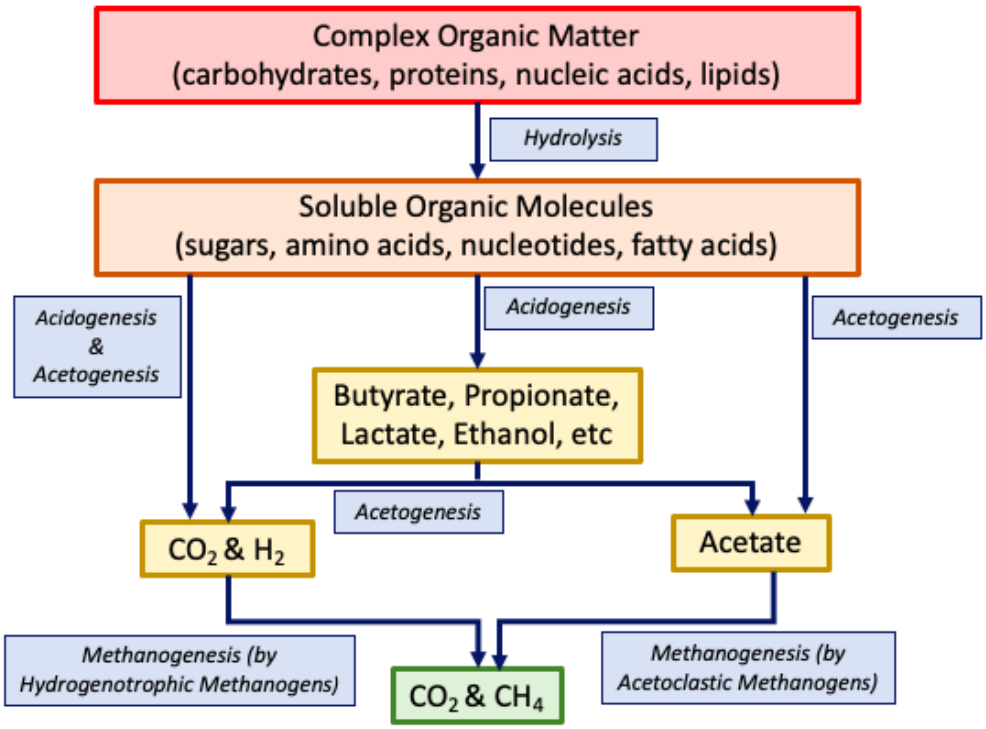

Figure 1.1. Anaerobic Digestion Process Diagram

A careful balance of substrates creates a healthy microbial community which is necessary for biogas production. However, the production of harmful intermediate compounds by the microbial community has the ability to offset the effectiveness of digestion. Food waste that is rich in carbohydrates is considered easily biodegradable, but can result in an increase in volatile fatty acid (VFA) accumulation and decrease pH below the optimal range for methanogens (Dalke et al., 2021). Ammonia and hydrogen sulfide are also produced by organic waste that is high in protein and can negatively impact the microbial community (Xu et al., 2018). Avoiding the production of harmful intermediates

is key to optimizing biogas production and ensuring an efficient, stable microbial community.

Organic loading rate (OLR) is a measurement of raw organic matter being fed to the digester on a daily basis and is considered an important operational parameter that impacts reactor stability and microbial activity (Duan et al., 2019). Typically, a range of 0.5 to 5 kg VS d^{-1} is recommended for organic loading rate (Gautam et al., 2022). While increased organic loading rates can increase the rate of biogas production, they risk inhibition and acidification due to overloading of organic matter (Gautam et al., 2022).

One proposed method to prevent accumulation of inhibitory byproducts in a digester is two-stage anaerobic digestion. A two-stage system separates acid production from methane production, allowing acid production to occur without inhibiting methanogenesis in the second stage. The acid production stage favors a more acidic pH typically around 5.5-6.5 with a short hydraulic retention of 1-2 days to allow for fermentation. The methane production stage typically favors a pH of 6-8 with a hydraulic retention time of 20-30 days (Xu et al., 2018). Separating the hydrolysis, acidogenesis, and acetogenesis phases can reduce accumulation of fermentation intermediates and can allow for the optimization of the fermenting microbial communities and methanogenic microbial communities separately.

A second proposed method is to feed multiple types of substrates with synergistic characteristics. In so-called “co-digestion,” multiple organic waste streams are digested together. As an example, animal manure has a high buffer capacity and low carbon to nitrogen (C:N) ratio (Mata-Alvarez et al., 2014). Food waste has a poor buffer capacity

and a high C:N ratio. Therefore, co-digesting animal manure and food waste can improve system stability and help prevent inhibitory conditions. Co-digestion can also allow for an increase in maximum allowable organic loading rate, allowing for a more efficient use of time and resources (Xu et al., 2018).

The use of external inoculum can also improve digester conditions, especially during the start-up phase. Inoculum is commonly anaerobic digester sludge from a well-functioning digester that is introduced to a digester during its start-up phase or when methane production is sub-optimal. The addition of inoculum can increase the active methanogenic population in a digester and has been proven to promote a stable start-up (Boulanger et al., 2012; Kobayashi et al., 2009). In fact, start-up of anaerobic digesters that do not use external inoculum can experience VFA accumulation and difficulty increasing their organic loading rate (Stroot, 2001).

Robust knowledge of the effects of environmental factors and different substrates on the microbial community is needed to achieve a stable, core microbial community. Studying the microbial community evolution and how it reacts to a two-stage process, co-digestion, inoculation, and other factors can provide insight into community dynamics and how each factor may impact the system's efficiency. While the composition of microbial communities in full-scale anaerobic digesters have been detailed in past studies, less is understood of the microbial evolution during the start-up phase of a full-scale digester. Microbial communities during start-up of bench-scale anaerobic digesters have been studied extensively (Goberna et al., 2015; Ziganshina et al., 2014), but studies of full-scale systems are limited (Campanaro et al., 2018; Goux et al., 2016;

Li et al., 2020). Further, 16S rRNA gene amplicon sequencing has been the most common sequencing used in anaerobic digestion research. This type of sequencing allows for taxonomic profiling of bacterial and archaeal species but the results are limited to defining the approximate abundance of organisms and their taxonomic classification. Further, archaeal and bacterial 16S rRNA gene sequencing require different primer sets, making it difficult to assess the relative abundance of bacterial and archaeal species.

MICROBIAL COMMUNITIES IN ANAEROBIC DIGESTERS

Past 16S rRNA gene amplicon sequencing studies have identified that the dominant bacterial phyla in most anaerobic digesters consist of *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes,* and *Chloroflexi* (Kirkegaard et al., 2017). *Methanosaeta* are classified as acetoclastic methanogens while *Methanoculleus, Methanobacterium, Methanobrevibacter, Methanospirillum, Methanomicrobium, Methanococcus,* and *Methanothermobacter* are classified as hydrogenotrophic methanogens. *Methanosarcina* are capable of both acetoclastic and hydrogenotrophic methanogenesis. These archaeal genera are common methanogens found in anaerobic digestion (Lim et al., 2020; Wang et al., 2018). While less research has been done on the Eukaryotic community, a study of a full-scale digester inoculated with run-off water and cattle manure and then fed plant biomass and cattle manure reported organisms of the division *Chlorophyta* during start-up (Goux et al., 2016). *Fungi* and *Ciliophora* appeared shortly after start-up (Goux et al., 2016).

Firmicutes and *Bacteroidetes* have been linked to parameters such as organic loading rate, volatile fatty acid concentration, and methane production (Chen et al., 2016). It has been reported that *Firmicutes* tend to dominate the community during stable performance at low organic loading rates while *Bacteroidetes* increase in abundance at higher loading rates (Chen et al., 2016). In many studies, *Firmicutes* and *Bacteroidetes* tend to be the most abundant bacterial phyla (Chen et al., 2016; Mata-Alvarez et al., 2014; Xing et al., 2020). *Firmicutes* are syntrophic bacteria which can degrade VFAs and produce H_2 which is used by hydrogenotrophic methanogens (Gannoun et al., 2016). On the other hand, *Bacteroidetes* are proteolytic bacteria which produce acetate from amino acids (Gannoun et al., 2016).

Methanosarcina are considered both acetoclastic and hydrogenotrophic methanogens as they can produce methane from acetate, methanol, monomethylamine, dimethylamine, trimethylamine, H_2, CO_2, and CO (Wang et al., 2018). *Methanosaeta* are specialized acetoclastic methanogens which can only use acetate. Comparatively, *Methanosarcina* have a greater maximum rate of acetate utilization and maximum growth rate, a greater half-saturation coefficient, and a greater yield coefficient compared to *Methanosaeta* (Conklin et al., 2006). For these reasons, higher acetate concentrations may favor *Methanosarcina* growth. *Methanosarcina* has reportedly been in lower abundance in anaerobic digesters with higher levels of ammonia, specifically in digesters being fed solely cow manure (Campanaro et al., 2018).

MICROBIAL COMMUNITIES IN FULL-SCALE ANAEROBIC DIGESTERS

The full-scale digestion of food waste has shown to be dominated by *Firmicutes, Bacteroidetes, Cloacimonetes,* and *Synergistetes* (Li et al., 2020). On the genus level, the bacterial community can be dominated by *Candidatus Cloacamonas* which has been found to be a hydrogen-producing syntroph that can oxidize propionate into acetate and CO_2 (Li et al., 2020). However, this reaction is only thermodynamically favorable when H_2 partial pressure remains low by coupling the syntrophic propionate oxidation with hydrogenotrophic methanogenesis (Razaviarani and Buchanan, 2015). The archaeal community has reportedly been dominated by *Methanomassiliicoccus* and *Methanoculleus*. *Methanoculleus* is a hydrogenotrophic methanogen which works well with the hydrogen-producing bacteria and has a tolerance of high salt concentrations (Li et al., 2020; Wang et al., 2018). *Methanothrix* is an acetoclastic methanogen which favors low acetate concentrations and has been reported in two-stage food waste digesters which separate acidogenesis from methanogenesis (Li et al., 2020).

A previous study sampled three full-scale mesophilic digesters in Vermont that use cow manure as their main substrate. *Methanosarcina thermophila* was the most highly represented species in two of the three digesters (St-Pierre and Wright, 2013). The third digester was most highly represented by *Methanoculleus bourgensis* followed by *Methanosarcina thermophila* (St-Pierre and Wright, 2013).

Few studies have monitored the full-scale co-digestion of food waste and cow manure in a mesophilic digester. One study followed the acclimation of co-digesting food waste and cow manure while using sludge from a full-scale mesophilic blanket reactor

from a brewery plant as inoculum. It was found that *Firmicutes, Bacteroidetes, Chloroflexi, Actinobacteria, Euryarchaeota,* and *Proteobacteria* were the most abundant (Xing et al., 2020). Specifically, the relative abundances of *Firmicutes, Bacteroidetes,* and *Actinobacteria* increased over time. At the genus level, the relative abundances of *Draconibacterium*, *Lentimicrobium*, and *Sunxiuqinia* genera belonging to *Bacteroidetes* increased overtime (Xing et al., 2020). Table 1.1 provides a comprehensive list of abundant microbes found in full scale anaerobic digesters.

Table 1.1. Abundant Microbes in Full Scale Anaerobic Digesters

Substrate Source	Inoculum Source	Reactor Type	Solids Retention Time	Abundant Microbes
Food Waste & Cow Manure	Sludge from Digester treating Brewery Waste	Dynamic Membrane Bioreactor	-	*Firmicutes, Bacteroidetes, Chloroflexi, Actinobacteria, Euryarchaeota, Proteobacteria (Xing et al., 2020)*
Dairy Cattle Manure & Whey	-	Plug Flow	21 days	*Methanosarcina Thermophila* (St-Pierre and Wright, 2013)
Dairy Cattle Manure & Ice Cream Factory Waste	-	Plug Flow	25-27 days	*Methanosarcina Thermophila (St-Pierre and Wright, 2013)*
Dairy Cattle Manure & Oil Waste	-	Completely Mixed Flow Reactor	30 days	*Methanosarcina Thermophila, Methanoculleus Bourgensis, Methanoculleus (St-Pierre and Wright, 2013)*
Food Waste	Sludge from Digester Treating Food Waste	Completely Stirred Tank Reactor	15 days	*Candidatus Cloacamonas, Saccharofermentans, Methanomassiliicoccus, Methanoculleus* (Li et al., 2020)
Plant Biomass & Cattle Manure	Cattle Manure & Run-off Water	Completely Stirred Tank Reactor	-	*Firmicutes, Proteobacteria, Tenericutes, Bacteroidetes, Methanobacterium, Methanoculleus, Methanocorpusculum* (Goux et al., 2016)

There are few full-scale studies of anaerobic digesters co-digesting food waste and cow manure, specifically using metagenomic sequencing. Further, there is a lack of comprehensive studies comparing the microbial community in the inoculum sludge, hydrolysis tank, and anaerobic digester from start-up through stable operation. The purpose of this study is to assess microbial community dynamics during the start-up phase of a full-scale anaerobic digester. The impacts of inoculating a full-scale digester with sludge from a separate full-scale will be assessed, along with impacts of feeding fermented food waste from a hydrolysis tank after an initial stabilization period with cow manure. Results from this study can inform future strategies for anaerobic digester start up and make connections between operating parameters and microbial community stability.

