
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHAPTER ONE

# The microbiome driving anaerobic digestion and microbial analysis

**Jun Wei Lim[a], Tansol Park[b], Yen Wah Tong[a,c], Zhongtang Yu[b,d,*]**

[a]NUS Environmental Research Institute, National University of Singapore, Singapore, Singapore
[b]Department of Animal Sciences, The Ohio State University, Columbus, OH, United States
[c]Department of Chemical & Biomolecular Engineering, National University of Singapore, Singapore, Singapore
[d]Environmental Science Graduate Program, The Ohio State University, Columbus, OH, United States
[*]Corresponding author: e-mail address: yu.226@osu.edu

## Contents



*Advances in Bioenergy*, Volume 5
ISSN 2468-0125
https://doi.org/10.1016/bs.aibe.2020.04.001

## Abstract

The microbiome residing in anaerobic digesters drives the anaerobic digestion (AD) process to convert various feedstocks to biogas as a renewable source of energy. This microbiome has been investigated in numerous studies in the last century. The early studies used cultivation-based methods and analysis to identify the four guilds (or functional groups) of microorganisms. Molecular biology techniques overcame the limitations of cultivation-based methods and allowed the identification of unculturable microorganisms, revealing the high diversity of microorganisms involved in AD. In the past decade, omics technologies, including metataxonomics, metagenomics, metatranscriptomics, metaproteomics, and metametabolomics, have been or start to be used in comprehensive analysis and studies of biogas-producing microbiomes. In this chapter, we reviewed the utilities and limitations of these analysis methods, techniques, and technologies when they were used in studies of biogas-producing microbiomes, as well as the new information on diversity, composition, metabolism, and syntrophic interactions of biogas-producing microbiomes. We also discussed the current knowledge gaps and the research needed to further improve AD efficiency and stability.

# 1. Introduction

## 1.1 A brief overview of the AD process and the guilds of microorganisms involved in AD

Biogas (comprising mainly of $CH_4$ and $CO_2$) produced through anaerobic digestion (AD) of various feedstocks (primarily organic wastes such as livestock manure, food wastes, sewage sludge, crop residues agricultural byproducts, and the organic fraction of municipal solid wastes (OFMSW)) is enabled by a complex community of microorganisms, or microbiome, present in AD bioreactors (or anaerobic digesters). The AD process can be conceptually divided into four phases: hydrolysis, acidogenesis, syntrophic acetogenesis, and methanogenesis (Lv et al., 2010). Each of these sequential phases is carried out by a unique functional group (or guild) of microorganisms. In the hydrolysis phase, polymeric substrates, primarily polysaccharides (cellulose, hemicellulose, starch), lipids, and proteins, are hydrolyzed by the

extracellular hydrolases (e.g., cellulase, xylanase, pectinase, amylase, lipase, and protease) secreted by hydrolytic bacteria, releasing monomers or oligomers, such as glucose and cellobiose from cellulose, glucose, and maltose from starch, xylose from hemicellulose, amino acids from proteins, and long-chain fatty acids (LCFA) and glycerol from lipids. The hydrolytic bacteria are phylogenetically diverse, but *Firmicutes* and *Bacteroides* are the two phyla containing most of the hydrolytic bacteria found in AD bioreactors. Hydrolytic bacteria, in general, can grow fast and are less sensitive to changes in environmental conditions, such as pH and temperature. Except for recalcitrant substrates, such as lignocellulose, the hydrolysis step is not rate-limiting in AD. All hydrolytic bacteria in AD bioreactors can utilize the hydrolysis products as growth substrates, primarily through fermentation, to produce short-chain fatty acids (SCFA).

The hydrolytic products are fermented to SCFA, with acetate, propionate, butyrate, valerate, and isobutyrate as the major SCFA, by acidogenic microorganisms (or acidogens, primarily bacteria) during acidogenesis. Carbon dioxide, hydrogen, ammonia, and sulfide are also produced during acidogenesis. Acetogens include both hydrolytic bacteria and fermentative bacteria that lack hydrolytic ability. *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, and *Atribacteria* are the major phyla that contain many species of acidogens reported in AD bioreactors. Acidogenesis is generally rapid, and it can cause accumulation of SCFA and concomitant sharp pH drop when AD bioreactors are overloaded with readily digestible feedstocks, such as food wastes. Accumulation of SCFA can cause upset or even failure of the AD process.

Acetate, formate, $H_2$, and $CO_2$, resulted from acidogenesis, can be directly utilized by methanogens for biogas production, but other acidogenesis products, including propionate, butyrate, isobutyrate, valerate, and isovalerate, cannot be utilized by any of the known methanogens. They need to be further degraded and transformed into the methanogenesis substrates through syntrophic acetogenesis, during which the above hydrolytic and acidogenic products are further degraded/oxidized into acetate, $H_2$, and $CO_2$. Syntrophic oxidation of propionate is particularly important because nearly 30% of the electrons generated from complex substrates flow through propionate during AD (Speece et al., 2006). Medium-chain fatty acids (MCFA) and LCFA from lipid hydrolysis also need to be oxidized to acetate, $H_2$, and $CO_2$ through syntrophic acetogenesis. Unless the $H_2$ partial pressure is kept very low ($>10^{-4}$ atm), syntrophic acetogenesis is thermodynamically unfavorable.

Hydrogenotrophic methanogens live in close proximity of syntrophic acetogens in AD bioreactors and consume the $H_2$ released from the syntrophic acetogens. This syntrophic relationship is based on interspecies hydrogen transfer (IHT) from hydrogen-producing bacteria (syntrophic acetogens) to hydrogenotrophic methanogens. Syntrophic acetogenesis is a critical process in maintaining the stable and robust operation of AD bioreactors because some of the SCFA, particularly propionate, are potent inhibitors of methanogens even at neutral pH.

Methanogenesis is carried out by methanogens, a specialized group of archaea. They can be categorized into three groups based on the methanogenesis substrates and pathways, (i) acetotrophic (or acetoclastic) methanogens, which use acetate to produce methane ($CH_4$) through the acetoclastic pathway; (ii) hydrogenotrophic methanogens, which use formate and $H_2$ to reduce $CO_2$ to $CH_4$ via the hydrogenotrophic pathway; and (iii) methylotrophic methanogens, which produce $CH_4$ from methyl compounds, such as methanol, methylamines, and methyl sulfides, through the methylotrophic methanogenesis pathway. Methanogens have also been divided into three classes (Anderson et al., 2009). Class I and II are hydrogenotrophic methanogens; they utilize formate, $H_2$, and $CO_2$ as their methanogenesis substrates and are important in the AD process owing to its ability to scavenge $H_2$ and keep the partial hydrogen pressure low. Class III methanogens possess the ability to utilize other substrates, such as acetate, methanol, and other $C_1$ compounds. In AD bioreactors, about two-thirds of the methane is produced from acetate, and about one-third produced from $H_2$ and $CO_2$, with minimal $CH_4$ production from methanol, methylamines, and methyl sulfides. Compared to other bacteria in AD bioreactors, methanogens grow the slowest and are more sensitive to environmental disturbances, such as pH decline and accumulation of SCFA or ammonia.

Methane can be produced through an alternative pathway under certain conditions. This pathway couples syntrophic oxidation of acetate to $H_2$ and $CO_2$ by syntrophic acetate-oxidizing bacteria and conversion of $H_2$ and $CO_2$ to $CH_4$ by hydrogenotrophic methanogens. This pathway is not a major pathway for biogas production in most AD bioreactors because syntrophic acetate-oxidizing bacteria are not as competitive as acetotrophic methanogens. However, when acetotrophic methanogens (primarily species of *Methanosaeta*) are inhibited under certain conditions, such as high

ammonia concentration and high operation temperature, this pathway is important to biogas production. This pathway is also favored when hydraulic retention time is long. Collectively, multiple trophic groups of microorganisms are involved in and important to biogas production. It is important to maintain a balance among the four trophic groups of microorganisms for a stable and robust AD process.

A recently found pathway of methanogenesis involves direct interspecies electron transfer (DIET), rather than interspecies hydrogen transfer. Some fermentative or syntrophic bacteria can directly transfer some of the electrons generated during fermentation or syntrophic oxidation of VFAs (exoelectrogenic bacteria) to methanogens that can accept electrons for methanogenesis (electrotrophic methanogens). Such direct interspecies electron transfer (DIET) is enabled cell-to-cell contact, including contact via conductive pili, and enhanced by conductive mediators such as metals (e.g., $Fe_3O_4$) and carbon (e.g., activated carbon) (Barua and Dhar, 2017). Besides hydrogenotrophic methanogens (e.g., species of *Methanobacterium* and *Methanospirillum*), obligate acetotrophic methanogens (e.g., *Methanosaeta harundinacea* and *M. concilii*) can also accept and utilize electron donated by electron-donating bacteria for methanogenesis. It has been well documented that DIET outperforms interspecies hydrogen transfer in supporting methanogenesis (Li et al., 2017).

## 1.2 The needs for identifying the microorganisms critical to AD efficiency and stability and the methodologies used

Most of the feedstocks used for biogas production, such as livestock manure, crop residues (mainly lignocellulosic), and municipal sludge, are complex and rather recalcitrant to microbial hydrolysis, the rate-limiting step inherent of these feedstocks. To achieve efficient AD, a diverse microbiome is required. On the other hand, when the feedstock is rich in readily hydrolyzable carbohydrates, such as feed wastes, acidogenesis can outpace syntrophic acetogenesis and methanogenesis, leading to accumulation of SCFA and pH drop, which then cause AD upset and even total failure. Process stability is crucial when it comes to the operation of AD bioreactors. Most of the AD bioreactors in operation are operated conservatively by limiting the organic loading rate because a failure of the operation is not only costly and takes a long time (up to several months) to restart or reestablish the microbial consortia and the process but also affects or disrupts the energy

supply. Therefore, improving AD stability without compromising biogas yield has always been a research focus. A stable AD process requires a delicate balance of microbial population dynamics and metabolic activities among the different guilds or trophic groups of the biogas-producing microbiome. Furthermore, different AD bioreactor designs (interested readers are referred to other relevant chapters of this book series) have been used to accommodate different engineering needs and handle different types of feedstocks. The design, the chemical and physical features of the feedstocks, and the operation of the AD bioreactors can profoundly affect the biogas-producing microbiomes. Therefore, it has been well recognized that understanding the microbial ecology of the AD process can help improve biogas production thereby and research on the biogas-producing microbiomes is needed. Indeed, such research has been the focus of numerous studies on AD since the early days of implementation of AD, with a hope to identify the diversity and composition of the individual microorganisms and better understand the metabolic activities so that AD efficiency and stability can be improved.

As AD is looked upon as a source of bioenergy, efficient biogas production from a wide array of feedstocks in a stable and reliable manner is critical. To this end, the vast diversity of the microbiome underpinning AD and the complexity of the interactions between different trophic groups and with the environmental conditions must be understood. Indeed, in the past century, many researchers have devoted their careers to research on the microbiome in various AD bioreactors using the best methodologies, techniques, and technologies available to them. These include the traditional cultivation-based methodologies, then the cultivation-independent molecular biology techniques to obtain an appraisal of the unculturable members of the biogas-producing microbiomes, and now the contemporary omics technologies. These increasingly powerful and enabling techniques and technologies have profoundly advanced our understanding of the microbiomes in various AD processes and contributed considerably to the advancement and development of AD as an increasingly robust and efficient technology for the production of biogas, a source of renewable energy from wastes. Here we will provide an overview of the main methodologies, molecular biology techniques, and the omics technologies that have been used in biogas-producing microbiome research, the utility of and limitations inherent to each approach (summarized in Table 1), the new insight gained from past studies, and the future perspectives.