CHAPTER 3

JOURNAL ARTICLE: METAGENOMIC INSIGHTS INTO THE FULL-SCALE ANAEROBIC DIGESTION OF COW MANURE AND PRE-TREATED FOOD WASTE

TO BE SUBMITTED TO: BIORESOURCE TECHNOLOGY

ABSTRACT

Anaerobic digestion provides many benefits, such as diverting organic waste from landfills, promoting nutrient recovery, reducing greenhouse gas emissions, and producing renewable energy. A complex community of microorganisms is responsible for the anaerobic digestion process that converts organic matter into biogas, a renewable energy source. The start-up phase of an anaerobic digester is considered a crucial time in solidifying the core microbial community that will ensure a stable process. Using metagenomics, a full-scale farm-based anaerobic digester that co-digests cow manure and other organic wastes pretreated in a hydrolysis tank was studied during its first year of operation.

The hydrolysis tank was operated at a low pH (3.5-4.4) and the microbial community was enriched with organisms known to ferment carbohydrates to acetate and lactate such as those related to genera *Lactobacillus*, *Prevotella*, and *Bifidobacterium*. The metagenomic analysis showed that genes for the Bifid shunt were abundant while those for hydrogen production and ferredoxin production and consumption were absent. This suggests that fermentation in the hydrolysis tank likely did not result in H_2 production.

The organic loading rate to the methane-producing anaerobic digester was slowly increased from 0 to 3 kg VS m^{-3} d^{-1} over the course of the study. The microbial community in the anaerobic digester exhibited a high ratio of archaea (up to 25% relative abundance), and the addition of inoculum sludge did not have a large impact on the abundant organisms, with *Methanoculleus* and an *unclassified Methanomicrobiales* genera persisting at high abundance both before and after inoculation. Unlike the bacterial community in the hydrolysis tank, bacteria in the anaerobic digester were likely producing H_2 based on the high abundance of genes involved in ferredoxin cycling and H_2 generation. Some genes involved in acetoclastic methanogenesis were present, but two diagnostic genes of this pathway (ack and pta) were absent. In total, this suggests that hydrogenotrophic methanogenesis was the primary route of methanogenesis.

INTRODUCTION

Over one third of all food produced in the United States goes unconsumed, resulting in food waste (Jaglo et al., 2021). Common disposal methods for food waste include landfilling and incineration. The anaerobic decomposition of food waste in landfills produces methane while the incineration of food waste releases carbon dioxide and other harmful gasses during combustion. Anaerobic digestion, an alternative process for food waste management, converts organic waste into biogas using a diverse community of microorganisms. Biogas, which consists of methane, carbon dioxide, and other trace gasses, can be combusted to create electricity or upgraded into pipe-line quality renewable natural gas.

Agricultural waste is another common organic waste byproduct of food systems. Manure is often stored in lagoons which are stagnant and open to the surrounding environment, allowing methane to be released directly to the atmosphere (Baral et al., 2018; Grant et al., 2015). While nutrient-rich manure is used as fertilizer, excess nutrients such as nitrogen and phosphorus can be transported by stormwater runoff into nearby waterways. This nutrient overload can cause algal blooms and eutrophication which harm watershed health (Li et al., 2021). Anaerobic digestion can alleviate agricultural pollution by directing carbon in manure to beneficial products and by directing nutrients in manure to a central processing location prior to field application.

Throughout the United States, farm-based anaerobic digesters that convert both manure and external substrates, such as food waste, are increasingly common. Co-digestion, the co-substrate with synergistic characteristics, can be implemented to reach a target carbon to nitrogen (C:N) ratio and supply missing nutrients if a single substrate is not optimal (El-Mashad and Zhang, 2010; Mata-Alvarez et al., 2000). Animal manure has a high buffer capacity and low C:N ratio (Mata-Alvarez et al., 2014). Food waste has a poor buffer capacity and a high C:N ratio. By combining these characteristics, co-digesting animal manure and food waste can improve system stability and help prevent inhibitory conditions. Co-digestion can also allow for an increase in maximum allowable OLR, allowing for a more efficient use of time and resources (Xu et al., 2018). As of May 2022, there are 416 farm-based digesters across the United States digesting manure and 218 of these perform co-digestion.

While full-scale anaerobic digestion of agricultural residues is becoming more common, there are very few studies monitoring the start-up of full-scale, on-farm anaerobic digestion facilities. An improved understanding of key operational parameters (e.g., organic loading rate, pH, gas production) and the underlying microbial communities could provide more informed decision making during the critical phase of digester start-up. Specifically, this study investigates the operational parameters and metagenomes of a two-phase, acid-gas digester system located on a farm in the eastern United States that is fed cow manure, food waste, and a variety of food processing byproducts. Using a combination of next generation sequencing tools and reactor operational parameters, we monitor the microbial community dynamics over time to assess (1) the impacts of using an external inoculum during the start-up phase; (2) the duration required to achieve a core microbial community; (3) the genetic stability of the digester; and (4) the relationship between abundant taxa and key operational parameters.

MATERIALS & METHODS

Full-Scale Digester Operations

This study focuses on a full-scale anaerobic digester co-digesting food waste and cow manure from start-up through the one year of operations. Initially, the digester was fed solely cow manure which was put through a screw press to remove solids prior to feeding. Sample collection began on day 0. The digester was then inoculated with sludge from another full-scale anaerobic digester on days 27 and 29. On day 104, food waste and other substrates processed through an on-site hydrolysis tank were introduced to the digester. Substrates brought to the on-farm digester, including food waste, go through

two-phase digestion by being fed to a hydrolysis tank operated at low pH prior to being fed to the digester. The separation of feeding steps allows for a comprehensive analysis on the impact of cow manure, the addition of inoculum, and the beginning of food waste co-digestion on the microbial community. Stage 1 consists of the digester solely containing cow manure. Stage 2 began when the digester was inoculated prior to food waste addition. Stage 3 began when the digester began accepting food waste from the hydrolysis tank.

Anaerobic Digester Data Collection and Analysis

Digester data including digestate pH, VFA concentration, OLR, and biogas composition was collected on a daily basis by the digester operators. Digestate pH was measured using a pH probe as part of the HANNA Instruments titrator system. VFA concentration was determined using a 2-step titration using 0.1N hydrochloric acid solution. The first and second titration endpoints were 5.00 and 4.40 pH respectively. 100 mL samples were collected directly from the digester and titrations were run in triplicate using 5 g of digestate for each measurement. The average total VFA concentration was recorded in mg/L as acetic acid. Biogas composition was measured with an in-line gas analyzer. A portable gas analyzer was used to verify the gas composition directly at the digester. Both analyzers were calibrated monthly. OLR was calculated using the weighed average of total volatile solids content of inbound feedstocks and manure (kg/m^3), the volume of organic feedstocks and manure fed to the digester on a daily basis (m^3/d), and the volume of the digester (m^3).

Sample Collection & Preservation

Samples were collected from the digester beginning on day 0 and from the hydrolysis tank beginning on day 210. Sample collection continued on a weekly basis for the duration of a year, until day 364. Inoculum sludge samples were collected on day 27 and day 29 prior to being added to the digester. Following sample collection, samples were centrifuged at 10,000 rpm for 10 minutes. The solid pellets were stored in a -80°F freezer until DNA extractions were performed.

DNA Extractions

DNA was extracted from the preserved biomass samples using the Qiagen PowerSoil DNA Extraction kits. The quality and quantity of DNA extractions was assessed using a NanoDrop spectrophotometer and a Qubit Fluorometer. DNA was diluted to 10 ug/mL for metagenomic sequencing. A Nextera XT library was constructed and next-generation sequencing was conducted by the Vermont Integrative Genomics Resource Massively Parallel Sequencing Facility on the Illumina HiSeq 1500 system to generate paired end 2 x 150bp reads.

Sequencing Data Analysis

Trimmomatic was used for adapter removal and quality trimming (Bolger et al., 2014). Leading and trailing bases with a Phred quality score of 3 or less were removed. FastQC was used to perform quality control checks on the merged sequence data (Andrews, 2010) and MultiQC was used to summarize the quality data from FastQC (Ewels et al., 2016). Due to poor read quality, digester samples from days 147, 154, 246, and 287 and a hydrolysis tank sample from day 258 were excluded from further analysis.

Downstream analyses including sequence assembly, annotation, and mapping were performed with the SqueezeMeta pipeline (Tamames and Puente-Sánchez, 2019). The SPAdes assembler was used for assembly and the minimap2-ont mapper was used for read mapping (Li, 2018; Nurk et al., 2017). SQMTools was used for downstream processing and data visualization (Puente-Sánchez et al., 2020). SqueezeMeta uses the DIAMOND software to compare gene sequences against taxonomic and functional databases (Buchfink et al., 2015). For taxonomic classification, the NCBI GenBank database was used (Clark et al., 2016). The KEGG database was used for gene annotation and all genes are identified by their KEGG identification numbers (Kanehisa, 2000). The abundance value of each gene was calculated from the mapped reads using the transcript per million (TPM) approach (Puente-Sánchez et al., 2020). For consistency with past publications, "TPM" is used as an abbreviation even though DNA reads, rather than transcripts, were used for mapping. As described in the original publication for SQM Tools, "the TPM of a feature (be it a transcript, a gene or a functional category) is the number of times that we would find that feature when randomly sampling 1 million features, given the abundances of the different features in our sample." Therefore, TPM normalizes the number of reads mapping to a specific gene based on the total number of reads in a sample allowing for comparison of samples with different sequencing depth. To calculate TPM, the reads per kilobase (RPK) is first calculated by dividing the number of reads mapped to a gene by the gene length, in kilobases. Then, TPM is determined by dividing the RPK by the sum of RPKs for all genes in a sample then multiplying by 1,000,000.

Statistical Analysis

The impact of operational parameters on the abundance of bacterial and archaeal genera was analyzed using Spearman's rank correlation coefficients and their corresponding *p*-values, which were considered statistically significant if less than 0.01. The R package "corrplot" was used for data visualization (Wei and Simko, 2021).

RESULTS & DISCUSSION

Operational parameters during start-up

The operational parameters collected from the methane-producing digester were monitored for 364 days (**Fig 1**). Early on in operations (Days 27 and 29), inoculum from a separate full-scale digester was added to the reactor. Further, addition of food waste from the hydrolysis tank began on Day 104. The hydrolysis tank enables "two-stage" anaerobic digestion of the non-manure portion of organic waste fed to the methane-producing digester. The acid production stage in the hydrolysis tank uses a below-neutral pH and a short hydraulic retention time of 1-2 days to allow for fermentation. Separating hydrolysis and the initial fermentation stage can reduce accumulation of fermentation intermediates and can allow for the separate optimization of the fermenting and methanogenic microbial communities.

The pH of the digestate was relatively stable throughout operation and ranged from 7.34 to 7.68 (**Fig. 1A**), indicating that the digester maintained a near-neutral pH appropriate for stable biogas production. The pH in the hydrolysis tank was maintained at low levels of 3.6 to 4.4. This is lower than typical "acid-stage" digestion processes that are maintained at a pH near 5.5 (Janesch et al., 2021). Although a stable pH was

maintained in the anaerobic digester, the VFA concentration of the digestate ranged from 1,242 to 3,833 mg L^{-1} (**Fig. 1B**). The VFA concentration gradually increased after inoculum sludge addition and steadily decreased after food waste addition from the hydrolysis tank. Around day 320, the VFA concentration experienced a sudden increase before steadily decreasing again. The reason for the spike is unknown, but may have been a result of non-farm substrates fed to the digester. Past studies have found that acetic acid concentrations of 2,400 mg L^{-1} were not significantly inhibitory to methanogens with the optimum acetic acid concentration being 1,600 mg L^{-1} (Wang et al., 2009). After the spike at Day 320, the VFA concentration decreased and was consistently less than 2,500 mg L^{-1}.

The organic loading rate (OLR) to the digester increased steadily throughout the course of the study, specifically after food waste addition (**Fig. 1C**). The OLR is a critical operational parameter that impacts reactor stability and microbial activity (Duan et al., 2019). Typically, a range of 0.5 to 5 kg VS d^{-1} is recommended and the OLR to the digester never exceeded the recommended maximum of 5 kg VS d^{-1} (Gautam et al., 2022). While increased OLR can produce higher biogas production rates, it risks inhibition and acidification due to overloading of organic matter (Gautam et al., 2022).

Another important parameter is the methane composition of biogas (**Fig. 1D**). Higher methane content increases the energy value of biogas and makes it easier to treat biogas for injection into a natural gas pipeline. The percent methane ranged from 52.5% to 70.7% (**Fig. 1D**). This is greater than other full scale anaerobic digesters co-digesting cow manure and wastes from the food, dairy, and beverage industry which reported

average methane percentages of 56.9-57.7% (Calbry-Muzyka et al., 2022) and from lab-scale reactors co-digesting cow manure and food waste which reported percentages of 57-69% (El-Mashad and Zhang, 2010). Percent methane reached a peak immediately after inoculum sludge addition before decreasing and again increasing over the coming weeks. After food waste was added, percent methane began to stabilize around 65%.

In total, the full-scale anaerobic digester maintained a stable pH throughout the first year of operation. This stable pH is likely attributable to the use of cow manure as a major substrate, which has a known capacity to buffer anaerobic digestion processes (Acosta et al., 2021). The OLR was steadily increased over the course of one year, from 0 to 3.5 kg VS d^{-1}. While VFA concentrations increased immediately after food waste addition, they steadily decreased over time even while the OLR increased. Initially fluctuating, the methane composition of biogas stabilized to 65%, which is higher than other reported full-scale digesters co-digesting cow manure and organic wastes. The pH of the hydrolysis tank was lower than typically used during the "acid-stage" of a two-stage digestion process, but this low pH did not impact the pH in the anaerobic digester.

Taxonomy of metagenomic reads

The abundance and make up of the microbial communities involved in anaerobic digestion can be impacted by process parameters and can determine process performance (Chen et al., 2016; Wang et al., 2018). A metagenomic approach was used to assess taxonomy of the microbial communities at multiple classification levels for the anaerobic digester, hydrolysis tank, and seed sludge. Initially, reads were classified at the phylum

level (**Fig. 2**). Across all samples, a total of 316,361,251 reads were mapped, which accounts for 83% of the total reads. Information on the total reads and mapped reads for each sample, as well as assembly statistics, can be found in Supplemental Materials. Bacteria was the dominant kingdom in all sampling locations, accounting for 50-70% of the digester, 24-75% of the hydrolysis tank, and 77% of the inoculum sludge DNA sequences. Archaea accounted for 5.21-23.59% of the digester and 5.08-5.17% of the inoculum sludge DNA sequences. Past studies have found the median "*Bacteria/Archaea"* ratio to be 14 across 18 experiments with 134 total samples (Campanaro et al., 2020). The median "*Bacteria/Archaea"* ratio in the digester and inoculum sludge was 3.8 and 15 respectively. This suggests a high abundance of archaea (Euryarchaeota) in the anaerobic digester relative to other full-scale digesters and a much higher abundance of archaea in the anaerobic digester compared to the inoculum seed sludge. Further, the hydrolysis tank was dominated by Bacteria, but one sample (Day 280) showed a sharp increase in archaea. This may be due to a substrate containing high amounts of archaea. While phyla-level classification provides an overview of abundant organisms, we also explored the genus-level classification of reads for both bacteria and archaea.

Bacterial Community

Bacterial DNA reads were classified at the genus level (**Fig. 3**). While separate genera were identified, most belong to "unclassified" genera based on NCBI Genbank annotations, suggesting these organisms belong to unisolated and/ or poorly-studied genera. Prior to inoculation, the most abundant bacterial genera consisted of *unclassified*

Gammaproteobacteria (0.13-7.38%), *unclassified Bacteria* (3.65-5.28%), *unclassified Spirochaetes* (3.14-4.21%), and *Fibrobacter* (2.68-7.53%). After Day 9, *unclassified Gammaproteobacteria* decreased significantly while *Fibrobacter* increased significantly. After inoculation, the *unclassified Spirochaetes* decreased.

Following inoculation and prior to food waste addition from the hydrolysis tank, several genera experienced a large increase in relative abundance. The abundance of *Bacteroidales bacterium* increased from 2.55-2.75% to 3.97-18.07% after inoculation. The relative abundance of this same *Bacteroidales bacterium* in the inoculum sludge was 6.29-7.33% which may explain the enhancement of the genus after inoculation. Similarly, the relative abundance of a *Firmicutes bacterium* which ranged from 0.10-0.29% prior to inoculation increased to 0.40-11.94% after inoculation. The relative abundance of this *Firmicutes bacterium* in the inoculum sludge was 2.68-3.03%. *Bacteroidetes* and *Firmicutes* have been found to be the most abundant bacterial phyla in many studies of anaerobic digesters (Chen et al., 2016; Mata-Alvarez et al., 2014; Xing et al., 2020), specifically studies co-digesting manure and food waste (Kim et al., 2022). *Bacteroidetes* and *Firmicutes* have been linked to parameters such as organic loading rate, volatile fatty acid concentration, and methane production (Chen et al., 2016). It has been reported that *Firmicutes* tend to dominate the community during stable performance at low organic loading rates while *Bacteroidetes* increase in abundance at higher loading rates (Chen et al., 2016). *Firmicutes* contain syntrophic bacteria which can degrade VFAs and produce H_2 which is used by hydrogenotrophic methanogens (Gannoun et al., 2016). On the other hand, *Bacteroidetes* contain proteolytic bacteria which produce acetate from amino acids

(Gannoun et al., 2016). Both phyla contain organisms that can ferment carbohydrates to a variety of end products.

Following the addition of food waste from the hydrolysis tank, the relative abundance of *Firmicutes bacterium* began to decrease overtime (1.55-14.13%) in the AD. The relative abundance of an unclassified *Proteobacteria* experienced a steady and significant increase within the range 2.01-15.75% whereas *Candidatus Cloacimonetes bacterium* (0.20-11.16%) increased after hydrolysis tank substrate addition before steadily decreasing. Throughout operation, the relative abundance of genera classified as an *unclassified Bacteroidales* and *an unclassified Bacteria* remained stable while *Bacteroidales bacterium* was dominant. After feeding from the hydrolysis tank, the bacterial community in the anaerobic digester remained largely the same. The hydrolysis tank bacterial community was dominated by organisms known to ferment carbohydrates to lactate and acetate, including *Lactobacillus*, *Limosilactobacillus*, *Prevotella*, and *Bifidobacterium* under low-pH conditions (Accetto and Avguštin, 2019; Watson et al., 2013). None of the abundant genera within the hydrolysis tank persisted in the anaerobic digester, suggesting the anaerobic digester bacterial community was resistant to migration from the hydrolysis tank, likely due to the higher pH and longer retention time.

Archaeal Community

The composition of archaeal genera with relative abundances of at least 0.25% in any given sample from the anaerobic digester and seed sludge inoculum were analyzed (**Fig. 4**) . All other archaeal genera were grouped into the 'other' category. Prior to inoculation, the most abundant archaeal genera were *Methanocorpusculum* (3.42-4.87%),

unclassified Methanomicrobiales (1.20-2.28%), and *Methanoculleus* (0.67-2.03%), all of which belong to the Methanomicrobiales order which are known to be hydrogenotrophic methanogens (Angelidaki et al., 2011). *Methanocorpusculum* has been found in similar environments including cattle slurry and manure (Goux et al., 2016; Habtewold et al., 2018; Yamamoto et al., 2011).

After inoculation, the relative abundance of the *unclassified Methanocorpusculum* in the digester decreased and was not highly abundant in the inoculum sludge. The most abundant genera in the inoculum sludge were *Methanoculleus* (2.37-2.79%) and an *unclassified Methanomicrobiales* (0.81-0.97%). Both genera were dominant in the digester prior to inoculation, but experienced an increase in relative abundance after inoculation and were dominant throughout operation. Specifically, the relative abundance of *Methanoculleus* and the *unclassified Methanomicrobiales* ranged between 2.30-5.65% and 1.12-4.74% after inoculation, respectively. Both the *Methanoculleus* and the *unclassified Methanomicrobiales* genera belong to the Methanomicrobiales order. Microorganisms belonging to the Methanomicrobiales order are hydrogenotrophic methanogens which are known to be more resistant to environmental stresses than other methanogens. Specifically, the *Methanoculleus* genus is known to utilize H_2 or formate and CO_2 for methanogenesis and has been found to resist ammonia toxicity (Abid et al., 2021). Following the addition of effluent from the hydrolysis tank, the relative abundance of an unclassified *Methanomicrobiales* (0.28-11.68%) and *Methanoculleus* (0.18-8.00%) increased significantly. However, the most abundant archaeal genera did not change.

In total, the taxonomic analyses reveal a stable methanogenic microbial community dominated by the *Methanoculleus* and an unclassified *Methanomicrobiales* genera. After inoculation, the *Methanocorpusculum* genera decreased in abundance and the overall abundance of methanogenic archaea decreased drastically, suggesting that inoculation did not enhance the methanogenic community. After feeding the anaerobic digester with effluent from the hydrolysis tank, the abundance of methanogens increased and the microbial community make-up was consistent throughout operation.

Relationship between taxa and digester operations.

To assess the relationship between key operational parameters (**Fig. 1**) and abundant genera (**Figs. 3 and 4**) within the anaerobic digester, Spearman correlation coefficients were calculated using a *p*-value of 0.01 (**Fig. 5**). Of the statistically significant results among the most abundant bacterial genera and digester operational parameters, there was significant variation in correlation. Few bacterial genera had a statistically significant correlation with pH. Of the genera that were significant, all were negatively correlated with pH except for an *unclassified Proteobacteria* which had a positive correlation. Many bacterial genera were negatively correlated with VFA concentration, specifically an unclassified *Proteobacteria*, *Candidatus Cloacimonetes bacterium*, an unclassified *Bacteroidetes*, an unclassified *Planctomycetes*, *Phycisphaerae bacterium*, and an unclassified *Clostridiaceae* while many others were positively correlated with VFA concentration. Unclassified *Proteobacteria* had the most negative correlation while *Firmicutes bacterium* and *Clostridiaceae bacterium* had the most positive correlation. Past studies have shown a positive correlation between VFA

production and the relative abundance of organisms from the *Firmicutes* phylum (Atasoy et al., 2019). Many bacterial genera had statistically significant correlations with OLR. Specifically, an unclassified *Planctomycetes* had the most positive correlation and an unclassified *Bacteroidia* had the most negative correlation with OLR.

Of the statistically significant results among the most abundant archaeal genera, all had a positive correlation with pH and a negative correlation with VFA concentration, except for *Methanocorpusculum* which had a positive correlation with VFA concentration. All genera except for *Methanocorpusculum* and *Thermoplasmatales archaeon* had a positive correlation with OLR. The relative abundance of *Methanocorpusculum* decreased significantly after inoculation. Therefore, the negative correlation between the relative abundance of *Methanocorpusculum* and OLR may be explained by the lack of *Methanocorpusculum* in the inoculum sludge. The most abundant archaeal genera all had a positive correlation with percent methane except for *Methanocorpusculum*. This may be explained by the percent methane increasing after inoculation and the relative abundance of *Methanocorpusculum* decreasing after introduction of seed sludge. Overall, the most abundant archaeal genera increased with pH and OLR and decreased with VFA concentration. This suggests VFA accumulation did inhibit methanogen growth, while higher pH promoted methanogen growth. The fact that methanogen abundance increased with the OLR suggests that the OLR was increased slowly enough for the methanogenic community to adapt to increased rates of substrate feeding.