**Table 1** Summary of the utility, pros, and cons of different methods, techniques, and technologies used in AD microbiome research.

| Methods or techniques | Applicability | Pros | Cons |
|---|---|---|---|
| Cultivation and isolation | Isolation of individual microbes; enumeration of functional groups of microbes | Definitive characterization of metabolism; abundance of functional groups; sources of reference genomes | Unable to analyze difficult-to-cultivate microbes; low throughput |
| Molecular fingerprinting | Microbiota profiling and changes | Rapid and low cost | Low resolution; unreliable microbe identification; unable to analyze microbes at low abundance |
| qPCR | Quantification of microbes of interest | Precise and accurate quantification | Only quantifying small numbers of microbes; requiring a known specific marker gene |
| FISH | Detection, quantification, and localization of specific microbes | Rapid, simple, and specific | Only feasible to analyze small numbers of microbes; poor precision and accuracy when used in heterogeneous samples |
| SIP | Detection of microbes capable of utilizing a specific substrate | Analysis of growing microbes of a specific guild | Requiring stable isotope and in vitro cultivation; unable to analyze slow growers |
| Microarrays | Analysis of taxonomic or functional diversity | Rapid and straightforward data analysis; able to achieve absolute quantification | Requiring known sequence for probe design |
| Metataxonomics | Analysis of taxonomic and functional diversity | High throughput, analyzing both known and new microbes; able to analyze microbes at low abundance; cost-effective | Prone to PCR bias; only determining relative abundance; poor below-genus resolution; not very precise or accurate; unable to exclude dead microbes |

*Continued*

**Table 1** Summary of the utility, pros, and cons of different methods, techniques, and technologies used in AD microbiome research.—cont'd

| Methods or techniques | Applicability | Pros | Cons |
|---|---|---|---|
| Metagenomics | Analysis of both taxonomic and functional diversity at the genetic level | Analyzing known and unknown microbes; below-genus taxonomic resolution; able to recover genomes of uncultured microbes | Unable to exclude dead microbes or detect minor microbes; only determining relative abundance; computing intensive (e.g., assembly); relatively high cost |
| Metatranscriptomics | Analysis of taxonomic and functional diversity at the transcription level | Analyzing both known and new microbes that are transcriptionally active | Maybe unable to detect transcripts at low abundance; only determining relative abundance; relatively high cost |
| Metaproteomics | Analysis of metabolic activity at the translation level | Analyzing proteins and enzymes from both known and new microbes including phages | Maybe challenged with AD sample matrixes, proteins at low concentrations, and redundancy of protein identification; maybe difficult to link the detected proteins to specific microbes due to lack of adequate databases |
| Metametabolomics | Analysis of functions at the metabolite level | Analyzing the outcomes of metabolism | Unable to analyze metabolites at low concentrations; difficult to link detected metabolites to producers; maybe challenged with AD sample matrixes |

## 2. Cultivation-based methods used and the microorganisms identified thereby

### 2.1 A brief history of the cultivation-based methods

The biogas-producing microbiomes were analyzed using only cultivation-based methods before the DNA-based molecular biology techniques became available in the later 1980s. The AD process was thought to include two phases: fermentation of feedstocks into SCFA in the acid-forming phase and then SCFA conversion to $CO_2$ and $CH_4$ in the methane phase. Based on our literature search, the very first study on the microorganisms in AD bioreactors was reported in a dissertation in 1906 (Söhngen, 1910), and the early isolates were published in 1924. The so-called methane bacteria received more research interest than bacteria in the 1930s and 1940s. The challenge of isolating individual methanogens was well recognized in early studies. The early isolates were reported in 1936, and the genera *Methanobacterium, Methanococcus*, and *Methanosarcina* were created for those methanogen isolates (Barker, 1936). The genus exclusively for the slow-growing obligate acetotrophic methanogens, *Methanothrix*, was established much later in 1984, and the latter was renamed as *Methanosaeta* (Boone and Kamagata, 1998; Patel, 1984). Söhngen (Söhngen, 1910) was also among the early pioneers who studied and isolated bacteria from AD bioreactors. Many anaerobic bacteria involved in the AD process were isolated using enrichment media in the 1960s and 1970s. Although time-consuming and labor-intensive, cultivation-based methods enabled the isolation of many bacteria and methanogens mediating most of the steps of the AD process. Studies on pure or axenic cultures obtained using the cultivation methods provided valuable information not only on the characteristics and fundamental metabolism of individual species but also on the syntrophic interspecies interactions. In addition to the isolation of bacteria and methanogens, cultivation-based methods were also used to estimate the abundance of specific groups of methanogens and bacteria. However, it was soon realized that the majority of any biogas-producing microbiome was not cultivatable or difficult to cultivate in the laboratory. Therefore, by the early 1990s, cultivation-based methods started to give way to molecular biology techniques when the latter became available to analyze methanogens and bacteria in various AD bioreactors.

### 2.2 Common cultivation-based methods traditionally used

Enrichment on specific substrates is the most effective approach to increase the abundance of the bacteria or methanogens of interest present in

biogas-producing microbiomes. Cellulose, starch, protein, and lipids have been used as the substrates to enrich the respective guild of bacteria, while acetate and other methanogenesis substrates ($H_2$ and $CO_2$, methylamines, and methanol) are used to enrich methanogens. After dilution in an anaerobic buffer, the bacteria or methanogens of interest can then be isolated on agar plates (or roll tubes) containing the substrates that support the growth of the bacteria or methanogens of interest but not the others (referred to as the "specific substrate" for the ease of reference) by forming individual colonies, characterized for their ability to utilize different substrates and produce end products, and finally identified through biochemical and morphological analyses. The biochemical characterization provides valuable information on the isolated bacteria and methanogens, particularly with respect to their roles in the AD process.

The population size or abundance of a specific guild of bacteria or methanogens can be estimated using plate counting or the most probable number (MPN) analysis. The former entails serial dilution (in an anaerobic buffer) of selected samples and plating the appropriate dilutions (three consecutive ones) on replicate agar plates (three or more) containing the specific substrate. In MPN analysis, the selected samples are also diluted. However, the dilutions, typically three, are inoculated into a broth medium that contains the specific substrate. Positive growth in the broth medium can be ascertained by increased turbidity. In both agar plate counting and MPN analyses, the population size is calculated based on the dilution factors and the numbers of colonies (for agar plate counting) or the number of culture tubes that have positive growth (for MPN).

## 2.3 The major microorganisms identified and characterized using cultivation-based methods

The first efforts to study biogas-producing microbiomes relied on cultivation-based approaches. To date, more than 150 species of microorganisms have been discovered in AD bioreactors (Söhngen et al., 2016). Many bacteria and methanogens have been cultured and characterized biochemically and morphologically. Species of hydrolytic and acetogenic bacteria have been taxonomically assigned to the genera *Acetivibrio*, *Clostridium*, *Bacteroides*, *Ruminococcus*, and *Thermotoga* (in the phylum *Thermotogae*). Non-hydrolytic acidogens have been assigned to *Bifidobacterium* (in the phylum *Actinobacteria*), *Lactobacillus*, *Anaerolineaceae* (in the phylum *Chloroflexi*), and a few thermophilic non-hydrolytic bacteria were found to be members of the phylum *Thermotogae*. Some species of

syntrophic acetogens have been identified from AD bioreactors. They include species of the genera *Smithllela*, *Syntrophobacter*, and *Pelotomaculum* for propionate oxidation and species in the genera *Syntrophus* and *Syntrophomonas* for the oxidation of butyrate and LCFA.

Common hydrogenotrophic methanogens found in AD bioreactors include species of *Methanoculleus*, *Methanobacterium*, *Methanobrevibacter*, *Methanospirillum*, and *Methanothermobacter*. Acetotrophic methanogens are only found in *Methanosaeta* (a genus of obligate acetoclastic methanogens) and *Methanosarcina* (a genus of facultative acetoclastic methanogens). *Methanosaeta* can only use acetate as the substrate, and it grows very slowly but has a high affinity for acetate, so it propagates and dominates when acetate concentration is low. *Methanosaeta* can have filamentous morphology (and thus it was initially named as *Methanothrix*) and play an important role in the granulogenesis of anaerobic granules or aggregates. *Methanosarcina* species can utilize a broader range of substrates, including methanol, methylamine, methyl sulfides, and $H_2$ and $CO_2$, in addition to acetate. Unlike species *Methanosaeta*, *Methanosarcina* species can grow fast but has a lower affinity for acetate so it can outcompete *Methanosaeta* when acetate concentration is high.

In recent years, new strains of hydrolytic/acidogenic bacteria such as *Clostridium bornimense*, *Herbinix hemicellulosilytica*, *Herbinix luporum*, *Herbivorax saccincola*, *Proteiniphilum saccharofermentans*, *Petrimonas mucosa*, *Fermentimonas caenicola*, and *Proteiniborus indolifex*, have been isolated and characterized owing to technical improvements in the anaerobic cultivation of microorganisms. New species for methanogenic archaea, such as *Methanobacterium aggregans* and *Methanosarcina flavescens* were also reported (Hassa et al., 2018).

## 2.4 Limitation of cultivation-based methods

The culture-dependent methods suffer from severe limitations that may lead to incomplete or even incorrect information. First, only a small fraction of the microorganisms in AD bioreactors can be cultivable because the artificial growth media may not adequately simulate the environment in the AD bioreactors or provide all the nutrients required for the growth of the microorganisms. Second, many microorganisms require syntrophic interactions with others, and thus they cannot be cultured individually. Third, some microorganisms share similar physiological, biochemical and/or morphological characteristics, and thus cannot be distinguished from one another with certainty. As a result, it has been estimated that no more than 1% of the microorganisms present anaerobic environments had been isolated or

characterized (Fang, 2010). Hence, the species of microorganisms identified do not reflect the actual composition or diversity of the biogas-producing microbiomes. According to BacDive—the Bacterial Diversity Metadatabase (http://bacdive.dsmz.de), which provides strain-level information about bacterial and archaeal biodiversity, about 150 species of biogas-producing microorganisms have been discovered, which is probably several orders of magnitude lower than the species richness likely present in any AD bioreactors (Söhngen et al., 2016). The sequenced genomes of biogas-producing microorganisms are therefore highly under-represented in public nucleotide sequence repositories. However, more reference genomes are needed to evaluate metagenome, metatranscriptome, and metaproteome data from biogas-producing microbiomes (Hassa et al., 2018). Therefore, there is still a need for continuous technical improvements in the cultivation of biogas-producing microorganisms.

## 2.5 New methods and techniques: Aerobic cultivation of anaerobic microorganisms and microbial culturomics

It's well recognized that the cultivation of anaerobic microorganisms, especially methanogens, is time-consuming due to the need to create and maintain anaerobic conditions and their slow growth. Dedicated apparatus and equipment, such as oxygen-free gases, gas manifolds, anaerobic jars, or anaerobic chambers, are also needed. Two recent studies showed that strictly anaerobic bacteria, methanogens, and fungi could be successfully cultured under aerobic conditions if antioxidants were included in the media (Dione et al., 2016; Khelaifia et al., 2016). When cysteine, glutathione, ascorbic acid, uric acid, and α-ketoglutarate were added to media together, they enabled the growth of 620 (out of 623 strains tested) microbial strains, including facultative anaerobic bacteria (154 species), strictly anaerobic bacteria (82 species), and fungi and methanogens. Two species of anaerobic rumen ciliate protozoa were also successfully cultured in aerobic media (Park and Yu, 2018). In the literature, no study has been reported that used this aerobic approach to cultivate the microorganisms of biogas-producing microbiomes. Given the ability to cultivate nearly all the tested strains of anaerobic microorganisms, it is certain that this aerobic method will greatly facilitate the cultivation of most, if not all, anaerobic microorganisms in biogas-producing microbiomes.

Microbial culturomics is a relatively new approach to extensively characterize the microbial composition by high throughput culturing. It was first used to study the gut microbiome composition of cultural microorganisms

in the human gut (Lagier et al., 2012). Using 212 different culture media and analyzing 32,500 different colonies, these authors successfully cultured 340 different species (including 32 new species) of bacteria as well as 5 species of fungi and the largest number of different viruses. The 32 new species equal approximately to one-third of all the new validated species recovered by culturing from the human gut in the last decade. To the best of our knowledge, only one study has used culturomics, together with metataxonomics and metatranscriptomics, in analyzing the microbiomes in AD bioreactors operated at 54 °C (Maus et al., 2016). Using 11 different cultivation strategies, 52 taxonomically different microbial isolates were obtained. These isolates were identified as one species of cellulolytic *Herbinix* (*H. hemicellulosilytica*), 4 species of cellulolytic *Clostridium* (*C. cellulosi*, *C. clariflavum*, *C. stercorarium*, *C. thermocellum*), 30 species of acidogens/acetogens (including six species of *Bacillus*; five species of *Clostridium*; three species of *[Clostridium]* in the family *Ruminococcaceae*; two species of *Tepidimicrobium*; one species each of *Aneurinibacillus*, *Defluviitalea*, *Defluviitoga*, *Desulfotomaculum*, *Geobacillus*, *Enterococcus*, *Lutispora*, *Paenibacillus*, *Proteiniborus*, *Sporanaerobacter*, *Tepidanaerobacter*, *Thermoanaerobacterium*, *Tissierella*, and *Ureibacillus*), and three species of methanogens (two species of *Methanothermobacter* and one species of *Methanoculleus*). Given the power of culturomics demonstrated in studies of the human gut microbiome, more culturomics studies are expected in the future to comprehensively characterize the biogas-producing microbiomes.

## 3. The molecular biology techniques used and the microbiome knowledge learned thereby

### 3.1 The coming of the molecular biology technique era

Thanks to the advancement and development in molecular biology techniques and technologies, many cultivation-independent methods are available and have become the preferable methods to study the microbial ecology of biogas-producing microbiomes. Most of these techniques and technologies analyze marker genes. Particularly, 16S ribosomal RNA (rRNA) genes have been almost exclusively used as a marker gene in studies of microbial communities, including biogas-producing microbiomes, either microbiome composition or population dynamics.

The 16S rRNA gene is a phylogenetic marker for both bacteria and archaea. This gene is composed of nine hypervariable regions interspersed with conserved regions. It was chosen as the marker gene over other

rRNA genes (i.e., 5S or 23S rRNA gene, about 120 bp, and 3000 bp, respectively) because its sequence (about 1600 bp) contains enough phylogenetic information needed to distinguish different bacteria and archaea, while it is still not too long for routine sequencing. In addition, the mosaic structure with both the conserved and the hypervariable regions allows phylogenetic comparison of different bacteria and archaea and the design of probes and primers specific at different taxonomic levels, respectively. Furthermore, because of the common use of 16S rRNA as the marker for phylogenetic studies of microbiomes, several large public databases dedicated to 16S rRNA genes have been constructed, including Greengenes (http://greengenes.lbl.gov/), Silva (http://www.arb-silva.de/), and RDP (http://rdp.cme.msu.edu/), which greatly facilitate archiving and phylogenetic analysis of 16S rRNA genes.