Gene Abundance

While the taxonomic analyses were able to identify changes in genus-level taxa during start-up, the genera identified are largely related to unisolated and uncharacterized genera. Therefore, the function of the abundant organisms can not be ascertained based on taxonomy alone. In addition to assessing taxonomic abundance, we investigated the abundance of functional genes related to key metabolic processes of anaerobic digestion including hydrolysis, fermentation, VFA production, hydrogen production, and methanogenesis.

Hydrolysis of complex carbohydrates and proteins

During hydrolysis, organic chemical polymers (e.g., complex carbohydrates, proteins, nucleic acids, and lipids) are broken down into monomers (e.g., sugars, amino acids, nucleotides, and fatty acids). Hydrolysis depends on microorganisms that produce extracellular and intracellular enzymes that cleave the chemical bonds between monomeric units of a polymer and is often the rate limiting step of anaerobic digestion (Mata-Alvarez et al., 2014; Xing et al., 2020). Because complex carbohydrates and proteins are expected to be major components of cow manure and other organic wastes fed to the anaerobic digester, we investigated the most abundant genes for protein-cleaving enzymes (peptidases) and complex carbohydrate degrading enzymes (glycosylases). Among the glycosylase-encoding genes, beta-galactosidase (K01190), beta-glucosidase (K05349), and endoglucanase (K01179) were the most abundant within the AD. Beta-galactosidase and beta-glucosidase hydrolyze terminal galactose and glucose monomers, respectively, from complex carbohydrates and endoglucanase cleave

internal sugar monomers (Gomes et al., 2000). Protein products of these three most abundant genes have been identified in anaerobic microbiomes degrading lignocellulosic materials (Chirania et al., 2022). The high abundance of cellulose-related degradation genes in the AD reactor is likely due to the high abundance of cellulosic material expected in manure, which is fed directly to the AD. The HT metagenomes showed similar abundant glycosylases, but there was higher variation between abundant glycosylases, suggesting gene abundance may have varied with differing complex feedstocks while abundance of glycosylases in the AD were more stable. Within the HT, genes encoding lysozyme (K01185) were at times very highly abundant (TPM > 3000) between Day 266 and Day 364. Lysozyme is involved in lysis of cell membranes, suggesting that microbial biomass may be abundant in some substrates (Lesnierowski and Kijowski, 2007). Genes encoding this enzyme were not present in the AD.

A variety of peptidase genes were also found (**Fig. 6**). The most abundant peptidase genes in the AD include a gene encoding peptidoglycan DL-protease (K21471) that is involved in the degradation of peptidoglycan, an abundant cell wall component of gram positive bacteria (Shin et al., 2020). These genes may be involved with the degradation of bacterial biomass grown in the hydrolysis tank, which is rich in gram-positive Firmicutes (**Fig. 2**). Genes encoding this enzyme were also highly abundant in the HT. ATP-dependent Lon protease genes were highly abundant in the AD but not typically abundant in the HT. These genes are largely involved in intracellular housekeeping and degrading mis-folded or damaged proteins. Genes that encode peptidases likely involved in degradation of extracellular proteins and polypeptides, such

as dipeptidase (K08659), amino peptidase N (K01256), and endopeptidase (K07386) were more abundant in the HT reactor. This is likely due to protein-rich food waste feedstocks being fed to the HT.

Fermentation of sugars and amino acids

Once carbohydrates and proteins are hydrolyzed, sugar and amino acid monomers are expected to be fermented. Therefore, we assessed the abundance of genes related to sugar and amino acid fermentation within the AD and HT (**Fig. 7**). Within the AD, genes encoding both glucokinase (glk; K00845) and xylulokinase (xylB; K00854) were highly abundant, suggesting both five- and six-carbon sugars were being fermented. The HT also had a high abundance of glucokinase and xylulokinase, but, unlike the AD, genes encoding phosphoketolase (xfp; K01621) were also highly abundant. This gene is diagnostic of the "Bifid shunt," a heterofermentative pathway that converts sugars to equimolar amounts of lactate and acetate and enables additional ATP conservation than conventional glycolysis and the pentose phosphate pathway (Gupta et al., 2017). Abundant amino acid degradation genes differed between the AD and HT. Among the amino acid degradation genes in the AD, genes involved in lysine (kamA; K01843) and histidine (hutH; K01843) degradation were most abundant and these genes were largely absent from the HT. In the HT, genes involved in arginine degradation (arcA; K01478) were more abundant than in the AD.

Carbohydrates and amino acids are expected to be directed into key intracellular intermediates (e.g., pyruvate, acetyl-CoA) that are further converted to fermentation products. We investigated the abundance of genes involved in the formation of a variety

of fermentation products, including lactate, acetate, ethanol, propionate, and butyrate. Within the HT, genes involved in lactate production (ldh; K00016) and ethanol production (adh; K00925) were highly abundant compared to the AD. The terminal gene for acetate production - acetate kinase - was abundant in both the AD and HT, but this gene also encodes the enzyme for acetate consumption during acetoclastic methanogenesis. Genes involved in the methyl-branch (aka, Eastern branch) of the Wood Ljungdahl pathway showed similar abundance within the HT and AD and many were absent. Interestingly, genes involved in propionate production via the acryloyl-CoA or methylmalonyl-CoA pathways were not highly abundant in either the AD or HT, suggesting propionate was not a major intermediate within the HT or AD. To investigate butyrate production, all genes of the reverse beta-oxidation pathway were investigated. In the AD, genes encoding butyryl-CoA dehydrogenase (bcd; K00248) were abundant, along with electron transfer flavoproteins (etfA; etfB; K03522; K03521) that form an electron-bifurcating enzyme complex involved in reverse β-oxidation (i.e., production of butyrate) (Demmer et al., 2017).

Routes of pyruvate oxidation, energy conservation, and redox balancing

Pyruvate oxidation pathways, energy conservation mechanisms, and hydrogen-generating enzymes all play an important role in redox balancing and ATP production in anaerobic microorganisms. Pyruvate oxidation genes in the AD were dominated by pyruvate ferredoxin/ flavodoxin oxidoreductase (por; K03737) (**Fig. 8**). This enzyme passes electrons from pyruvate to oxidized ferredoxin, resulting in production of highly electronegative reduced ferredoxin. Reduced ferredoxin can be used to conserve energy

via the RNF complex (Buckel and Thauer, 2018) and the genes encoding this enzyme complex (RnfABCDEG) were also enriched in the AD. For hydrogen production and/or consumption, genes encoding the Hnd complex were most highly abundant, especially those for subunits hndD and hndC. This hydrogenase complex is an electron bifurcating complex that relies on both NADH and ferredoxin to reduce H^+ to H_2 when producing H_2 and produces reduced ferredoxin and NADH when consuming H_2 (Kpebe et al., 2018). Genes encoding Hnd were the most abundant hydrogenase encoding genes in the metagenomes. Within the HT, genes encoding components of a NAD^+-reducing pyruvate dehydrogenase were more highly abundant than pyruvate oxidation genes producing ferredoxin. Further, genes encoding the RNF complex in the HT were much less abundant than in the AD and hydrogen-producing genes were less abundant in the HT than the AD. In total, this suggests that the HT microbial community is unlikely to produce H_2 as a major fermentation intermediate. Taken together with the high abundance of the phosphoketolase enzyme (xfp; K01621) (**Fig. 7**) in the HT, this suggests that acetate production is not coupled with H_2 production. Further, genes encoding ferredoxin-producing and -utilizing energy conserving mechanisms such as Por, Hnd and the RNF complex are much more abundant in the AD samples.

Methanogenesis

Methane production is achieved through multiple routes, with acetoclastic and hydrogenotrophic methanogenesis regarded as the most common during anaerobic digestion. Taxonomic analysis revealed an abundance of archaea in the AD that are closely related to known hydrogenotrophs, such as Methanomicrobiales. To further assess

the routes of methane production, we investigated the abundance of genes related to the two expected routes of methane production (**Fig. 9**). Genes were grouped into three categories: (1) associated with acetoclastic methanogenesis; (2) associated with both acetoclastic and hydrogenotrophic methanogenesis; and (3) associated with hydrogenotrophic methanogenesis.

In acetoclastic methanogenesis, acetate is first converted to acetyl-CoA via three possible routes: a two step pathway with acetate kinase (ackA) and phospho-acetyltransferase (pta); a single-step ADP forming acetate-CoA ligase (acdA and acdB); and an AMP forming acetate-CoA ligase (acs). The first two pathways require one mole of ATP per mole acetate, while the last requires the equivalent of two moles of ATP per mol acetate. Genes predicted to encode acetyl-CoA synthetase (acs), are highly abundant in the AD across the entire start-up phase, while genes for acdA and acdB are less abundant. Genes for acetate kinase (ackA) and phospho-acetyltransferase (pta) are not abundant. Genomes for species within *Methanosarcina* are the only known archaea to contain ackA and pta (Fournier and Gogarten, 2008), and this genus was not highly abundant (< 1%) in the anaerobic digester (**Fig. 4**). *Methanothrix*, another known acetoclastic methanogen, which was also present at low abundance has been shown to use an acetyl-CoA synthetase to convert acetate to acetyl-CoA (Teh and Zinder, 1992).

After conversion of acetate to acetyl-CoA, a methylated Co(I)corrinoid protein is produced along with carbon monoxide. This step can be catalyzed by multiple enzyme complexes with CdhC, CdhE, and CdhD forming an acetyl-CoA decarbonylase (i.e, converts acetyl-CoA to CO) and CdhA and AcsA forming the carbon monoxide

dehydrogenase (i.e., converts CO to CO_2). The subunit encoded by acsB may allow for a complex to form that combines all three steps of acetoclastic methanogenesis (Doukov et al., 2002). Genes encoding all these subunits were present in all of the AD samples with varying abundance.

Both hydrogenotrophic and acetoclastic methanogenesis rely on tetrahydromethanopterin S-methyltransferase (MtrABCDEFGH, E.C. 2.1.1.86), ethyl-coenzyme M reductase (McrABC, E.C. 2.8.4.1), and a heterodisulfide reduction reaction. The heterodisulfide reduction reaction can be catalyzed by five known enzyme complexes (Buan and Metcalf, 2010; Hedderich et al., 1990; Mander et al., 2004; Stojanowic et al., 2003). Genes were abundant for two: a complex containing HdrA2, HdrB2, HdrC2 (E.C. 1.8.98.4), and a complex containing HdrA2, HdrB2, HdrC2, MvhA, MvhD, and MvhG (E.C. 1.8.98.5). The key difference between these complexes is that the complex containing Mvh can directly accept electrons from H_2, while the HdrA2B2C2 complex without Mvh relies on coenzyme F420 as an intermediate electron carrier and HdrDE relies on Coenzymes B and M.

All genes needed for hydrogenotrophic methanogenesis are present across the AD samples. The first step of hydrogenotrophic methanogenesis is fixing CO_2 onto a methanofuran via formylmethanofuran:ferredoxin oxidoreductase (Fwd, E.C. 1.2.7.12). This enzyme complex contains 5 subunits (FwdABCDE) and one iron-sulfur cluster of which three types have been identified: FwdF, FwdG, and FwdH. Both fwdF and fwdG were abundant, but fwdH was not. In the fourth step, a methenyl group is reduced to a methylene group via two possible routes. 5,10-methenyl tetrahydromethanopterin is

either reduced directly with H_2 via 5,10-methenyl tetrahydromethanopterin hydrogenase (hmd; E.C. 1.12.98.2) or with F420 via 5,10-methenyl tetrahydromethanopterin dehydrogenase (mtd; E.C. 1.5.98.1). In the AD metagenomes, mtd is the dominant gene, suggesting that F420 is an intermediate electron carrier for this reductive step.

In total, the metagenomic and taxonomic results suggest that hydrogenotrophic methanogenesis was the dominant route of methane production, but acetoclastic methanogenesis may have occurred as well. Although acs is highly abundant, the protein encoded by this gene requires the equivalent of two ATP to convert acetate to acetyl-CoA and would result in net consumption of ATP based on known acetoclastic methanogenesis pathways (Dyksma et al., 2020). Therefore, it is expected that acetoclastic methanogens do not use AMP-forming acs to form acetyl-CoA, but genes for ADP-producing routes were much less abundant. Given the high VFA concentration, it is expected that some acetate may also have been oxidized by syntrophic acetate oxidizing bacteria to form H_2 and CO_2 using the Wood Ljungdahl pathway; however, these genes were not highly abundant (**Fig. 7**) and only one taxa related to known syntrophic acetate oxidizers (**Fig. 3**, *Syntrophamonadaceae bacterium*) was identified and present at low abundance. Thus, despite the high abundance of genes for producing acetate in the HT, the ultimate route for conversion of acetate to methane in the AD remains unknown.

CONCLUSIONS

After addition of the inoculum seed sludge, the bacterial community experienced some changes, but the archaeal community was largely unchanged. Interestingly, the most abundant archaea within the seed sludge were already abundant in the anaerobic

digester prior to inoculation. After inoculation, *Methanocorpusculum* decreased but other Methanomicrobiales remained largely unchanged. None of the monitored digester parameters changed significantly after inoculation, suggesting that inoculation had little impact on the digester performance or abundant microbial community members. Likewise, the abundance of key genes involved in hydrolysis, fermentation, hydrogen production, and methanogenesis remained largely unchanged after inoculation. In total, this suggests that a resilient methanogenic microbial community had already taken hold in the anaerobic digester and inoculation had little impact on the overall process. This suggests that so long as a stable pH is maintained and the OLR is increased slowly, cow manure itself may be a suitable inoculum for cow manure and food waste co-digestion.

Differences in gene abundance across the HT and AD also suggest major differences in the fermentative pathways within these two reactors. First, the HT metagenomes contained abundant genes encoding the phosphoketolase enzyme. Second, genes encoding hydrogen-producing enzymes were much less abundant in the HT than the AD. Third, pyruvate-ferredoxin oxidoreductase (por) was much more abundant in the AD than the HT. Combined, these results suggest that acetate production in the HT is largely through the phosphoketolase pathway and is not coupled with H_2 production. For this specific system, this suggests that a major drawback of two-stage acid-gas digestion, electron loss due to H_2 generation in the first "acid-stage," may not be an issue (Janesch et al., 2021).

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FIGURE CAPTIONS

Figure 1. Operational parameters during the first 364 of operations. (A) pH for both the anaerobic digester and the hydrolysis tank; (B) organic loading rate to the anaerobic digester; (C) percent methane and carbon dioxide in the biogas; and (D) total concentration of volatile fatty acids.

Figure 2. Classification of metagenomic reads at the phylum level for (A) the anaerobic digester; (B) the hydrolysis tank; and (C) the inoculum seed sludge. All reads, including unmapped and unclassified reads, are included in these plots.

Figure 3. Classification of bacterial metagenomic reads at the genus level for (A) the anaerobic digester; (B) the hydrolysis tank; and (C) the inoculum seed sludge. Relative abundance calculations are based on the total reads, including archaeal reads, unclassified reads, and unmapped reads. The top 25 most abundant bacteria genera from each location are displayed. All other genera were grouped into the 'other' category.

Figure 4. Classification of archaeal metagenomic reads at the genus level for the anaerobic digester and the inoculum seed sludge. The composition of archaeal genera with relative abundances of at least 0.25% in at least one sample from each sampling location are shown. All other genera were grouped into the 'other' category. Relative abundance calculations are based on the total reads, including bacterial reads, unclassified reads, and unmapped reads.

Figure 5. Spearman coefficients for abundant genera and operational parameters. Red intensity indicates a negative correlation and blue intensity indicates a positive correlation. Coefficients with a p-value greater than 0.01 are marked with an X.

Figure 6. Abundance of genes related to hydrolysis, including glycosylases and peptidases. Shading intensity represents the TPM of each gene. A full list of glycosylases and peptidases included in the analysis are provided in Supplemental Materials. For clarity, only the 27 most abundant glycosylases and 38 most abundant peptidases are

included in the figure. KEGG and Enzyme Commission identification numbers are provided and the full names are available in Supplemental Materials.

Figure 7. Abundance of genes related to sugar degradation, amino acid degradation, simple fermentation (e.g., conversion of pyruvate to lactate, acetate, and ethanol), homoacetogenesis via the Wood Ljungdahl pathway, propionate production via the methylmalonyl-CoA and acrylyl-CoA pathways, and production of butyrate and other carboxylic acids via reverse β-oxidation. The genes for reverse β-oxidation may also be used for oxidation of fatty acids.

Figure 8. Abundance of additional genes involved in hydrogen production and consumption, pyruvate oxidation, and energy conservation via the RNF complex.

Figure 9. Abundance of genes involved in acetoclastic methanogenesis, hydrogenotrophic methanogenesis, and both types of methanogenesis in the anaerobic digester and inoculum seed sludge.