Although the 16S rRNA gene has been the most commonly used phylogenetic marker in analyzing biogas-producing microbiomes, several genes encoding enzymes have also been used as marker genes. The *mcr*A gene that encodes the α subunit of methyl-CoM reductase of the methanogenesis pathway has been widely used in studying methanogens as a guild. The *pct* gene coding for the propionate-CoA transferase of the syntrophic propionate oxidation pathway has also been used to investigate the syntrophic propionate-oxidizing bacteria in temperature-phased AD bioreactors (Li et al., 2013). Using T-RFLP fingerprinting of *mcr*A transcripts, a correlation was found between ammonia concentration and the abundance of different methanogens (Zhang et al., 2014). The *fhs* and the *acs*B genes, which code for the formyltetrahydrofolate synthetase and the acetyl-CoA synthase, respectively, of the homoacetogenesis pathway, have also been used as useful markers in analyzing homoacetogens in anaerobic environments. Functional genes as phylogenetic markers have a few advantages. First, they support the analysis of the phylogenetic diversity and population of a particular guild, such as homoacetogens, methanogens, and sulfate-reducing bacteria. Second, most functional genes in bacteria and archaea are mostly single-copy genes, and thus they can support more accurate quantification than the 16S rRNA genes, which can exist in multiple varying copy numbers in different species.

## 3.2 PCR, cloning, and sequencing of marker genes

A variety of 16S rRNA-based techniques have been developed and applied to microbial ecological studies of biogas-producing microbiomes.

Cloning of PCR-amplified 16S rRNA gene, a fragment or the entire gene, followed by sequencing of individual clones with the Sanger sequencing technology has been used for decades in the analysis of microbiomes, including biogas-producing microbiomes. Domain-specific primers allow a broad analysis of microbiome, while primers specific to a lower taxon, such as a genus, allow for a detailed analysis of its species. Most of the information on microbial composition in AD bioreactors were produced by this approach (Nelson et al., 2011). However, because this traditional sequencing approach can only sequence individual clones one by one, which is costly and time-consuming, it does not support detailed analysis to reveal the true microbial composition and diversity, especially when multiple samples are analyzed. Nonetheless, this culture-independent approach has greatly expanded the knowledge of the diversity of both bacteria and methanogens in different AD bioreactors. More than 5900 operational taxonomic units (OTUs, based on $\geq$97% sequence identity) of bacteria representing 28 known bacterial phyla had been documented, with *Proteobacteria* (nearly 1600 OTUs), *Firmicutes* (more than 1350 OTUs), *Bacteroidetes* (more than 700 OTUs), and *Chloroflexi* (nearly 700 OTUs) being predominant. The archaeal sequences were assigned to 296 OTUs, primarily *Methanosaeta* and the uncharacterized WSA2 group. More importantly, nearly 60% of all the sequences could not be classified into any established genus. Rarefaction analysis suggested that approximately 40% of the bacterial and 10% of the archaeal diversity in AD bioreactors remained to be identified. Interested readers are referred to the meta-analysis by Nelson et al. (2011) for a detailed description of the biogas-producing microbiomes identified with the cloning and sequencing approach. Of course, the recent advancement and decreasing cost of the next-generation sequencing (NGS) technologies (see below) have made this traditional method obsolete.

## 3.3 Molecular fingerprinting of biogas-producing microbiomes

In the molecular biology technique era, microbiome fingerprinting was also frequently used to evaluate and compare different microbiomes, including biogas-producing microbiomes. The ones that have been used include terminal restriction fragment length polymorphism (T-RFLP), single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and automated ribosomal intergenic spacer analysis (ARISA). All these microbiome fingerprinting methods entail PCR amplification of a hypervariable region of the 16S rRNA gene using universal or

taxa-specific primers and separation, detection, and quantification of individual PCR products. In T-RFLP, one or both of the primers are labeled with a fluorescent dye at 5′ end. The PCR products are digested using one or two restriction endonucleases, and the terminal fragments with the fluorescent label are size separated and detected using Sanger sequencers. Differences in the occurrence and locations of the cutting sites of the restriction endonucleases produce different terminal restriction fragments among different microorganisms. The terminal restriction fragments can be quantified based on the intensity of the fluorescence signal, but such quantification is not accurate because of PCR bias. T-RFLP fingerprinting has been used to investigate both the archaeal (e.g., Witarsa et al., 2016) and bacterial communities (e.g., Acs et al., 2015) in different AD bioreactors. Automated ribosomal intergenic spacer analysis (ARISA) is another fingerprinting method similar to T-RFLP. The main difference is that (i) the target of the analysis is the ribosomal intergenic spacer (RIS) between the 16S and the 23S rRNA genes; (ii) the primers anneal to the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene; (iii) the PCR-amplified RIS are size separated and detected using Sanger sequencers based on RIS length differences.

Both SSCP and DGGE are polyacrylamide gel-based fingerprinting techniques. SSCP relies on different migration of single strands of DNA fragments (PCR-amplified hypervariable region of 16S rRNA genes) with different sequences in a polyacrylamide gel (single-strand DNA fragments of the same length but different sequences can form different secondary structures, which affect migration during gel electrophoresis). SSCP has been used to investigate the methanogenic community in several AD bioreactors (e.g., Kampmann et al., 2012), but it is not used as frequently as the other techniques. With DGGE, a 40 bp GC clamp is added to one of the two primers and the PCR products with different sequences are resolved in a polyacrylamide gel containing a gradient of denaturants. Different PCR products have different denaturing behaviors and thus form different banding patterns, reflecting the composition of the microbiome. Bands of interest can be excised and sequenced to identify the microorganisms. Because of its technical simplicity, rapidness, and low cost, DGGE has been used more frequently than T-RFLP or SSCP in the analysis of biogas-producing microbiomes. DGGE has been used in numerous studies assessing the effect of feedstock and operation conditions on biogas-producing microbiomes, including recent studies (e.g., Vasconcelos et al., 2019). It should also be noted that DGGE profiles concurred with the detailed profiles

of the microbiomes in several AD bioreactors that were determined using 454 pyrosequencing, and it was recommended as a preliminary screening method to identify representative samples from a large number of samples for detailed analysis using NGS technologies (Nelson, 2011). Similarly, β diversity analysis using non-metric multidimensional scaling (NMDS) showed a similar clustering profile for the Illumina, bacterial T-RFLP, and archaeal T-RFLP data (De Vrieze et al., 2018b).

The major limitations of SSCP, T-RFLP, ARISA, and DGGE include low resolution and the inability to reliably identify the microorganisms represented by the band or chromatogram peaks because it is difficult to obtain sequence information of the 16S rRNA gene fragments. To putatively identify the bands on either SSCP or DGGE gels, a set of amplicons from known species can serve as references, but such identification is not reliable. As for DGGE, individual DNA bands in the gel could be excised out, re-amplified, and sequenced directly or after cloning, but multiple sequences can result from one band, making it difficult to reliably identify the bacteria or methanogens represented by individual bands. For T-RFLP, terminal restriction fragments typically are compared to databases to identify the bacteria or methanogens, but individual terminal restriction fragments nearly always match multiple species of microorganisms. Nevertheless, despite their limitations, these microbiome fingerprinting or profiling methods can still be useful to obtain a snapshot of biogas-producing microbiomes from large numbers of samples. Indeed, they have been used in recent studies on biogas-producing microbiomes (most recent studies were found in 2019 for DGGE and T-RFLP, 2016 for ARISA, and 2015 for SSCP).

## 3.4 Quantification of individual taxa or guilds of microorganisms using qPCR and droplet digital PCR

Different populations of biogas-producing microbiomes can respond differently to changes in feedstock, design, and operation conditions of AD bioreactors, and the differential responses are reflected by changes in their population sizes. Quantitative PCR (qPCR) is the most commonly used technique to reliably and accurately quantify populational changes of select microorganisms, particularly methanogens and key guilds of bacteria. By quantifying the accumulation of PCR amplicons in real-time at each PCR cycle, qPCR can accurately quantify the copy number of the gene of interest in a sample. By comparing to a standard curve prepared with serial dilutions of known concentration of the target gene, absolute quantification can be achieved. Relative quantification of a target group of microorganisms

is determined using a reference target, mostly total 16S rRNA genes or a house-keeping gene. Primers and/or probes with specificity at different taxonomic levels (from domain to genus) have been designed to quantify the population of interest, such as the genera *Methanoculleus*, *Methanosarcina*, and *Methanothermobacter* (Franke-Whittle et al., 2009b). Genus-specific primers targeting the 16S rRNA of hydrolytic bacteria *Clostridium*, *Peptostreptococcus*, and syntrophic acetogen *Syntrophomonas* were also used in qPCR to investigate the spatial distribution of these genera in different compartments of a plug flow digester (Talbot et al., 2010). Although metataxonomics empowered by NGS can comprehensively characterize the diversity and composition of biogas-producing microbiomes, it cannot accurately quantify the population sizes of individual microorganisms. qPCR can be used to confirm the populational difference or dynamics of taxa or guilds of microorganisms as they respond to changes in AD processes. In addition to qPCR, droplet digital PCR (ddPCR) (Kim et al., 2015), which is a relatively new technology to precisely quantify a target gene without an external stander or reference in nanoliter droplets, has also been used in quantification of individual taxa of biogas-producing microbiomes (Kim et al., 2015). Interested readers are referred to the above citation.

## 3.5 Fluorescent in situ hybridization (FISH)

FISH is a visualization technique based on microscopic examination of a given species or groups of bacteria or archaea after staining their cells with specific fluorogenic oligonucleotide probes that bind the RNA molecules inside their cells. FISH probes are short sequences of single-stranded DNA (about 16–20 nucleotides) labeled with one or two fluorescent dyes. The probe specifically hybridizes in situ with 16S rRNA, 23S rRNA, or mRNA inside microbial cells according to DNA-RNA complementary matching. A list of the probes commonly used on biogas-producing microbiomes is available in the probeBase 2016 database (www.probebase.net), while ARB/SILVA is a good tool to perform online analysis and design of probes. The FISH process can be divided into four stages: (A) sampling and immediate fixation in formaldehyde to preserve the integrity of the cells, especially the ribosomes; (B) hybridization with a specific probe labeled with a fluorescent dye at its 5′ end; (C) counterstaining with a universal dye (mostly DAPI, which binds non-specifically to DNA molecules) or a general probe labeled with a different fluorescent dye; and (D) visualization via fluorescence microscopy.

This technique has become the method of choice for reliable and rapid identification of microorganisms in a microbiome sample since DeLong et al. (1989) reported the suitability of fluorescently labeled rRNA-targeted oligonucleotide probes as phylogenetic stains for cultivation-independent identification of microorganisms. A group of microorganisms of interest can also be quantified by manual counting the hybridized cells under a microscope (an epifluorescence or a laser confocal microscope), by analysis of microscope images, or by automated counting with a flow cytometer. The ratio of cells that have hybridized with the specific probe to the total cell count reflects the relative abundance of the target microorganisms. FISH has its limitations in quantifying individual groups of microorganisms. First, homogenous samples are required for accurate and precise quantification. Second, microscopic cell counting is prone to variations among researchers and can be tedious and time-consuming when large numbers of samples need to be analyzed. Third, background fluorescence can interfere with cell counting. Fourth, probes are designed based on known sequences, and thus FISH may not allow the detection of novel microorganisms.

FISH is a relatively simple and rapid technique that allows for the direct visualization of microorganisms. As a method without the need for DNA extraction or PCR, FISH overcomes some of the issues associated with PCR-based molecular methods, such as DGGE, T-RFLP, cloning, and sequencing. Furthermore, FISH can help localize the specific group of interest in anaerobic granules formed in some AD bioreactors (e.g., up-flow anaerobic sludge blanket reactors, UASB) and on the surface of insoluble feedstocks (Angenent et al., 2004; Yamada et al., 2005). Furthermore, a method that combines microautoradiography and FISH (MAR-FISH) was successfully developed and used in investigating the microbial species that are involved in syntrophic propionate oxidation and non-acetoclastic acetate oxidation in AD bioreactors (Ariesyady et al., 2007; Ho et al., 2013). With metataxonomics being the primary technology to detail the diversity and composition of biogas-producing microbiomes, FISH can still be a useful tool, as demonstrated by the studies mentioned above.

## 3.6 Microarrays

Microarrays are a powerful and high throughput tool that allows for the detection and quantification of thousands of genes or RNA transcripts simultaneously. The core innovation of the microarray technique is the

ability to attach oligonucleotide probes onto a solid matrix to create a densely packed array. DNA microarrays offer the possibility to determine the occurrence of an entire array of microorganisms in a microbiome, including that in AD bioreactors. Several types of microarrays have been developed for the analysis of microbiomes in different environments. Phylogenetic arrays have probes specific for the 16S or 18S rRNA genes or other conserved functional genes and are used to examine species diversity and composition of microbiomes. The ANAEROCHIP is a specialty phylogenetic array developed specifically for the examination of the methanogenic community present in AD bioreactors (Franke-Whittle et al., 2009a). It contains 103 probes specific for different groups of methanogens commonly found in biogas-producing microbiomes. Community genome arrays have whole genomic DNA from microbial isolates as "probes," and they can be used to identify or screen for species of interest. Functional gene arrays (FGAs) contain probes specific for genes encoding proteins or enzymes involved in functions of interest. Unlike phylogenetic arrays or community genome arrays, FGAs can help examine the potential functional capabilities of microbiomes and link microbial diversity to ecophysiological processes (Van Nostrand et al., 2010). However, one of the greatest challenges in using FGAs to detect functional genes and/or microorganisms is the design of oligonucleotide probes specific to the target genes. Another challenge is the lack of arrays that contain a comprehensive set of probes specific for most, if not all, the genes (Zhang et al., 2019a). GeoChip is the most comprehensive FGA available to date, and the latest generation of GeoChip (GeoChip 5.0) has 167,044 distinct gene probes (including 455 phage probes). The raw microarray data can be processed with the GeoChip Microarray Data Manager pipeline (http://ieg.ou.edu/microarray/).