FIGURES

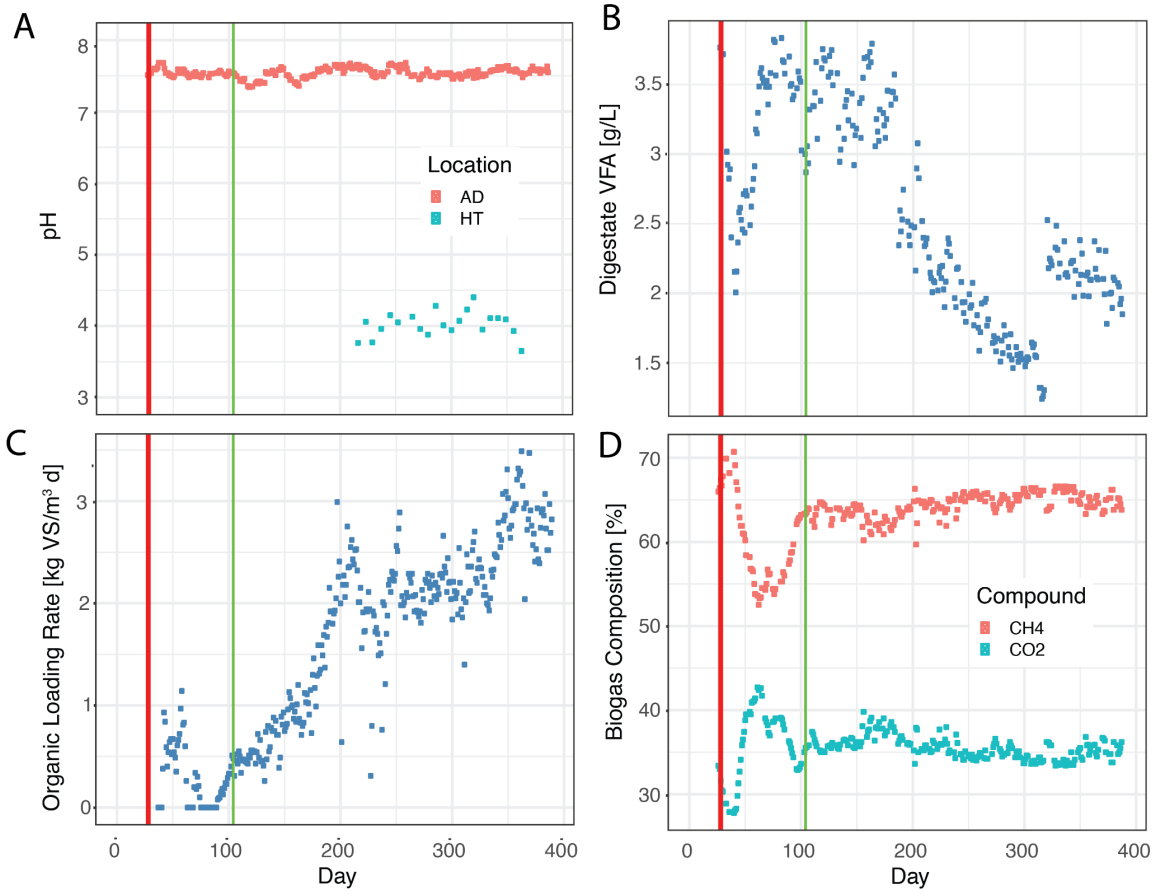

Figure 1. Operational Parameters

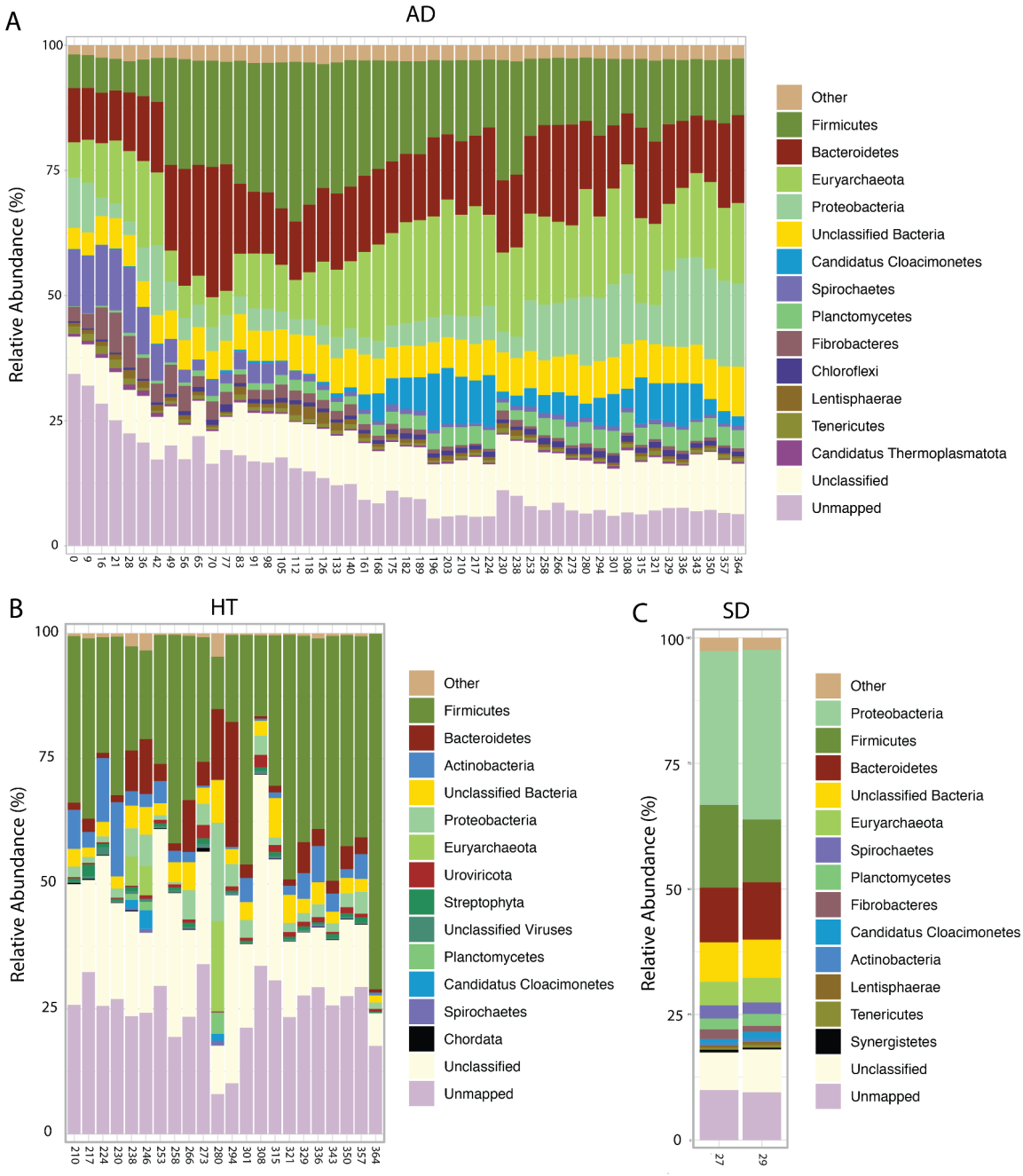

Figure 2. Taxonomic Classification at Phylum Level

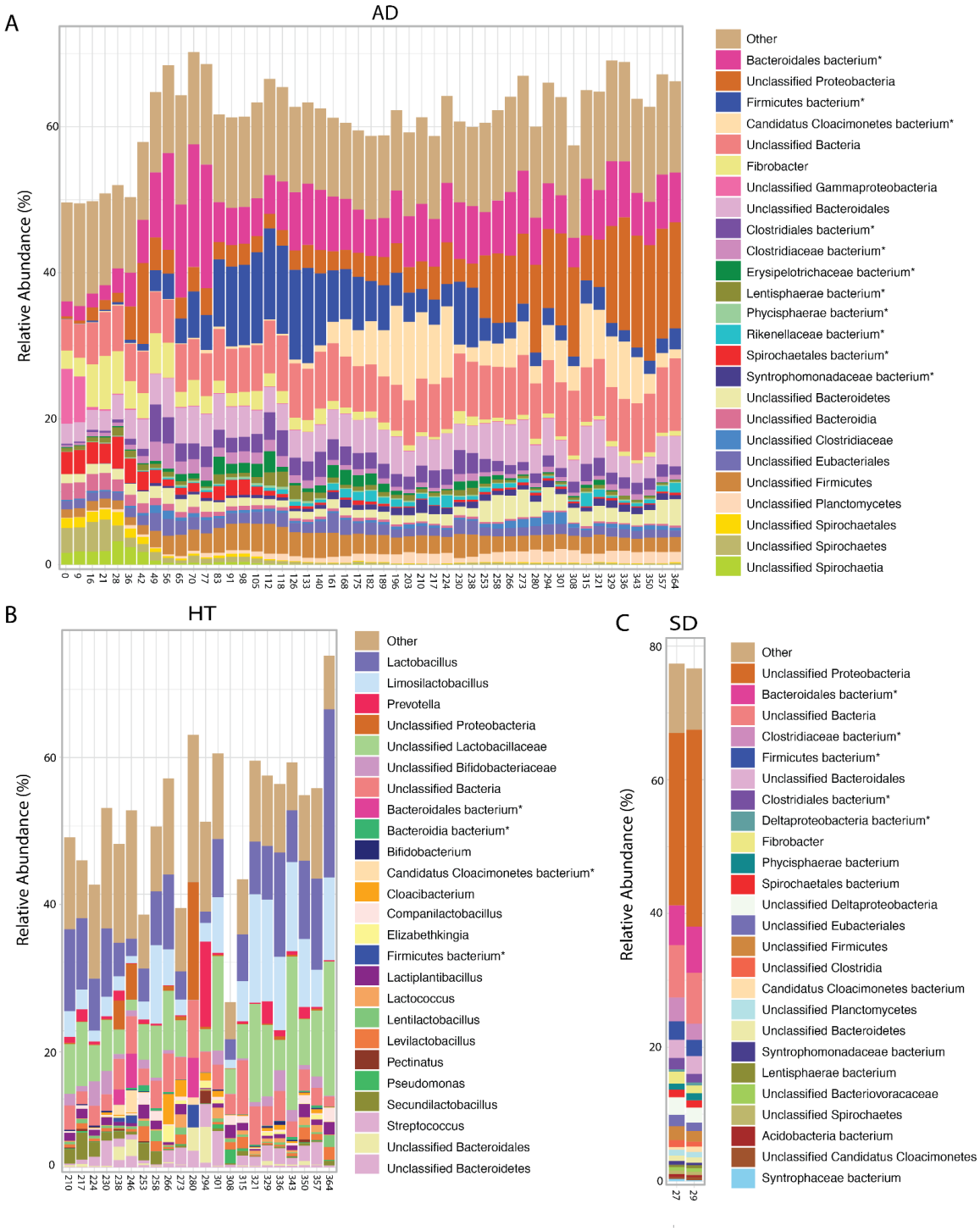

Figure 3. Taxonomic Classification at Genus Level for Bacterial Community

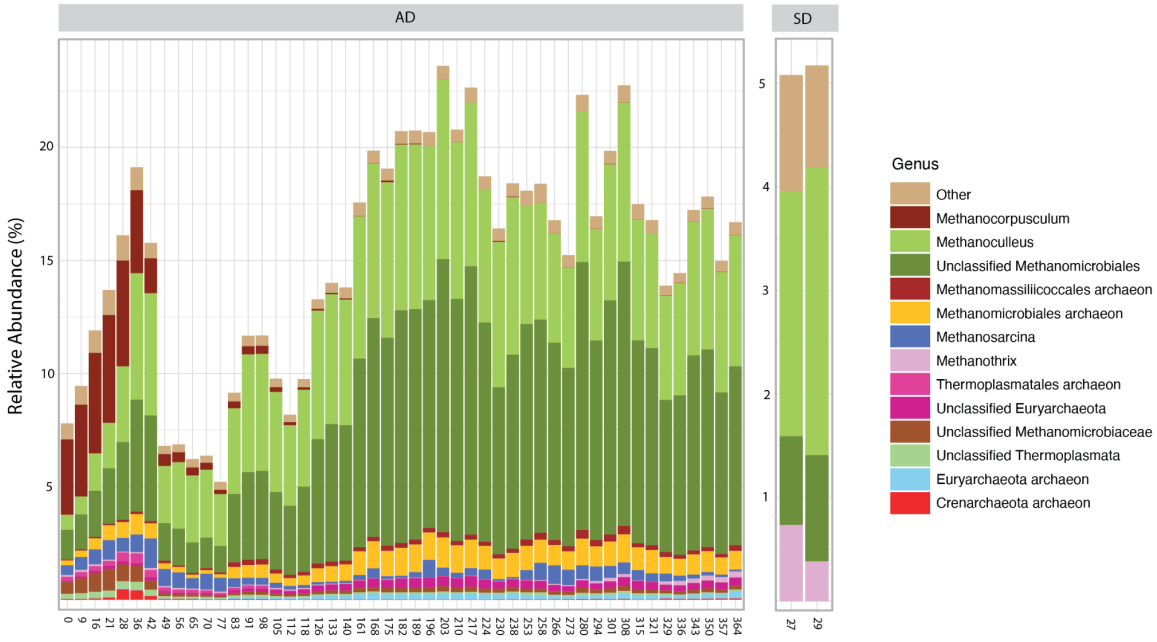

Figure 4. Taxonomic Classification at Genus Level for Archaeal Community

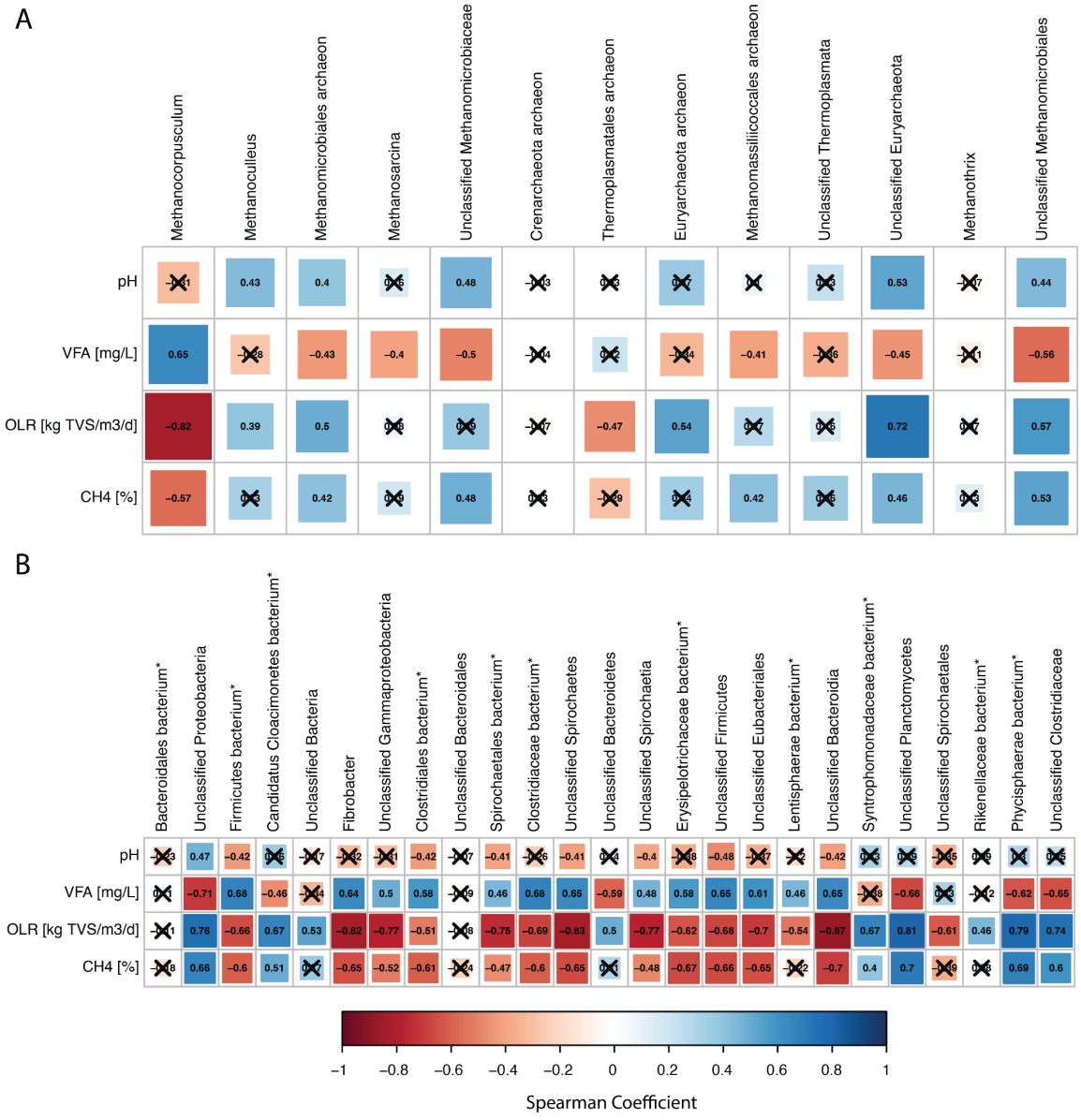

Figure 5. Correlation Plots for Abundant Genera and Operational Parameters

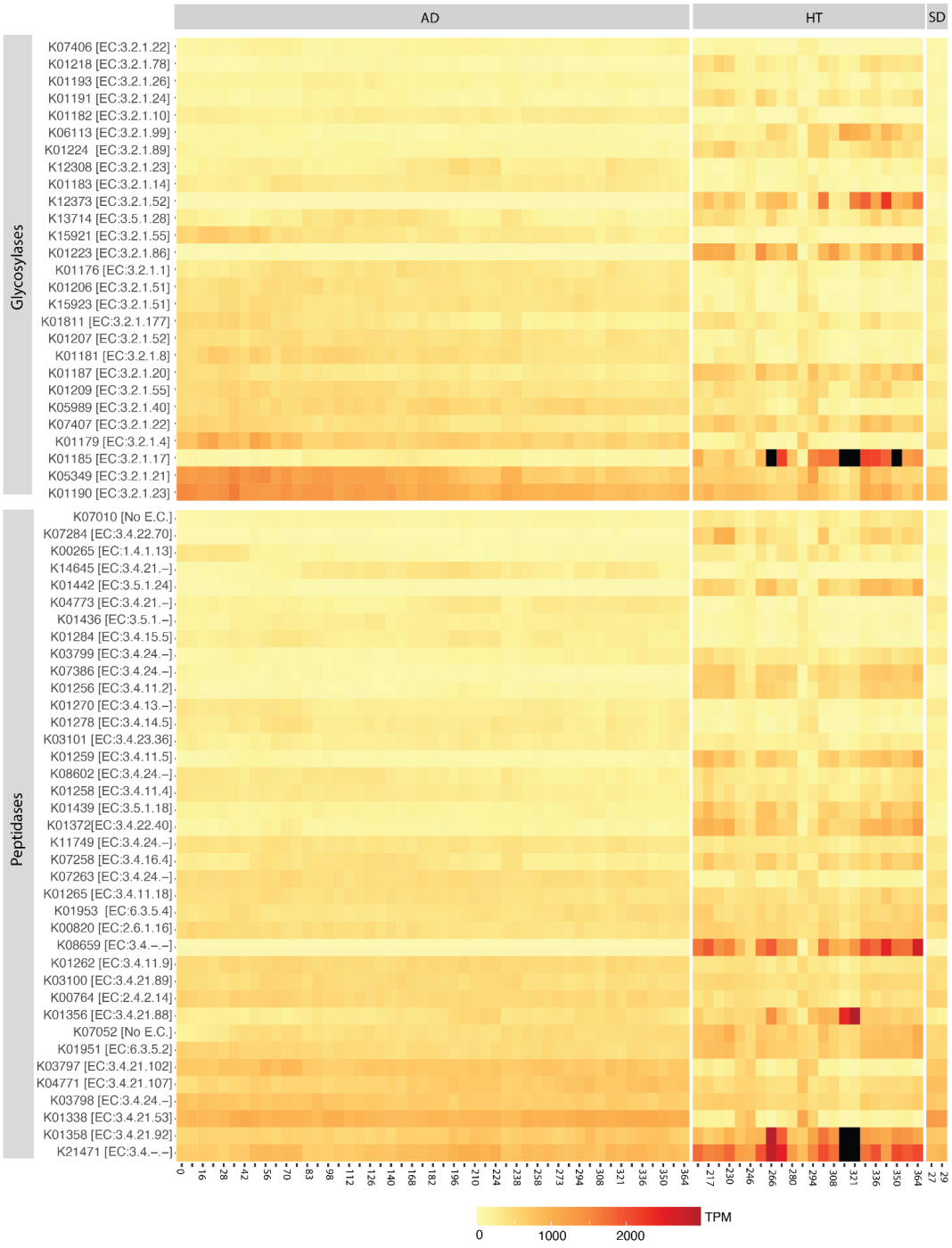

Figure 6. Hydrolysis Heat Map - Glycosylases & Peptidases

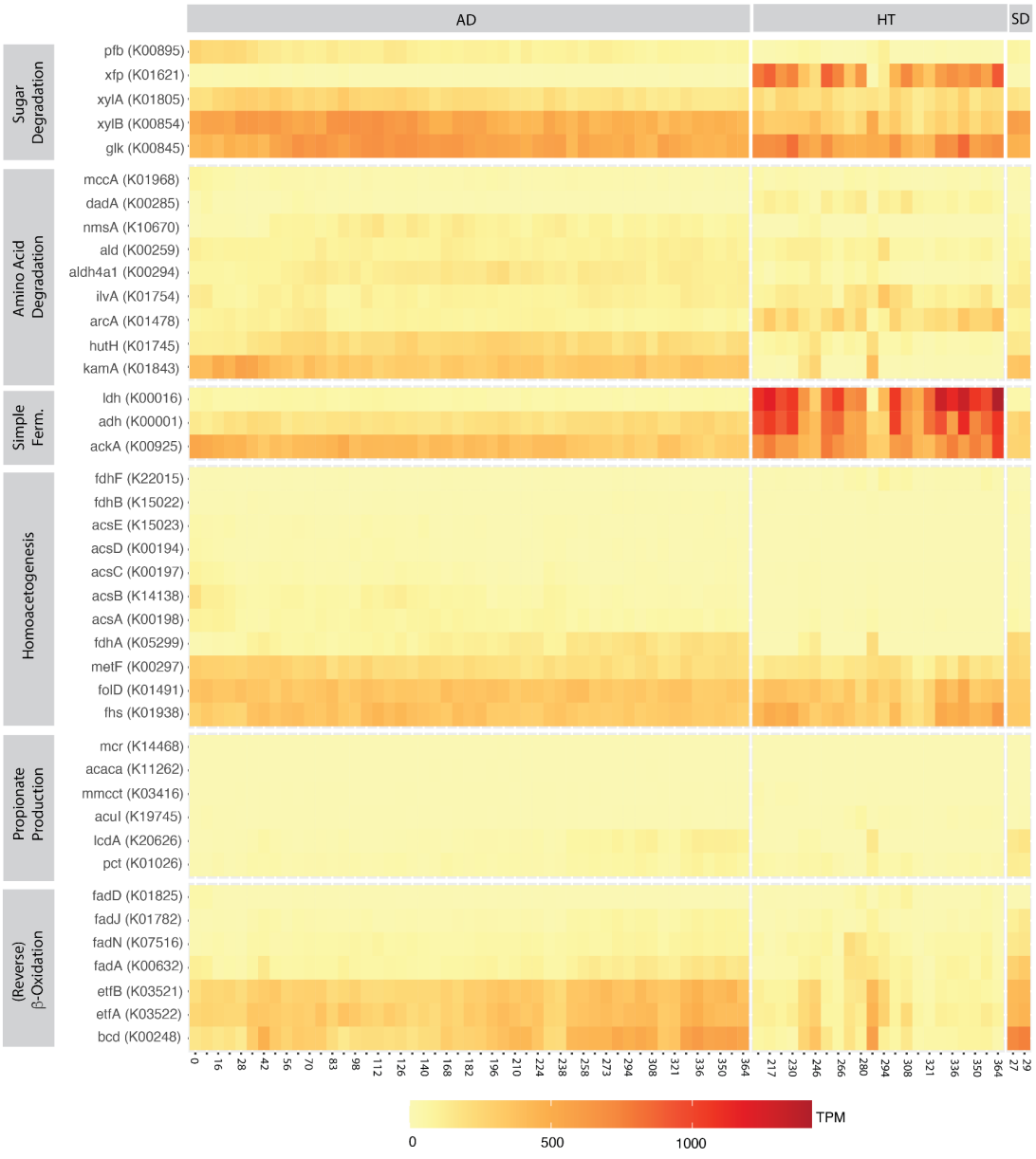

Figure 7. Fermentation Heat Map

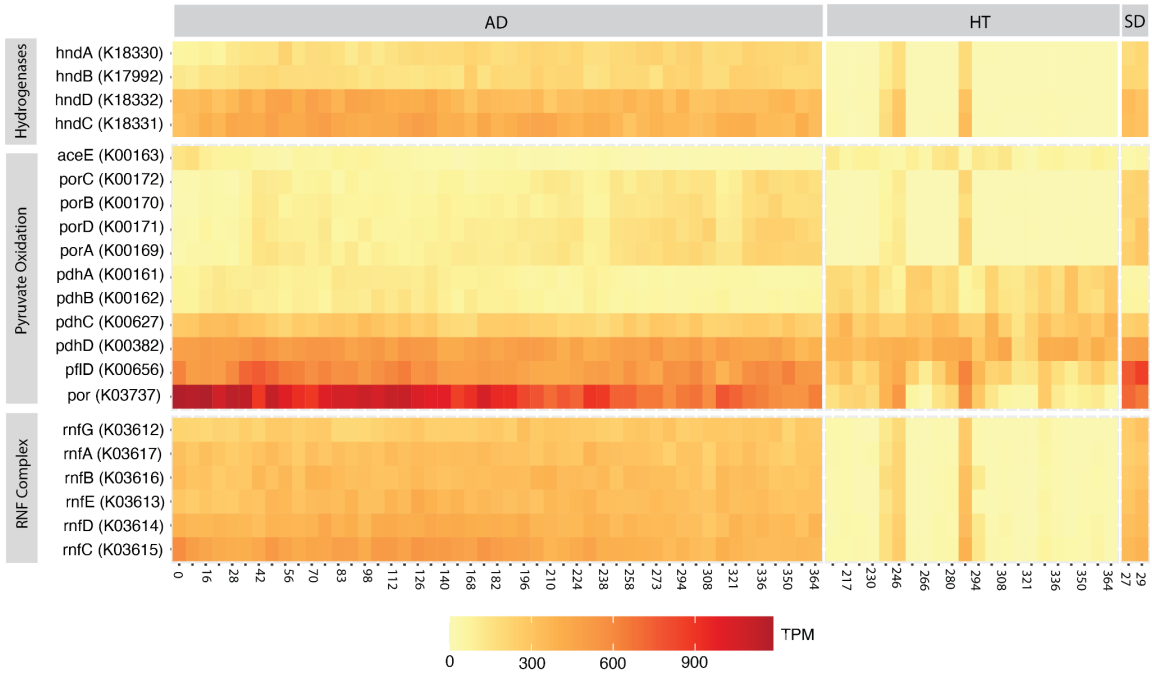

Figure 8. RNF Complex, Pyruvate Oxidation, & Hydrogenases Heat Map

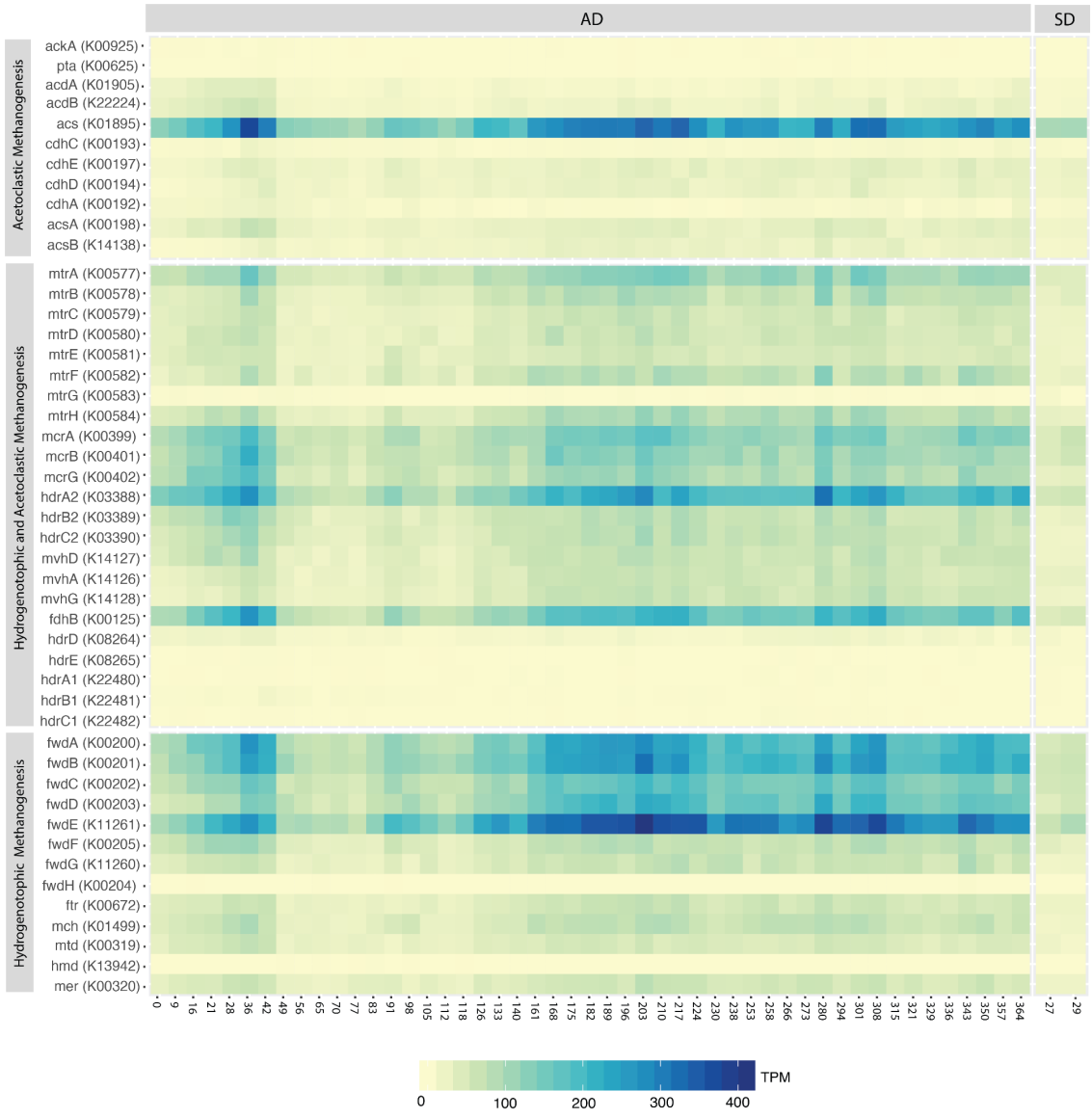

Figure 9. Methanogenesis Heat Map

SUPPLEMENTAL MATERIALS

Table 1. Sequencing Data for Anaerobic Digester Samples

Day	Total Reads	Mapped Reads	Percent Mapped (%)	Total bases	Day	Total Reads	Mapped Reads	Percent Mapped (%)	Total bases
0	6,592,816	4,329,287	65.67	854,637,697	189	16,484,452	14,940,105	90.63	2,210,353,209
9	4,669,406	3,174,460	67.98	628,999,456	196	3,402,658	3,216,165	94.52	482,630,220
16	4,771,922	3,417,472	71.62	641,429,316	203	4,581,460	4,311,054	94.10	643,339,612
21	6,469,062	4,844,096	74.88	876,261,767	210	4,307,360	4,043,860	93.88	602,017,195
28	2,703,220	2,095,739	77.53	378,184,714	217	3,869,276	3,643,963	94.18	549,704,098
36	5,385,028	4,272,213	79.34	713,366,715	224	4,443,418	4,180,504	94.08	624,710,580
42	3,710,790	3,070,309	82.74	526,067,829	230	5,326,470	4,735,252	88.90	757,172,097
49	2,890,282	2,311,095	79.96	390,976,708	238	4,646,640	4,181,754	90.00	664,798,619
56	3,662,508	3,028,776	82.70	518,425,712	253	3,861,228	3,554,887	92.07	530,373,320
65	4,173,742	3,258,049	78.06	585,090,880	258	4,185,266	3,887,033	92.87	580,414,863
70	6,668,866	5,574,743	83.59	927,586,751	266	5,155,874	4,710,176	91.36	674,251,508
77	4,659,220	3,766,056	80.83	643,722,073	273	4,473,352	4,156,573	92.92	617,953,093
83	3,987,162	3,266,439	81.92	528,452,477	280	2,913,056	2,725,926	93.58	404,660,691
91	1,310,530	1,089,219	83.11	182,440,389	294	3,729,108	3,461,108	92.81	519,149,660
98	1,785,066	1,487,966	83.36	248,200,143	301	6,448,568	6,061,569	94.00	907,746,259
105	2,542,930	2,093,274	82.32	346,247,819	308	3,942,156	3,677,901	93.30	556,178,363
112	4,454,836	3,762,483	84.46	612,815,923	315	2,507,222	2,349,415	93.71	359,376,843
118	3,424,090	2,914,902	85.13	471,984,214	321	3,944,674	3,666,057	92.94	564,598,483
126	3,523,262	3,045,842	86.45	481,429,068	329	7,551,416	6,982,198	92.46	1,023,250,197
133	3,236,190	2,844,350	87.89	461,181,227	336	4,300,466	3,972,600	92.38	581,921,745
140	3,356,762	2,942,326	87.65	476,670,220	343	2,539,386	2,363,506	93.07	353,481,548
161	5,073,900	4,607,440	90.81	709,637,347	350	3,984,782	3,697,929	92.80	548,853,152
168	2,747,072	2,513,719	91.51	379,505,484	357	3,431,768	3,205,224	93.40	487,746,403
175	6,283,586	5,589,480	88.95	841,000,293	364	5,359,570	5,018,899	93.64	749,200,643
182	21,083,642	19,035,548	90.29	2,800,763,892					

Table 2. Sequencing Data for Hydrolysis Tank Samples

Day	Sample Location	Total Reads	Mapped Reads	Percent Mapped (%)	Total bases
210	HT	5,870,864	4,355,394	74.19	741,531,610
217	HT	6,436,120	4,351,832	67.62	770,434,966
224	HT	5,921,736	4,404,549	74.38	708,829,577
230	HT	1,965,974	1,435,341	73.01	215,015,969
238	HT	8,362,730	6,392,779	76.44	995,203,646
246	HT	8,449,612	6,400,658	75.75	975,001,092
253	HT	5,095,724	3,588,025	70.41	559,710,702
266	HT	6,207,180	5,002,649	80.59	716,687,993
273	HT	4,287,398	3,282,393	76.56	525,176,283
280	HT	3,102,624	2,049,312	66.05	379,663,038
287	HT	6,635,568	6,106,095	92.02	857,309,262
294	HT	6,740,722	6,056,280	89.85	862,719,133
301	HT	5,411,562	4,260,751	78.73	632,755,888
308	HT	6,299,200	4,181,771	66.39	654,769,802
315	HT	10,800,464	7,484,919	69.30	1,143,083,096
321	HT	5,191,050	3,977,705	76.63	517,869,731
329	HT	10,306,078	7,451,308	72.30	1,086,698,398
336	HT	10,525,742	7,437,534	70.66	1,118,055,666
343	HT	4,566,150	3,392,896	74.31	513,319,831
350	HT	4,381,900	3,173,111	72.41	521,362,741
357	HT	6,230,848	4,399,144	70.60	702,507,402
364	HT	3,376,432	2,781,017	82.37	426,597,853

Table 3. Sequencing Data for Inoculum Seed Sludge Samples

Day	Sample Location	Total Reads	Mapped Reads	Percent Mapped (%)	Total bases
27	SD	6,279,290	5,653,919	90.04	882,807,120
29	SD	4,049,670	3,662,928	90.45	573,079,394

Table 4. Assembly Statistics

Number of contigs	6,375,882
Total length	2,467,908,761
Longest contig	516,062
Shortest contig	200
N50	343
N90	222
Contigs at superkingdom (k) rank	4,926,823 (77.3%), in 4 superkingdoms
Contigs at phylum (p) rank	4,437,607 (69.6%), in 210 phyla
Contigs at class (c) rank	3,454,819 (54.2%), in 207 classes
Contigs at order (o) rank	2,847,060 (44.7%), in 454 orders
Contigs at family (f) rank	1,879,798 (29.5%), in 889 families
Contigs at genus (g) rank	1,083,721 (17.0%), in 3131 genera
Contigs at species (s) rank	1,555,937 (24.4%), in 5636 species
Congruent	6,373,117 (100.0%)
Disparity >0	2,766 (0.0%)
Disparity >= 0.25	2,197 (0.0%)

Table 5. Glycosylase Genes

KEGG Number	Enzyme Commission ID	Full Name
K07406	3.2.1.22	alpha-galactosidase
K01218	3.2.1.78	mannan endo-1,4-beta-mannosidase
K01193	3.2.10.26	beta-fructofuranosidase
K01191	3.2.10.24	alpha-mannosidase
K00182	3.2.10.1	wingless-type MMTV integration site family, member 2
K06113	3.2.10.99	arabinan endo-1,5-alpha-L-arabinosidase
K01224	3.2.10.89	arabinogalactan endo-1,4-beta-galactosidase
K12308	3.2.10.23	beta-galactosidase
K01183	3.2.1.14	chitinase
K12373	3.2.1.52	hexosaminidase
K13714	3.2.1.28	bifunctional autolysin
K15921	3.2.1.55	arabinoxylan arabinofuranohydrolase
K01223	3.2.1.86	6-phospho-beta-glucosidase
K01176	3.2.1.1	alpha-amylase
K01206	3.2.1.51	alpha-L-fucosidase
K15923	3.2.1.51	alpha-L-fucosidase 2
K01811	3.2.1.177	alpha-D-xyloside xylohydrolase
K01207	3.2.1.52	beta-N-acetylhexosaminidase
K01181	3.2.1.8	endo-1,4-beta-xylanase
K01187	3.2.1.20	alpha-glucosidase
K01209	3.2.1.55	alpha-L-arabinofuranosidase
K05989	3.2.1.40	alpha-L-rhamnosidase
K07407	3.2.1.22	alpha-galactosidase
K01179	3.2.1.4	endoglucanase
K01185	3.2.1.17	lysozyme
K05349	3.2.1.21	beta-glucosidase
K01190	3.2.1.23	beta-galactosidase

Table 6. Peptidase Genes

KEGG ID	Enzyme Commission ID Number	Full Name	KEGG ID	Enzyme Commission ID Number	Full Name
K07010	No E.C.	putative glutamine amidotransferase	K11749	3.4.24.-	regulator of sigma E protease
K07284	3.4.22.70	sortase A	K07258	3.4.16.4	serine-type D-Ala-D-Ala carboxypeptidase (penicillin-binding protein 5/6)
K00265	1.4.1.13	glutamate synthase (NADPH) large chain	K07263	3.4.24.-	zinc protease
K14645	3.4.21.-	serine protease	K01265	3.4.11.18	methionyl aminopeptidase
K01442	3.5.1.24	choloylglycine hydrolase	K01953	6.3.5.4	asparagine synthase (glutamine-hydrolysing)
K04773	3.4.21.-	protease IV	K00820	2.6.1.16	glutamine---fructose-6-phosphate transaminase (isomerizing)
K01436	3.5.1.-	amidohydrolase	K08659	3.4.-.-	dipeptidase
K01284	3.4.15.5	peptidyl-dipeptidase Dcp	K01262	3.4.11.9	Xaa-Pro aminopeptidase
K03799	3.4.24.-	heat shock protein HtpX	K03100	3.4.21.89	signal peptidase I
K07386	3.4.24.-	putative endopeptidase	K00764	2.4.2.14	amidophosphoribosyltransferase
K01256	3.4.11.2	aminopeptidase N	K01356	3.4.21.88	repressor LexA
K01270	3.4.13.-	dipeptidase D	K07052	No E.C.	CAAX protease family protein
K01278	3.4.14.5	dipeptidyl-peptidase 4	K01951	6.3.5.2	GMP synthase (glutamine-hydrolysing)
K03101	3.4.23.36	signal peptidase II	K03797	3.4.21.102	carboxyl-terminal processing protease
K01259	3.4.11.5	proline iminopeptidase	K04771	3.4.21.107	serine protease Do
K08602	3.4.24.-	oligoendopeptidase F	K03798	3.4.24.-	cell division protease FtsH
K01258	3.4.11.4	tripeptide aminopeptidase	K01338	3.4.21.53	ATP-dependent Lon protease
K01439	3.5.1.18	succinyl-diaminopimelate desuccinylase	K01358	3.4.21.92	ATP-dependent Clp protease, protease subunit
K01372	3.4.22.40	bleomycin hydrolase	K21471	3.4.-.-	peptidoglycan DL-endopeptidase CwlO

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