The specialty ANAEROCHIP has been used in several studies of biogas-producing microbiomes. In a recent study, ANAEROCHIP was used to investigate how the methanogenic community would respond to changes in the SCFA levels in AD bioreactors (Franke-Whittle et al., 2014). The authors reported that the dominant archaeal populations were not influenced by changes in the SCFA levels. However, real-time PCR results conducted in parallel to the ANAEROCHIP analysis revealed greater diversity in methanogens than ANAEROCHIP. Apparently, the probe set of ANAEROCHIP needs to be expanded to include more probes targeting the diverse methanogens found in AD bioreactors.

High-density microarrays have also been used in many studies on the biogas-producing microbiomes. One of the most recent studies used

GeoChip 5.0 in investigating the functional genes of the methanogenic pathways (Zhang et al., 2019a) and examining the bacteriophage diversity in full-scale AD bioreactors in China (Zhang et al., 2017). A total of 334 probes from 18 genes associated with methanogenesis were detected using the GeoChip 5.0 (Zhang et al., 2019a). By correlating the process performance data with the taxonomic data from amplicon sequencing (performed on a MiSeq platform) and gene data from GeoChip 5.0, the authors concluded that high variations in methanogenic traits (i.e., taxa or genes) were responsible for variations in biogas production in full-scale AD bioreactors. They also found that hydrogenotrophic methanogens, especially members of the order *Methanomicrobiales*, were correlated with biogas production performance and nearly all the methanogenic genes detected. High variations in bacterial community compositions were also observed, with only a few abundant OTUs (e.g., OTUs assigned to *Clostridiales*, *Anaerolineaceae*, and *Methanosaeta*) being persistently present across different AD bioreactors at different sampling time. Functional redundancy, which is defined as multiple species representing a variety of taxonomic groups sharing similar, if not identical, roles in ecosystem functionality, can lead to a decoupling of taxonomic composition from functional structure because functionally similar but taxonomically distinct species can replace one another. Lower functional redundancy index, which is calculated as the ratio of functional diversity to taxonomic diversity, for methanogens than for fermentative microorganisms, explained the high consistency between taxonomy and function in methanogens. That study also revealed several syntrophic associations, including that between hydrogenotrophic methanogens and the population of *Clostridia* (it contains known acetate-oxidizing bacteria converting acetate to hydrogen), between acetate producer *Anaerolineaceae* and acetoclastic methanogen *Methanosaeta*, and between hydrogen-producing *Syntrophomonas* and *Smithella* and hydrogen-scavenging *Methanomicrobiales* and *Methanobacteriales*.

Bacteriophages infect bacteria, while archeophages can infect archaea. These prokaryotic phages can thus affect the population dynamics of bacteria and archaea, playing important roles in shaping the microbiome composition and ecosystem functions in AD bioreactors. Using GeoChip 5.0 together with MiSeq sequencing, Zhang et al. (2017) examined the phage and prokaryotic communities and the interactions between the two communities in four full-scale AD bioreactors in China. It was shown that despite the relatively stable biogas production, populations of some phages and prokaryotes fluctuated considerably. Significant correlations were also found between phages and prokaryotes with respect to $\alpha$- and $\beta$-diversity.

The phages explained 41% of the total variations of the prokaryotic community composition and were significantly linked to parameters related to process performance, including biogas production and volatile solid concentrations. That study demonstrated that phages could be a major biotic factor shaping the biogas-producing microbiomes and therefore affecting process performance in AD bioreactors (Zhang et al., 2017).

Although microarrays have been used successfully in the analysis of gene expression in pure cultures, its adaptation to the analysis of diverse and complex microbiomes, including those in AD bioreactors, still presents numerous challenges in terms of probe design, coverage of gene sequences, specificity, sensitivity, and quantitation. With the continued improvement in sequencing technologies and decrease in cost, DNA and RNA sequencing with NGS or the third-generation sequencing technologies may be preferred over microarrays.

## 3.7 Stable isotope probing (SIP)

Coupling of molecular biological techniques with stable isotope probing (SIP) of biomarkers has provided a cultivation-independent in vitro approach to linking the identity of microorganisms with their functions in microbiomes. In SIP, one substrate labeled with a heavy isotope, such as $^{13}C$, $^{15}N$, or $^{18}O$, is added to in vitro cultures of interest that are incubated. The genomes (DNA), transcriptomes (RNA), and proteomes (protein) of actively growing microorganisms that can utilize the substrate are labeled with the isotope. The labeled "heavy" DNA or RNA can be separated from the "light" counterparts using density gradient centrifugation and then analyzed using several molecular biology techniques, primarily sequencing of the heavy DNA (DNA-SIP) or RNA (RNA-SIP). The labeled "heavy" protein can be identified using liquid chromatography-tandem mass spectrometry (protein-SIP). The light DNA and RNA can also be sequenced to examine the microorganisms that cannot utilize the substrate. Thus, these three SIP approaches can be used to investigate the microorganisms of particular guilds at DNA, RNA, and protein levels.

DNA-SIP has been used in many studies to identify and discover new microorganisms important to the AD processes, especially the microorganisms that are involved in the degradation of SCFA and LCFA. Syntrophic fatty acid-oxidizing bacteria grow very slowly, and they are difficult to isolate because they need a hydrogenotrophic partner, mostly hydrogenotrophic methanogen, for their growth. However, they constitute a key group of

microorganisms that affects the rate and stability of biogas production in AD bioreactors. Using DNA-SIP ($^{13}$C-acetate and pyrosequencing), Hao et al. (2015) found that taxon OPB54 (assigned to *Clostridia*) and *Methanosarcina thermophila* were the active predominant bacterial and archaeal population, respectively, at both low ($0.26\,g\,L^{-1}$) and high ($7.00\,g\,L^{-1}$) total ammonia nitrogen concentrations in a thermophilic AD bioreactor. These researchers also determined the stable isotopic signature of biogas to evaluate the pathway dynamics of acetoclastic methanogenesis. In another study using DNA-SIP ($^{13}$C-acetate, $^{13}$C-lactate, and Illumina sequencing), the population of *Clostridium*, *Hydrogenophage*, *Fervidobacterium*, *Spirochaeta*, *Limnohabitans*, and *Rhodococcus* was shown to increase by $^{13}$C-acetate, while that of *Anaeromyxobacter*, *Desulfobulbus*, *Syntrophus*, *Cystobacterineae*, and *Azospira* was elevated by $^{13}$C-lactate in thermophilic AD (Sun et al., 2018). Using $^{13}$C-oleate and genome-centric metagenomics, Ziels et al. found that *Syntrophomonas* was the major LCFA-degrading populations in AD bioreactors (Ziels et al., 2018b), while *Syntrophomonas* was probably the major syntrophic oxidizer of butyrate and *Methanothrix* the acetoclastic methanogen utilizing the acetate produced by *Syntrophomonas* (Ziels et al., 2019). In contract to DNA-SIP, RNA-SIP has only been used in a few studies to examine the metabolic active microbes in biogas-producing microbiomes. Using RNA-SIP and T-RFLP, Hori et al. (2014) identified one acidogenic bacterium in the genus *Thermoanaerobacterium* as the potential acidogen responsible for the acidification resulting from an increase in glucose feeding. In a recent study using RNA-SIP and metatranscriptomics (Wang et al., 2018), magnetite was shown to reduce the accumulation of SCFA and accelerate methane production by enhancing DIET. The metatranscriptomic analysis, coupled with analysis of the key enzymes involved in methanogenesis, also showed that IHT could be partially substituted by enhanced DIET, and acetotrophic methanogenesis could be improved after the blockage of electron transfer caused by SCFA accumulation was alleviated. Furthermore, it was shown that magnetite could downregulate the expression of both pili and c-type cytochromes while maintaining DIET, indicating that magnetite could replace their roles for efficient electron transfer between acetogens and methanogens.

The first and the only study that used protein-SIP investigated the syntrophic acetate-oxidizing bacteria in AD bioreactors (Mosbaek et al., 2016). By mapping the $^{13}$C-labeled peptides onto a binned metagenome, the authors showed that five subspecies of *Clostridia* and the genera *Methanosarcina* and *Methanoculleus* were the key microorganisms involved in methane production from acetate. All the acetate-consuming

microorganisms affiliated to *Clostridia* contained the FTFHS gene coding for the formyltetrahydrofolate synthetase, which is a key enzyme of reductive acetogenesis. Those five subspecies of *Clostridia* are possible syntrophic acetate-oxidizing (SAO) bacteria that can facilitate acetate consumption via syntrophic acetate oxidation.

As discussed above, SIP represents a powe approach to identify or discover bacteria and methanogens of interest without the need for isolation. However, it may be difficult to successfully identify all the important bacterial or archaeal taxa in any AD bioreactors because some of them only grow slowly or co-exist synergistically with other microorganisms, making enrichment of their DNA, RNA, or protein labeled with $^{13}C$ or other stable isotopes, very low. More efficient separation and collection of "heavy" DNA, RNA, protein or more sensitive analysis techniques are needed to overcome these challenges. Future studies may also investigate the bacteria that are important to digestion and degradation of recalcitrant feedstock, such as lignocellulose, the most abundant feedstock for biogas production.

## 3.8 Qualitative and quantitative perspectives of select groups of microorganisms

### *3.8.1 Methanogens*

Methanogens play a key role in biogas production during AD. Changes in the dynamics of methanogens may provide important information to better understand biogas production, and thus methanogens have been analyzed in most of the studies that involve microbial analyses. Methanogen sequences constituted over 91% of the archaeal 16S rRNA sequences recovered from each of three full-scale AD bioreactors treating municipal wastewaters and solid wastes, with *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* being represented by most of the methanogen OTUs. The acetoclastic genus *Methanosaeta* was the most abundant methanogenic genus in one of the three full-scale AD bioreactors (accounting for 33.7–67.9% of total methanogens), while *Methanolinea*, a hydrogenotrophic genus belonging to the newly established family *Methanoregulaceae*, is the most predominant genus of methanogens in the other two AD bioreactors (10–41% in the second, and 22–88% in the third AD bioreactor). Hydrogenotrophic methanogen *Methanobacterium* accounted for 34–79% of the total methanogen abundance of the third digester. *Methanosarcina* is another predominant genus of methanogen detected in these digesters (Zhang et al., 2019a).

The occurrence of methanogenic archaea in 78 full-scale AD bioreactors described in 17 publications (dated from 2008 to 2017) was recently

reviewed (Hassa et al., 2018). Feedstock appeared to have a stronger impact on the methanogenic communities than the operating temperature or hydraulic retention time in those AD bioreactors. Hydrogenotrophic methanogens were the major producers of methane from agricultural material or municipal bio-wastes (usually high in concentrations of salt and ammonium), while acetoclastic methanogens, namely *Methanosaeta*, dominated in AD bioreactors receiving municipal sludge. Hydrogenotrophic methanogens in *Methanoculleus*, *Methanomicrobiales* spp. and *Methanobacteriales* spp. are relatively tolerant of ammonia toxicity, while acetoclastic methanogen *Methanosaeta* is usually absent in AD bioreactors with a high concentration of ammonia. In the absence of acetoclastic methanogens, acetate is converted to methane through SAO coupled with hydrogenotrophic methanogenesis (SAO-HM), in which acetate is oxidized to $CO_2$ and $H_2$ by SAO bacteria, such as some members of the class *Clostridia*, and the hydrogen produced is then consumed by hydrogenotrophic methanogens in methane production. Positive correlations between hydrogenotrophic methanogens and *Clostridia* populations were observed in full-scale AD bioreactors (Zhang et al., 2019a). The functional redundancy index also revealed that *Methanomicrobiales* could replace *Methanobacteriales* without changing the formate consumption rate, and *Methanoregula* could replace *Methanosaeta* while maintaining methanogenic community diversity (Zhang et al., 2019a). *Methanosarcina* is a genus of versatile methanogens capable of utilizing acetate, methylamines, methanol, and $H_2/CO_2$, and it is robust when ammonia or salt concentrations are high or when operational conditions (e.g., temperature) fluctuate. It is puzzling that it is not a predominant genus of methanogens in most AD bioreactors.

### 3.8.2 Syntrophic fatty acid-oxidizing bacteria

Many feedstocks fed to AD bioreactors contain lipids. The LCFA released from lipolysis of feedstock lipids, together with some of the SCFA (e.g., propionate, butyrate, and valerate) formed during acidogenesis, must be first converted to methanogenesis substrates (primarily acetate, $H_2$, and $CO_2$) by syntrophic fatty acid-oxidizing bacteria before they can be used to produce biogas by methanogens. Additionally, the accumulation of SCFA or LCFA can lead to upset or even failure of AD bioreactors. Thus, many studies have focused on important syntrophic fatty acid-oxidizing bacteria in biogas-producing microbiomes. These syntrophic bacteria need to live in syntrophy with hydrogenotrophic methanogens through IHT. In three full-scale AD bioreactors, the genera *Syntrophomonas* of *Firmicutes*

(5% relative abundance) and *Smithella* of *Proteobacteria* (8% relative abundance) were found to be the major syntrophic fatty acid-oxidizing bacteria (Zhang et al., 2019a). Both *Syntrophomonas* and *Smithella* are well known syntrophic bacteria that degrade SCFA to $H_2$, formate, and acetate. Species of *Syntrophomonas* are syntrophic butyrate-oxidizing bacteria (SBOB), while *Smithella* is syntrophic propionate-oxidizing bacteria to produce acetate and butyrate. They live in syntrophy with hydrogen-scavenging species of *Methanomicrobiales* or *Methanobacteriales*. Acetate producer *Anaerolineaceae* and acetoclastic methanogen *Methanosaeta* were another two core genera fount to live in syntrophy. This syntrophic relationship was inferred from the positive correlation between the genus *Leptolinea* of the family *Anaerolineaceae* and *Methanosaeta* (Zhang et al., 2019a). Other syntrophs that live in syntrophy with hydrogenotrophic methanogens include the genera *Syntrophus*, *Pelobacter*, *Syntrophorhabdus*, and *Syntrophobacter*, all of which belong to the phylum *Proteobacteria* (Hassa et al., 2018). Some new species of syntrophic fatty acid-oxidizing bacteria were found in the families *Thermoanaerobacteriaceae*, *Costridiaceae*, and *Syntrophomonadaceae* in the class *Clostridia*.

### 3.8.3 Pathogenic bacteria and indicator bacteria

Besides biogas, AD generates residual digestate that is commonly used as fertilizer. Digestate is often used to replace inorganic fertilizers because it is nutrient-rich and inexpensive for growers and farmers. However, digestate may contain pathogens when the feedstocks fed to AD bioreactors contain wastes of human or animal origin. Such pathogens can be spread to the environment and food chain when the digestate is applied to agricultural soil (Maynaud et al., 2016). Potential pathogens in digestate include bacteria (e.g., *Salmonella*, pathogenic *E. coli* strains, *Campylobacter jejuni*), viruses (e.g., adenovirus, rotavirus, and hepatitis A), protozoa (e.g., *Cryptosporidium* spp., *Entamoeba histolytica*, and *Giardia lamblia*), and helminths (e.g., *Ascaris lumbricoides*, *Ascaris suum*, and *Trichuris trichiura*) (Yergeau et al., 2016). Various regulatory agencies often use indicator organisms (e.g., fecal coliforms, *Bacteroides*, *E. coli*, and *Salmonella*) to assess the adequacy and efficiency of the treatment process in reducing pathogen loads in the final product. For example, *Salmonella* must be absent (non-detectable), and fecal coliform levels must not exceed 1000 MPN/g of dry weight in biosludge (Federal Canadian Food Inspection Agency, CFIA).

Culture-based methods (e.g., the MPN method) are the simplest, most inexpensive, and most common methods used to detect live pathogens.

However, they cannot detect non-indicator pathogens or viable but non-culturable (VBNC) bacteria. In contrast, qPCR can detect non-culturable bacteria and could be and has been used to detect the presence of specific pathogens in the digestate. However, the wide range of possible pathogens renders qPCR a highly laborious and costly method. Sequencing-based methods, such as amplicon sequencing of 16S rRNA and the *cpn*60 genes of the pathogens and shotgun metagenomics, are potential alternatives to culture-based and qPCR approaches to detect pathogens in biosludge. Shotgun metagenomics is not limited to the detection of targeted pathogens. It has detected scores of potential pathogens that other methods cannot detect in digestate. For example, viruses, which can serve as an indicator of biosludge treatment efficiency, can represent up to 10–14% of the total sequences in metagenomic datasets but were not detected using other methods (Yergeau et al., 2016). DNA-based methods can determine the presence of pathogens, but they cannot distinguish dead from live pathogens. Pretreatment of samples with propidium monoazide (PMA) can help exclude dead bacteria from being detected by DNA-based methods, but its use in digestate samples is challenging due to the presence of the complex matrix.

Molecular biology techniques and technologies have detected the presence of a wide range of pathogens in digestate, including pathogenic species of *Aeromonas*, *Clostridium*, *Enterococcus*, *Corynebacterium*, *Klebsiella*, *Legionella*, *Mycobacterium*, *Salmonella*, *Streptococcus*, *Vibrio*, *Yersinia*, and parasitic geohelminths or helminths. Among these detected pathogens, *E. coli*, *Salmonella*, *Yersinia enterocolitica*, and *Enterococcus* were reported to be vulnerable to AD. On the other hand, *C. jejuni*, *Streptomyces*, *Collinsella aerofaciens*, *Streptococcus salivarius*, and *Gordonia Bronchialis* can survive AD. In addition, some spore-forming pathogens, such as species of *Clostridium* and *Bacillus*, have high resistance to acute stresses and can survive mesophilic and even thermophilic AD (Zhao and Liu, 2019). When the digestate is stored after the AD process (due to varying demands in different seasons), such as the digestate from AD bioreactors on many confined-livestock farms, different pathogens can also have different survivability. For example, *Listeria monocytogenes* demonstrated greater persistence in digestate as compared to *E. coli* and *Salmonella enterica* Derby during digestate storage. It should be noted that some pathogens can enter the VBNC stage during AD or digestate storage, and they can still pose a health risk because they can regain growth under permissive conditions (Maynaud et al., 2016).

### 3.8.4 Viruses

Digestate generally have DNA viruses (e.g., adenovirus, herpesvirus, papillomavirus, and bocavirus) and RNA viruses (e.g., coronavirus, klassevirus, and rotavirus). Viruses were more resistant than coliform bacteria, but less resistant than bacterial spores, during AD. DNA viruses were also found to be more persistent than single-stranded RNA viruses (Zhao and Liu, 2019). Bacteriophages and archeophages can affect the composition, dynamics, and functions of microbiomes, including the ones in AD bioreactors. Thus, these phages have been included in some studies on biogas-producing microbiomes. Together with a large number of prokaryotic OTUs, 183 phage genes belonging to 78 phages were detected with GeoChip 5.0 from four full-scale AD bioreactors sampled monthly over 1 year (total of 48 samples) (Zhang et al., 2017). The α diversity of phages showed considerable temporal fluctuation, and about half of the phage communities was comprised of "common" phages across the four digesters, while 45 phages were only detected in one of the AD bioreactors. Biochemical pathway analysis linked the phage and the prokaryotic communities to the process performance of the AD bioreactors, and one of the pathways showed the infection of phages on prokaryotic hosts. The relative abundance of the phage families *Myoviridae*, *Siphoviridae*, and *Podoviridae* was significantly correlated with the relative abundance of the class *Methanomicrobia*, revealing a linkage between phages and methane production. Another pathway showed that the lysis of prokaryotes by phages, also known as viral shunt, increased the supply of organic matter in the AD bioreactors, resulting in a positive feedback of net primary productivity. Most enterobacteria phages showed predominantly positive links to prokaryotic OTUs, while several enterobacteria phages showed links to *Proteobacteria* species. On the other hand, *Aeromonas* phage phiO18P, *Aeromonas* phage Aeh1, and *Mycobacterium* phage Che12 showed almost exclusively negative links to prokaryotic OTUs. Future research is needed to investigate the relationship between key guilds of bacteria and methanogens and their phages, and how the phage-host dynamics affect AD performance.

### 3.8.5 Antibiotic resistance

Antibiotic residues and antibiotic-resistant bacteria find their ways to AD bioreactors. Therefore, the microorganisms (both pathogenic and commensal) in anaerobic digestate are also an important source of antibiotic resistance genes (ARGs). ARGs in municipal wastewater were shown to be transferrable to bacteria in biosludge and crops fertilized with the digestate of human wastes (Zhao and Liu, 2019). Bacterial pathogens are potential hosts of ARGs encoding resistance to multi-drugs and macrolide-lincosamide-streptogramin

(MLS). ARGs can spread through mobile genetic elements, such as integron, plasmids, and transposon, with transferrable genes encoding pathogenicity factors. Thermophilic AD has been commonly used to destruct integrons and ARGs in sewage sludge.

### 3.9 New perspectives on biogas-producing microbiomes learned in the molecular biology technique era

The use of molecular biology techniques discussed above revealed the great diversity of the biogas-producing microbiomes. As documented by the 16S rRNA gene sequences produced from clone libraries, the microbiomes underpinning AD collectively can have 5926 OTUs of bacteria representing 28 known bacterial phyla, with *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chloroflexi* being the predominant phyla (Nelson et al., 2011). Nearly 300 OTUs of methanogenic archaea were also documented, and the class *Methanomicrobia* was one of the major groups of methanogens. Based on rarefaction analysis, approximately 40% of the bacterial and 10% of the archaeal diversity in AD bioreactors remained to be identified. It is also found that more than half of the bacterial OTUs cannot be assigned to any known genera. Another important new finding enabled by molecular biology techniques is SAO bacteria and their important role in methane production from acetate in thermophilic AD bioreactors or at high ammonia concentrations. The finding of six clusters of *pct* genes (Li et al., 2013) suggests the presence of a diverse group of syntrophic propionate-oxidizing bacteria in AD bioreactors.

Omics technologies have replaced most of the molecular biology techniques in studies on biogas-producing microbiomes. However, high-density microarrays, qPCR including droplet digital PCR, and SIP can still be very useful because they can complement omics technologies by accurately quantifying the taxa or guilds of microorganisms important to certain aspects of biogas production, such as rate and process stability, or by unearthing the metabolism of important substrates or intermediates during AD.

## 4. The omics technologies used and the microbiome knowledge learned thereby

### 4.1 The unprecedented opportunities provided by omics technologies

The rapid advancement and improvement of omics technologies, especially in the past decade, have provided unprecedented opportunities to characterize the diversity, composition, gene expression, and metabolism of various

microbiomes, including the ones in AD bioreactors, in a comprehensive manner. These omics technologies include metataxonomics metagenomics, metatranscriptomics, metaproteomics, and metametabolomics. Metataxonomics can help identify the bacteria, archaea, fungi, and protozoa by sequencing an amplicon library of a marker gene (mostly a marker gene). Therefore, it is a powerful tool to comprehensively examine the diversity, composition, and structure of biogas-producing microbiomes. Metagenomics, or shotgun metagenomics, can potentially reveal all the genes present in a microbiome by sequencing the DNA extracted from a microbiome sample. Metatranscriptomics analyzes the RNA (either total RNA or mRNA) transcribed in a microbiome, and thus it provides a snapshot of the gene expression at the time of sampling. All these three sequencing-based omics technologies are empowered by the NGS technologies, which include 454 pyrosequencing (no longer used), Illumina sequencing, and Ion Torrent sequencing, all of which afford high throughput sequencing cost-effectively. The third-generation sequencing technologies, including Single Molecule Real-Time (SMRT) sequencing (Pacific Biosciences), the Tru-seq Synthetic Long-Read sequencing (Illumina), and the MinION (Oxford Nanopore Technologies) can generate long sequence reads, facilitating (meta)genome sequence assembly. These technologies have not been used commonly in microbiome research because of their high cost. Unlike the above sequencing-based omics, metaproteomics and metametabolomics are chemical technologies that involve a comprehensive analysis of the proteins and metabolites, respectively, in microbiome samples. They complement the sequencing-based omics by directly analyze the products of gene expression (metaproteomics) or metabolic activities (metametabolomics). These omics technologies, their application to studies of biogas-producing microbiomes, and the new information learned using these omics are discussed in this section.

## 4.2 Metataxonomics

Metataxonomics can comprehensively detail the taxonomic diversity and composition of a microbiome. By eliminating the needs for cloning and by sequencing hundreds of millions of a phylogenetic marker gene, it substantially improves upon the cloning and sequencing approach (Fig. 1). In most reported metataxonomic studies on AD bioreactors, one amplicon library is prepared for each microbiome sample by PCR amplifying one or multiple (two or three) hypervariable regions of the 16S rRNA gene using a pair of prokaryotes- or domain-specific primers. One unique barcode

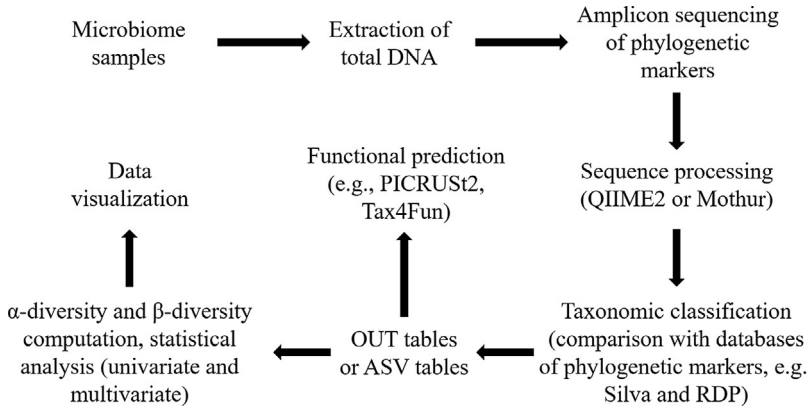


**Fig. 1** Workflow of metataxonomic analysis of microbiomes.

(a short unique sequence region) is added to the 5′ end of the primers to "label" individual samples for multiplexing and simultaneous sequencing in a single sequencing run, primarily on the Illumina MiSeq platform. The sequencing reads are quality checked, processed, and phylogenetically analyzed for taxonomic classification by comparison with reference gene database, primarily Greengenes (https://greengenes.secondgenome.com/), Silva (https://www.arb-silva.de/), RDP (https://rdp.cme.msu.edu/), and NCBI RefSeq (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/). The fungal community in AD bioreactors is analyzed similarly, but one of the two internal transcribed spacers (mostly ITS1) was used because the fungal 18S rRNA gene does not provide enough phylogenetic resolution to distinguish fungal species. The UNITE database is a specialty database for fungal identification based on ITS1 sequences (Nilsson et al., 2018). For analysis of the protozoal community, a region of their 18S rRNA gene is sequenced. Several metataxonomic analysis pipelines have been developed and used in metataxonomics, with QIIME (with QIIME2 being the most recent version) being the most commonly used, followed by Mothur and MG-RAST. Both the α diversity, which describes the diversity and structure of microbiomes, and β diversity, which compares the overall diversity of two microbiomes, can be calculated. Then, univariate and multivariate statistical analyses (e.g., principal component analysis, principal coordinates analysis, non-metric multidimensional scaling, partial least squares discriminant analysis, and LEfSe) are used to compare multiple microbiomes or to identify individual taxa of microorganisms that are affected or changed by feedstocks or operating conditions.