In total, it was found that a resilient methanogenic microbial community was present in the anaerobic digester during start-up and throughout stable operation. Though the abundance of methanogenic archaea decreased after inoculation, the make-up of the community was largely unchanged as the most abundant archaea in the inoculum sludge were also the most abundant archaea in the anaerobic digester prior to inoculation, with the exception of *Methanocorpusculum* which decreased in abundance after inoculation. These most abundant methanogenic genera consisted of *Methanoculleus* and an *unclassified Methanomicrobiales*, both of which belong to the Methanomicrobiales order which consist of hydrogenotrophic methanogens. Similarly, the abundance of key genes involved in hydrolysis, fermentation, hydrogen production, and methanogenesis remained largely unchanged after inoculation. Given this stability within the microbial community prior to and after inoculation, cow manure may be a suitable inoculum for cow manure and food waste co-digestion without the need for external inoculum sludge, if a stable pH is maintained and OLR is increased slowly.

Differences in gene abundance across the hydrolysis tank and anaerobic digester suggest major differences in fermentative pathways within the two reactors. First, hydrolysis tank metagenomes contained abundant genes encoding the phosphoketolase enzyme. Second, genes encoding hydrogen-producing enzymes were much less abundant

in the hydrolysis tank than the anaerobic digester. Third, pyruvate-ferredoxin oxidoreductase (por) was much more abundant in the anaerobic digester than the hydrolysis tank. Overall, these results suggest that acetate production in the hydrolysis tank is largely through the phosphoketolase pathway and is not coupled with H_2 production. For this specific system, this suggests that a major drawback of two-stage acid-gas digestion, namely electron loss due to H_2 generation in the first "acid-stage", may not be an issue (Janesch et al., 2021).

RECOMMENDATIONS FOR FUTURE WORK

Raw metagenomic reads are to be made available through the National Center for Biotechnological Information (NCBI) sequencing reads archive. Results from this study are prepared as a manuscript for submission to Bioresource Technology to inform future strategies for successful start-up of full-scale anaerobic digesters.

Additional research is needed to verify the relationship between inoculation and the methanogenic microbial community, specifically for full-scale anaerobic digesters co-digesting cow manure and food waste. Though inoculation is known to increase the active methanogenic community, in this study the abundance of total methanogenic microbes decreased after inoculation, only to increase after co-digestion began.

This work used a "gene-centric" approach to identify abundant genes within the microbial community and assess how the abundance of these genes changed overtime. Future work with this dataset could include a "genome-centric" approach where contigs are binned into draft genomes that represent individual populations of organisms to assess the abundance of specific organisms and how these abundances changed overtime.

Initially, binning was performed which resulted in 283 bins. However, there were only 5 high-quality bins with a completeness greater than 90% and contamination less than 10%. Due to the poor binning statistics, the "genome-centric" approach was not pursued. Future work may include performing long-read (Nanopore) sequencing to improve the recovery of draft genomes; however, the work performed still shows the utility of using a "gene-centric" metagenomics approach with a relatively shallow sequencing depth for genomic and taxonomic analyses.

A significant amount of contigs went unclassified. An alternative way to perform classification and work towards a higher percentage of classified contigs would be to use the Genome Taxonomy Database (GTDB) (Parks et al., 2022) instead of the GenBank database. Taxonomy changes significantly over time; therefore, reclassification in the future may give more specific results than seen in this study. As these tools to study microbes improve, we should adapt them for environmental engineering applications.

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