Metataxonomics can identify the microorganisms present in microbiome samples but reveal little information on their metabolism or functions. For some of the detected microorganisms that are closely related to known species, their potential functions can be inferred from the genomes and functions of the known microorganisms. Four bioinformatics tools have been developed and used for such a functional prediction: PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013), Tax4Fun (Aßhauer et al., 2015), Piphillin (Iwai et al., 2016), and PanFP (Jun et al., 2015). Most studies used PICRUSt or its newest version PICRUSt2 (Douglas et al., 2019). In PICRUSt, 16S rRNA gene copy number-based normalized taxonomic abundance information is used to link the taxonomic IDs and the KEGG ortholog database (KEGG, https://www.genome.jp/kegg/) following ancestral-state reconstruction. PICRUSt2 can use KEGG and other databases, including COG (http://eggnogdb.embl.de/), ExPASy (https://enzyme.expasy.org/), PFAM (https://pfam.xfam.org/), and TIGRFAM (https://www.jcvi.org/tigrfams). PICRUSt has only been used to predict potential function from 16S rRNA gene sequences. PICRUSt2 can be used to predict functions from 18S or ITS sequences retrieved from fungi using the 1000 Fungal Genomes Database (http://1000.fungalgenomes.org). It should be noted that such a functional inference is only a prediction and will not provide a reliable functional profile.

The metataxonomic data can also be further analyzed to answer other important ecological questions. It is important to identify the taxa that contribute to the functional profiles and shifts in a microbiome. FishTaco was developed to identify taxa that drive functional shifts in the human gut microbiomes associated with diseases (Manor and Borenstein, 2017). It is an analytical and computational framework that integrates taxonomic and functional comparative analyses to accurately quantify taxon-level contributions to disease-associated functional shifts. It has been used in studies on other microbiomes, but not the microbiomes in any AD bioreactors yet. It shall be useful in identifying the bacteria and methanogens that contribute to functional shifts when AD is affected.

Microorganisms interact with each other, either positively or negatively. Conceptually, positive interaction results in positive correlation in their abundance, while negative interaction leads to negative correlation. In many microbiome studies, co-occurrence patterns of genera or OTUs were used to infer positive or negative interactions. Gephi (Bastian et al., 2009) and Cytoscape (Shannon et al., 2003) are two commonly used software tools

to depict co-occurrence networks. CoDiNA has been recently developed to allow users to compare differential co-occurrence networks (Gysi et al., 2018). Thus, it can determine how changing feedstock and other operating conditions may affect the interactions among different microorganisms in future studies.

Metataxonomics has been used in numerous studies in the past decade. These studies provided new insight into the diversity and composition of biogas-producing microbiomes. For example, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Chloroflexi* were confirmed to be the predominant phyla of bacteria. Microbial successions during the startup of AD bioreactors can also be detailed using metataxonomics. The feedstocks fed to AD bioreactors can induce the emergence of different active bacterial and archaeal assemblages (Lu et al., 2013). In a relatively large-scale study that comparatively examined 32 full-scale AD bioreactors fed wasted activated sludge over a 6-year period using 16S rRNA gene amplicon sequencing, it was found that several of the most abundant populations were likely inactive and immigrating with the influent (Kirkegaard et al., 2017). Apparently, the failure to exclude nonactive microorganisms immigrating with feedstocks, especially feedstocks that contain dense microorganisms (e.g., wasted activated sludge and livestock manure), can interfere with correlation analysis between microbial and AD performance data. A core microbiome was also identified among these AD bioreactors. Most of the biogas-producing microbiomes have a high diversity at species or below levels, suggesting functional redundancy in AD bioreactors. *Anaerolineaceae* is a group of novel abundant primary acidogens in AD bioreactors treating waste activated sludge (McIlroy et al., 2017). Proteolytic bacteria are quite abundant in full-scale AD bioreactors fed municipal solid waste (Cardinali-Rezende et al., 2016). Stage-specific bacterial and archaeal populations were found to reside in thermophilic vs. mesophilic AD bioreactors (Li et al., 2015; Shaw et al., 2017). Based on rRNA/rRNA gene ratio, some rare taxa were found to have disproportionate activities in AD bioreactors (Jia et al., 2019). By comparing the metataxonomic data from DNA and RNA, it is found that a significantly greater diversity results from DNA than from RNA for archaea, but not for bacteria, and β-diversity analysis showed a significant difference in microbiome composition for both bacteria and archaea between the DNA- and RNA-based analysis (De Vrieze et al., 2018a). The same authors also showed in another study that DNA- and RNA-based metagenomic analyses revealed different responses of biogas-producing microbiomes to salt perturbation (De Vrieze et al., 2016). Unique bacteria

and archaea were also found in solid-state AD bioreactors, probably attributable to the physical and chemical features in solid-state AD bioreactors (Li et al., 2015). High concentrations of ammonia led to an increased predominance of genera that contain syntrophic bacteria (e.g., *Clostridium*, *Bellilinea*, *Longilinea*, and *Bacteroides*) and shift of methanogens from acetoclastic *Methanosaeta* to hydrogenotrophic *Methanoculleus* and the poorly understood methylotrophic *Methanomassiliicoccus* (Ruiz-Sanchez et al., 2019). New SAO bacteria also increased at high ammonia concentrations. Co-occurrence network analysis of rRNA, not rRNA gene, can identify active populations, and syntrophic and methanogenic taxa are highly represented within the networks, indicating that the obligate energy-sharing partnerships between syntrophic bacteria and methanogens play critical roles in stabilizing biogas production (Ziels et al., 2018a). The combination of SIP and metataxonomics allows identification of bacteria that mediate the conversion of particular substrates in AD bioreactors (Limam et al., 2014).

Metataxonomics enables comprehensive analysis of complex microbiomes by potentially identify all their members of prokaryotic and eukaryotic microorganisms. This is demonstrated by the numerous studies reported in the literature. However, metataxonomics has its limitations and can result in biases. First, DNA extraction methods can have different efficiency and bias of DNA recovery. Second, variations can be introduced by the selection of marker genes (including the region to PCR amplify, PCR primers, number of PCR cycles, etc.) in preparation of amplicon libraries (Bonnet et al., 2002; Walker et al., 2015). Third, different sequencing platforms, sequence analysis pipelines, and databases affect the analysis results, especially with respect to minor taxa. Fourth, only a short region (<500 bp) of a marker gene is sequenced in most studies, and such short sequences may not have the sufficient phylogenetic information needed to detect microorganisms at the species level. Fifth, the detection of a taxon does not necessarily reflect its activity, and DNA- and RNA sequencing can produce different results. Additionally, in metataxonomic studies, the relative abundance of individual taxa is used to reflect their richness, but the relative abundance of unchanging taxa is affected by that of changed taxa. Co-occurrence network analysis only reflects a mathematical relationship between two taxa, but not biological interaction. Furthermore, different amplicon libraries need to be prepared for prokaryotes, fungi, and protozoa.

Metataxonomic studies on biogas-producing microbiomes will further improve with the advancement of sequencing technologies, improvement

of phylogenetic analysis pipelines, and expansion of databases. The most commonly used phylogenetic marker 16S rRNA genes can be sequenced to full length using the third-generation sequencing technologies. The biogas-producing microbiomes have their unique features with respect to diversity and functions. Dedicated databases for the microorganisms involved in AD will greatly help robust analysis of metataxonomic data of biogas-producing microbiomes. In most studies, results are often compared with those from other studies. Because results can vary because of differences in DNA extraction and other analysis methods, a standardized protocol is required. A standardized analysis protocol will also enable the compilation of datasets from different studies to develop more robust anaerobic digestion models.

## 4.3 Metagenomics

Shotgun metagenomic sequencing refers to the sequencing of metagenomic DNA extracted directly from microbiome samples such as samples from AD bioreactors. Metagenomics goes beyond metataxonomics based on the sequencing of amplicons of phylogenetic markers such as 16S rRNA genes because it provides insight into the taxonomic diversity and functional diversity as well as the physiological potential of microbiomes (Vanwonterghem et al., 2014). Metagenomics entails high throughput sequencing of metagenomic DNA, sequence assembly, gene prediction and annotation, and binning of contigs into metagenome-assembled genomes (MAGs) or genome bins (GBs) (Fig. 2). Shotgun metagenomic sequencing is most commonly done using the Illumina HiSeq platform because of its low cost and high throughput capacity. The de novo assembly of metagenomic sequence reads is often the most challenging step because it is computing demanding and time-consuming. Many algorithms and bioinformatic tools have been developed to improve the speed and the accuracy of de novo assembly, such as MEGAHIT, MetaVelvet, metaSPAdes, IDBA-UD, Genovo, and SOAPdenovo2. De novo assembly is computing intensive and prone to errors. The quality of assembled contigs needs to be assessed by mapping the sequence reads to the contigs or comparing metagenome assemblies to close references using MetaQuast. Binning can be performed using CONCOCT, MetaBAT2, or MaxBIN2.

Annotation of metagenomic genes is achieved by comparison to databases. The commonly used databases include Gene Ontology (GO, http://geneontology.org/), KEGG, COG, IMG/M (https://img.jgi.doe.gov/), SEED (http://pubseed.theseed.org/), and Pfam. Metabolic pathways can

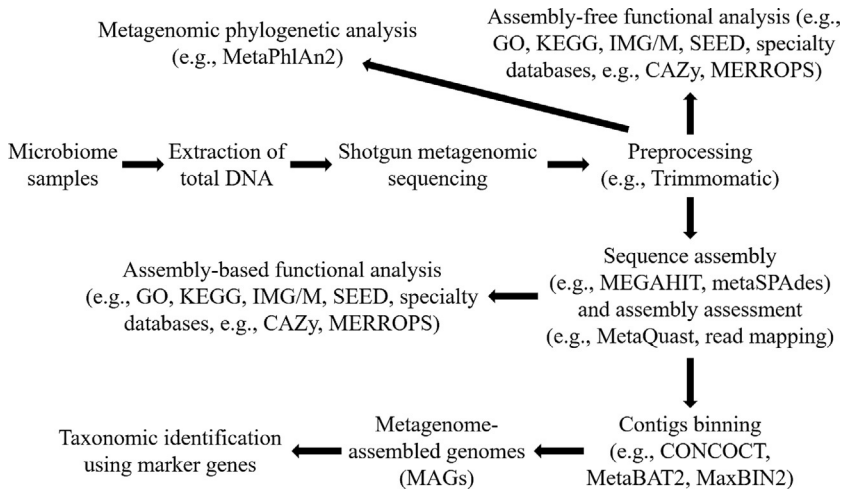


**Fig. 2** Workflow of metagenomic analysis of microbiomes. Functional and taxonomic features are statistically analyzed using univariate and multivariate analyses as done in metataxonomic analysis.

be reconstructed and or modeled from the genes annotated to metabolic pathway databases, such as the KEGG Pathway database (https://www.genome.jp/kegg/pathway.html), the MetaCyc Metabolic Pathway Database (https://metacyc.org/), and the BRENDA (BRaunschweig ENzyme DAtabase) database (http://www.brenda-enzymes.org). The metagenomic sequences can also be annotated by comparison with "specialty databases," such as the CAZy database (http://www.cazy.org/) dedicated to carbohydrate-active enzymes, the MEROPS database (https://www.ebi.ac.uk/merops/) for peptidases. Therefore, metagenomics can potentially recover the genomes and predict the metabolism of novel and unculturable microorganisms. Metagenomics has been used in many studies on the biogas-producing microbiomes. This has led to the compilation of hundreds of MAGs or GBs. The Biogas Microbiome Database (https://biogasmicrobiome.env.dtu.dk/) is one repository that contains a collection of reference microbial genomes (1635 MAGs, at the time of writing) recovered from biogas-producing microbiomes (Campanaro et al., 2019). The metagenomic data provided new information on the functional diversity of biogas-producing microbiomes and the metabolic pathways that underpins the AD process.

In the past decade, many studies have investigated the biogas-producing microbiomes using metagenomics. The first metagenomic study was

reported in 2008 that used the 454 pyrosequencing technology and examined the functional diversity of the microbiome in a full-scale AD bioreactor fed with maize silage, green rye, and some chicken manure (Schlüter et al., 2008). Besides house-keeping genes, genes involved in the degradation of sugars and amino acids were found. With a 64% depth coverage of the genome of *Methanoculleus marisnigri* JR1, the genus *Methanoculleus* was thought to play a dominant role in methanogenesis in that AD bioreactor. Moreover, numerous contigs were assigned to clostridial genomes, including genes encoding cellulolytic functions, suggesting that clostridia are important for the hydrolysis of cellulosic plant biomass in that AD bioreactor. One of the most ambitious metagenomic sequencing efforts was made to characterize the metagenomes of four full-scale biogas plants (three mesophilic and one thermophilic) digesting maize silage together with manure (Stolze et al., 2016). From the 348 Gb of sequence data obtained, de novo assembly yielded a total of 1.5 Gb of contigs and supercontigs. About 1.6 million genes were predicted from the four metagenomes. Binning of these (super)contigs resulted in 532 MAGs, with five of them meeting the stringent quality requirements and being assigned to the taxa of interest (in the phyla *Thermotogae*, *Fusobacteria*, *Spirochaetes*, and *Cloacimonetes*). Most of the MAGs represent novel and uncharacterized species, corroborating the premise that most of the microorganisms remain unknown. The metabolism of these five MAGs was deduced from the MAG sequence information. The MAGs assigned to *Fusobacteria* and *Cloacimonetes* appeared to be from amino acid-fermenting and $CO_2$/$H_2$-producing bacteria, while those assigned to *Thermotogae* and *Spirochaetes* were probably recovered from bacteria that utilize sugar to produce acetate, $CO_2$, and $H_2$. However, the nearly 1.6 million hypothetical genes identified in that study remain to be further analyzed. New cellulolytic bacteria and glycoside hydrolase (GH) genes were also abundant in AD bioreactors (Wei et al., 2015).

The biogas-producing microbiomes are very diverse and contain thousands of species of microorganisms. However, the number of niches is much smaller than the taxonomic species richness. Indeed, several metagenomic studies revealed high functional redundancy in AD bioreactors (Bertucci et al., 2019; De Vrieze et al., 2017; Langer et al., 2015). Such functional redundancy explains taxonomic variations under certain changing operational conditions, while biogas production remaining relatively stable. It also explains the recovery of upset AD bioreactors without external inoculation. Some of the metagenomic studies focused on the genetic potential of cellulose degradation, which is often the bottleneck step in biogas production

from cellulosic feedstocks. In one metagenomic study, 19,335 CAZymes were identified from four full-scale mesophilic AD bioreactors in Denmark (Wilkens et al., 2017). About 30% of these CAZymes showed 50% or lower sequence similarity to known proteins, demonstrating the presence of novel CAZymes in these AD bioreactors. Many different α-glucan-acting CAZymes were identified, with GH13 being most abundant. Cellulase genes were also abundant in the four metagenomes, which include almost exclusively endoglucanases and beta-glucosidases. No dockerin domains were identified, suggesting that the cellulolytic enzymes in those AD bioreactors are not organized into cellulosomes. Xylanase genes were also abundant, and most of them encode xylanases and β-xylosidase. A battery of accessory enzymes was also found. Given the importance of CAZymes in biogas production from agricultural feedstocks, more studies with a focus on CAZymes are expected.

The major methanogenesis pathways in AD bioreactors have been identified by assigning metagenomic sequences to the KEGG Pathway database. The functional potential of metagenomes in AD bioreactors depends strongly on the feedstocks. The genes involved in acetoclastic methanogenesis are more abundant in AD bioreactors fed with sewage sludge. The most abundant genes of the acetoclastic pathway include the genes encoding acetyl-CoA synthetase and the acetyl-CoA decarbonylase/synthase complex. These genes are essential in the synthesis of acetyl-CoA from acetate. However, these genes are also present in bacteria. Therefore, the identification of major methanogenesis pathways based on the abundance of these genes needs to be interpreted with caution (Hassa et al., 2018). On the other hand, the genes involved in the hydrogenotrophic pathway are at high abundance in AD bioreactors fed agricultural residues and livestock manure. The most dominant genes of the hydrogenotrophic pathway include those encoding formate dehydrogenase and formylmethanofuran dehydrogenase. Genes involved in the methylotrophic pathway are at low abundance, indicating that this methanogenesis pathway is generally of less importance in AD bioreactors.

Nearly complete genomes of dominant bacteria can be assembled from shotgun-sequenced metagenomes, but MAGs cannot be obtained from bacteria or methanogens that have a low relative abundance (typically $<1\%$) owing to insufficient sequencing depth or difficulty in binning (classification) and assembly of individual genomes. Rapid improvements in sequencing throughput, read length, and quality have made it theoretically possible to assemble genomes from low abundance populations in "deep" (tens of Gb per sample) metagenomic data sets. Indeed, genomes

of rare and uncultured bacteria inhabiting an AD bioreactor fed activated sludge were obtained by differential coverage binning of multiple metagenomes (Albertsen et al., 2013). A total of 31 GBs was generated that could be assigned to five different phyla, and they included many rare members with a relative abundance as low as 0.02% in the microbiome. Thirteen of these GBs were further refined into complete or nearly complete MAGs, with four being assigned to the candidate phylum TM7. The nearly complete TM7 genomes (average genome size of 1 Mb) contain all the key genes of the pentose phosphate pathway and the heterolactic fermentation pathway, but not any of the key genes of the Embden-Meyerhof-Parnas pathway (i.e., the phosphofructokinase gene) or the Entner-Doudoroff pathway (i.e., the KDPG aldolase gene). This suggests that members of TM7 probably use only the pentose phosphate and heterolactic fermentation pathways. The differential coverage binning approach can be used in recovering the genomes of candidate taxa and other under-represented or rare taxa.

Many MAGs and GBs were recovered from recent metagenomic studies that used deep sequencing. The initial Biogas Microbiome database contained 236 GBs that were assembled from sequences generated using the Illumina NextSeq 500 platform. A core microbiome for biogas production was suggested by a comparison between two datasets (Campanaro et al., 2016; Treu et al., 2016). This core microbial group was found to be present independently from the operational conditions and contain members of *Methanoculleus*, *Methanothermobacter*, *Syntrophomonas*, and *Proteobacteria*. The composition of the archaeal community was also found to be resilient, and the bacterial community was more diverse due to higher functional variability. In addition, only 13% of the GBs can be identified at the genus level (30 GBs) and 3% (8 GBs) at the species level. The taxonomic assignment of the remaining 84% (198 GBs) was possible only at higher taxonomic levels, confirming that most biogas-producing microorganisms have not been characterized.

By compiling 134 publicly available metagenomic datasets derived from different AD bioreactors, 1635 MAGs were obtained recently (Campanaro et al., 2019). These MAGs were estimated to be >50% complete, and nearly half of them were ≥90% complete with ≤5% contamination. Of the abundant MAGs (each with a relative abundance >1%), only 25 were identified in more than 10% of the datasets. Four of these prevalent MAGs were singularly assigned to Candidatus *Methanoculleus thermohydrogenotrophicum*, *Methanosarcina thermophila*, *Methanothrix soehngenii*, and *Methanoculleus thermophilus*. The remaining 21 MAGs were assigned to the phyla

*Firmicutes* (14 MAGs), *Bacteroidetes* (2 MAGs), *Synergistetes* (2 MAGs), *Thermotogae* (1 MAG), and *Coprothermobacterota* (1 MAG). The abundant *Thermotogae* MAGs were classified as *Defluviitoga tunisiensis*, potentially reflecting its central role in thermophilic AD. The minor MAGs were assigned to *Chloroflexi*, *Elusimicrobia*, *Firmicutes*, and *Planctomycetes* among other bacterial phyla. Analysis (based on distribution and completeness) of the MAGs for KEGG pathway modules showed that only a very small number of modules was widespread among the MAGs, with only 15 modules present in more than 90% of the phyla, 108 in 50–90% and 434 in less than 50% of the phyla. This suggests a partitioning of microorganisms according to their predicted substrate utilization capacities. Additionally, many of the modules showed a remarkable association with specific taxa. Some of the crucial AD processes, such as the conversion of acetate to $CO_2$, may be carried out by a small number of species. It was also concluded that the biogas-producing microbiomes are flexible at the species level, allowing for adaptation to different conditions, including a wide range of operational temperatures and feedstocks. This adaptation is facilitated by the presence of functional redundancy in biogas-producing microbiomes. It should be noted that the complied datasets were generated using different methods, including DNA extraction, sequencing, bioinformatic analysis (databases and software programs used), and metadata registration. The high heterogeneity of the methods used probably affected the analysis results. A standardized set of methods and analyses are required to compare different datasets.

The application of metagenomics has provided a deeper mechanistic understanding of AD microbiomes and substantially extends the existing repository of genomes. Metagenomics enables the identification of previously unknown but abundant species featuring important functional potential in the context of the AD process (Maus et al., 2017). Novel and rare species residing in AD bioreactors have also been discovered. The established database represents a useful resource for future studies related to AD bioreactors. However, detection of particular genes does not inform if they confer any metabolic activity (De Vrieze et al., 2016). The complexity and diversity of biogas-producing microbiomes make it difficult to identify the rare species, which may be important to the AD process by affecting other keynote species. Additionally, the analysis of metagenomic sequencing data, especially the sequence assembly step, is time-consuming and requires bioinformatics expertise in microbiology. The limited reference genomes of biogas-producing microorganisms also hinder accurate assembly and

annotation of metagenomic sequences from biogas-producing microbiomes (Bharagava et al., 2019). Genomic sequencing and studies of major microorganisms involved in each of the four phases of AD, at least representative ones, will help future metagenomic and other omic studies of biogas-producing microbiomes. Continued improvement in DNA sequencing technologies, including the third-generation sequencing technologies, will further lower the cost while increasing the read length and throughput and the identification of microorganisms at low abundance. Furthermore, novel binning methods, such as the differential coverage binning method (Albertsen et al., 2013), can also help identify rare microorganisms in biogas-producing microbiomes. Of course, metagenomic data do not provide any information on the gene expression. This is exemplified by the significant differences in the relative abundance of methanogens found in metagenome (4% of total 16S rRNA sequences) and metaproteome (20–30% of total identified proteins) (Hanreich et al., 2013). To further elucidate the metabolic activities critical to biogas production, metatranscriptomics and/or metaproteomics are required.

## 4.4 Metatranscriptomics

In microbiome studies, it is essential to link microorganisms to their specific activities. Metatranscriptomics offers the ability to determine RNA sequences matching a specific functional activity or category and the microorganisms produce those RNA sequences. Metatranscriptomics involves the sequencing of all the transcripts of a given microbiome and bioinformatic analysis of the transcripts (Fig. 3), thus providing insights into its transcriptional activity by revealing what genes are expressed at RNA level at a microbiome scale. Therefore, metatranscriptomics can capture a snapshot of the gene expression of a chosen microbiome at a given moment and under specific conditions by sequencing the total mRNA. It can complement metagenomics by revealing the microorganisms that are actively growing and the genes that are expressed. In order to analyze a metatranscriptome, the first step is to isolate high-quality RNA. It is more difficult to isolate representative RNA than to extract representative DNA from microbiome samples that contain complex matrix such as AD bioreactor samples. Additionally, different isolation methods may yield different isolation efficiencies, leading to nonrepresentative RNA. In general, isolation of RNA is less efficient from Gram-positive than from Gram-negative bacteria, and thus transcripts from Gram-positive bacteria may be under-represented,

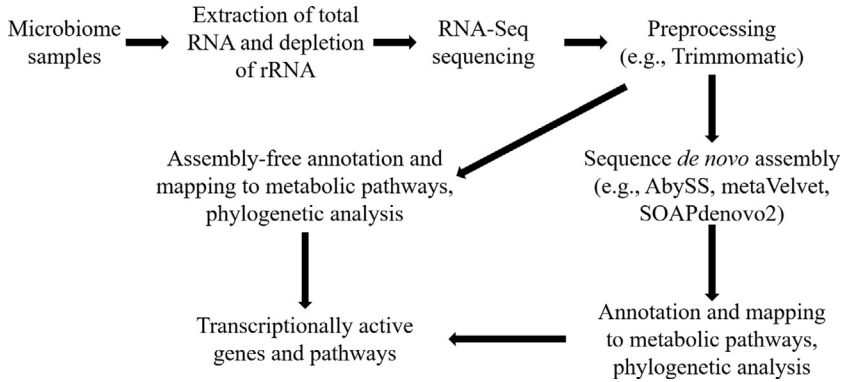


**Fig. 3** Workflow of metatranscriptomic analysis of microbiomes. Functional and taxonomic features are statistically analyzed using univariate and multivariate analyses as done in metataxonomic analysis. Specialty bioinformatic pipeline, such as MetaTrans and SAMSA2, can also be used (see Section 4.4 for details).

whereas transcripts from Gram-negative bacteria may be over-represented (Stark et al., 2014). Special caution must be taken when interpreting metatranscriptomics data or using such data for certain purposes, such as modeling of the AD process. Total RNA can be sequenced so that the diversity and composition of a microbiome can be examined from the rRNA sequences, while the functional genes that are expressed can be determined from the mRNA sequences. Because about 85% of the total RNA is rRNA, mRNA is under-represented. Alternatively, mRNA can be enriched by depleting rRNA. RNA can be reverse transcribed to the corresponding cDNA and sequenced as DNA on Illumina platforms or directly sequenced on the Nanopore platform. The first approach requires reverse transcription and amplification and produces short sequencing reads, whereas the direct RNA sequencing on the Nanopore platform has a high error rate. Continued improvement of direct RNA sequencing will greatly facilitate robust transcriptomics.

The bioinformatic analysis of a transcriptome includes the following steps. The first step is to filter out low-quality raw sequences to improve the accuracy of the metatranscriptome. The quality-checked sequences are either subjected to bioinformatic analyses directly or de novo assembled into transcript contigs using bioinformatic tools that are adapted to de novo assembly of RNA sequencing data, such as AbySS, MetaVelvet, Oases, SOAPdenovo2, Trans-Abyss, and Trinity. De novo assembly can improve annotation, but it is computing demanding and requires powerful sequence

assembler programs and computers. The quality-checked sequences or the transcript contigs can be annotated by mapping to reference genome databases. Such an approach can produce accurate annotation, but it is limited by the lack of reference genomes of new and novel microorganisms. In most studies, the quality-checked sequences or transcript contigs are annotated by comparison to reference databases, which are the same as the one used in the annotation of metagenomic sequences. Specialty bioinformatic tools are available to annotate transcript sequences or contigs. MetaTrans is a pipeline that exploits multi-threading computers to analyze metatranscriptomic data. It can analyze both 16S rRNA for taxonomic diversity and composition and mRNA for gene expressions. Analysis using MetaTrans involves four major steps. First, sequence reads are quality-filtered. Second, the sequences are sorted into rRNA and mRNA. Third, 16S rRNA sequences are mapped against the Greengenes database using SOAP2 to determine taxonomic diversity. Fourth, mRNA sequences are mapped against a functional database (e.g., MetaHIT-2014) using SOAP2 to identify the gene expressed. MetaTrans is highly flexible and offers the possibility to use third-party tools. However, it relies on rRNA sequences for the identification of microorganisms.

SAMSA2 is a standalone metatranscriptome analysis pipeline that is designed specifically for metatranscriptomic data analysis. It can perform preprocessing (merging of paired-end reads with PEAR, removing low-quality sequences with Trimmomatic, and removing rRNA sequences with SortMeRNA), annotation of transcript sequences with DIAMOND against custom DIAMOND-searchable databases, which can be created from NCBI's RefSeq database, the SEED Subsystems Hierarchical database, and other databases including the CAZy database. SAMSA2 can sort metatranscriptome data by microorganisms or by function or functional category. One metatranscriptome can be subdivided to examine only the sequences annotated to a single genus or species so to determine which microorganisms produce a specific group of transcripts. Therefore, SAMSA2 can help obtain both the taxonomic and functional results for each specific sequence in an entire metatranscriptome. Web-based servers, such as COMAN (http://sbb.hku.hk/COMAN/) and MG-RAST (https://www.mg-rast.org/) can also be used to analyze metatranscriptomics data. These web servers eliminate the need to install programs into local computers and are simpler to use. However, they are not as flexible as standalone pipelines and often limit the size of data that can be processed.

Metatranscriptomics has been used in many recent studies to investigate how some of the most important factors affect the performance of AD bioreactors. Fischer et al. (2019a) examined the response of biogas-producing microbiomes to different ammonia nitrogen concentrations (4.9 vs. $8.0\,g\,L^{-1}$) with respect to microbiome composition using metataxonomics and the transcriptional activity using metatranscriptomics. It was found that *Clostridia* and *Methanomicrobiales* dominated the microbiome persistently at both ammonia concentrations, while *Methanosarcinales* was only present at low relative abundance. The genes encoding enzymes of the hydrolysis step (e.g., cellulase, EC 3.2.1.4) were expressed at high levels, and they were assigned to *Clostridia*. The genes encoding the key enzymes of the methanogenic pathway (e.g., methyl-CoM reductase, EC 2.8.4.1; heterodisulfide reductase, EC 1.8.98.1) were also expressed at high levels. At the high ammonia concentration, expression of the genes involved in cellulose hydrolysis and methanogenesis were significantly down-regulated. It was concluded that besides methanogenic archaea, hydrolytic cellulose-degrading microorganisms were also negatively affected by high ammonia concentrations. Both *Acholeplasma* and *Erysipelotrichia*, both of which showed lower abundance under increased ammonia concentrations, might serve as indicator species for earlier detection of ammonia shock. In another study (Fischer et al., 2019b), the same authors showed that the microbiome in a full-scale mesophilic AD bioreactor was highly flexible to changing ammonia concentration and feedstocks by changing its composition and transcriptional activities over nearly 600 days of operation. As ammonia concentration increased, *Methanosarcina* gave way to *Methanomicrobiales* and the genes involved in acetoclastic methanogenesis were down-regulated, while those involved in syntrophic acetate oxidations and hydrogenotrophic methanogens were up-regulated. It was concluded that the overall process performance could maintain stable during increased ammonia concentration by shifting the microbiome composition and transcriptional activities.

Anaerobic digestion of waste sludge is slow because of the limited amounts of readily fermentable carbohydrates present, and thus it necessitates long retention time. Different pretreatments have been evaluated to increase the AD rate by enhancing the hydrolysis of polymeric feedstocks such as plant cell wall materials and microbial cellular proteins. In a study evaluating the efficacy of pretreatment using part-stream low-frequency ultrasound (Xia et al., 2018), the pretreatment resulted in a gradual increase of *Bacteroidales* and its transcriptional activity in hydrolyzing cellulosic biomass. *Thermotogales* with high cell mobility was also highly active transcriptionally. Hydrogenotrophic methanogenesis was the major pathway,

and it was mostly mediated by *Methanomicrobiales*. More interestingly, it was found that the genus *Methanoculleus* was the major contributor to methanogenesis within *Methanomicrobiales* and a major group of protein hydrolyzers. Cellulosic biomass from agriculture is an abundant feedstock for the production of biogas as a renewable energy source. To understand the cellulosic biomass-degrading potentials in two full-scale AD bioreactors, Jia et al. (2018) used both metagenomics and metatranscriptomics to analyze the active microbial populations. *Clostridium cellulolyticum*-related, *Clostridium leptum*-related, and *Ruminococcus*-related bacteria were identified as major hydrolyzers and primary fermenters. The authors maintained that their results could facilitate the development of potential biomarkers and the rational design of the microbiome in AD bioreactors (Jia et al., 2018). Other transcriptomic studies revealed that (i) *Thermotogae* and *Spirochaetes* were important to sugar metabolism, while *Fusobacteria* and *Cloacimonetes* contributed significantly to amino acid metabolism (Stolze et al., 2018); (ii) *Defluviitoga*, *Clostridium* cluster III, and *Tepidanaerobacter* were highly transcriptionally active bacteria, while *Methanoculleus* is the most transcriptionally active methanogens in three mesophilic and one thermophilic AD bioreactors, whereas *Hallocella*, *Tepidimicrobium*, and *Methanothermobacter* were transcriptionally less active (Maus et al., 2016); (iii) AD process upset and failure could impact the transcriptional activities of many bacteria and methanogens (Grohmann et al., 2018); and (iv) syntrophic and methanogenic taxa were highly correlated in co-occurrence network. These metatranscriptomic data not only shine new light on the microorganisms that are important to the AD process and their response to variation in feedstock and operational conditions but are also useful to improve Anaerobic Digestion Model 1 (Weinrich et al., 2019).

## 4.5 Metaproteomics

Metaproteomics is the identification and quantification of the microbial enzymes and proteins of microbiomes. By linking the function of proteins to a taxon, it is possible to draw correlations between the presence of proteins and metabolic activities. While metatranscriptomics provides insight into gene expression and activity, cellular localization and regulation of enzymatic activities occur at the protein level. In metaproteomics, proteins are extracted from a mixed microbiome sample, followed by protein purification, fractionation, separation using liquid chromatography (LC), and detection with tandem mass spectrometry (MS/MS) (Heyer et al., 2015; Vanwonterghem et al., 2014). Peptide and proteins are then identified through a comparison of fragment spectra against theoretical spectra from

a database, such as UniProt/Swiss-Prot (https://www.uniprot.org/), KEGG, and GO. Specialty database, such as PRIDE Archive (https://www.ebi.ac.uk/pride/archive/), and algorithms and pipelines, such as MetaProteomeAnalyzer (Muth et al., 2015), are also available for annotation of metaproteomes. The number of correctly identified peptides and proteins and the reliability of their identification can be increased by combining multiple algorithms (Heyer et al., 2015).

Metaproteomics has been applied to various AD bioreactors to identify key enzymes important to the metabolic pathways and novel functional proteins (Vanwonterghem et al., 2014). Combining isoelectric focusing (IEF) and GeLC-MS/MS, Kohrs et al. (2014) identified up to 1000 proteins that were mapped to the main steps of hydrolysis, acidogenesis, acetogenesis, and methanogenesis in an AD bioreactor fed a mixture of whole crop silages of maize, forage rye, cattle manure and slurry and operated at mesophilic and then thermophilic temperatures. Using a combined metagenomic and metaproteomic approach, the microbiome in an AD bioreactor degrading plant carbohydrates was analyzed at both the gene and protein level (Hanreich et al., 2013). About 98% of the metagenomic sequences were identified to be bacterial and the remaining 2% archaeal. The orders *Clostridiales* and *Bacteroidales* were predominant and were thought to play important roles in the degradation of plant carbohydrates. The metaproteomic data revealed several GHs expressed by *Firmicutes*, suggesting *Firmicutes* as the main degraders of cellulose. Metaproteomic analyses also detected a large number of sugar transporters, expressed by members of the *Bacteroidetes*, which probably takes up various glycans efficiently and digests some of the plant polysaccharides.

Significant differences in the relative abundances of both bacteria and methanogens were reported between metagenomic and metaproteomics analyses. While metagenomic data showed that methanogens represented only 2% of the microbiome, 20–30% of the proteins identified through metaproteomics were of archaeal origin (Hanreich et al., 2013). These results suggest a posit that methanogens were disproportionally active. In another study, the discrepancy in relative abundance of metagenomic and metaproteomic data was highlighted once again when proteins for both hydrogenotrophic and acetoclastic methanogens were detected, even though 99% of the *Euryarchaeota* sequences were represented by hydrogenotrophic methanogens and 1% by acetoclastic methanogens (Hagen et al., 2017). The discrepancy between metagenomic and metaproteomic data was also reported for the bacterial genera, which was highly abundant in the pyrosequencing data

but not on the metaproteomic data (Lü et al., 2014). Therefore, future research needs to use multiple meta-omic approaches when investigating biogas-producing microbiomes.

The main microorganisms and metabolic pathways driving the different steps of AD of food wastes at thermophilic temperature (60 °C) and elevated levels of free ammonia were investigated using metataxonomics, metagenomics, and metaproteomics by Hagen et al. (2017). Several bacteria were identified to actively produce enzymes needed for the degradation of proteins, amino acids, and polysaccharides (the major components of food waste). The metaproteomic analysis suggested that acetate turnover occurred predominantly via syntrophic acetate oxidation in coexistence with hydrogen-consuming *Methanothermobacter thermoautotrophicus*-like methanogens. The metaproteomic data also suggested acclimation of a member of *Methanosaeta* to high ammonia levels. The metaproteomic data corroborated shift from acetoclastic methanogenesis to hydrogenotrophic methanogenesis at high ammonia levels and at thermophilic temperature. *Methanosaeta* was at low abundance, but several enzymes involved in its acetoclastic methanogenesis pathway were detected in the metaproteome, indicating that *M. thermophila* is metabolically active in the thermophilic AD of food wastes. Based on the identified enzymes required for glycolysis, the pentose phosphate pathway, methanogenesis pathways, Abram et al. (2011) proposed a metabolic model for AD of synthetic glucose-based wastewater. Because cellulosic biomass and livestock manure are the major feedstock for biogas production, similar research is needed to model the metabolic pathways for the degradation of these feedstocks to biogas.

Infection with phages can lead to lysis of host microorganisms and significant microbial process disturbances due to the removal of essential microbial groups. However, phages are difficult to examine owing to their small size, low biomass, and the lack of a universal phylogenetic marker. Metaproteomics offers an ability to detect and quantify phages present in microbiomes through the identification and quantification of phage proteins. Through the examination of microbial functional networks of 10 agricultural biogas plants and one laboratory-scale AD bioreactor using metaproteomics, Heyer et al. (2019) found that biogas-producing microbiomes could be affected by host-phage interactions besides other microbial interactions such as syntrophy and competition. Mapping of the proteins identified using metaproteomics to different metabolic pathways also confirmed the main assumptions used in the Anaerobic Digestion Model 1 and revealed some indications for additional metabolic

pathways (e.g., syntrophic acetate oxidation) and microbial interactions. Some antimicrobial peptides and proteins were also detected at high abundance. These antimicrobial peptides and proteins, as well as the phages observed in thermophilic biogas plants, were factors contributing to the less stable operation therein. Additionally, each biogas plant appeared to have its own protein signature, and protein patterns could be linked to process disturbances. Some enzymes were even identified as potential biomarkers for process monitoring (Heyer et al., 2015). Therefore, metaproteomics can be used to monitor the changes of the AD process at the functional level and can potentially provide information on the metabolic activities of individual groups of microorganisms in AD bioreactors (Hanreich et al., 2013).

The main advantage of metaproteomics, as compared to metataxonomics, metagenomics, and metatranscriptomics, is the detection and quantification of expressed enzymes and proteins, which directly contributes to the phenotypes of the microorganisms. It can also detect phages by identifying phage proteins. Thus, metaproteomics greatly complements the other -omics technologies. However, metaproteomics is challenged by sample impurities, sample complexity, redundancy of protein identifications, and the lack of adequate genome sequences required for accurate protein identifications (Heyer et al., 2015). Additionally, dedicated algorithms and software tools are required to process and analyze the large amounts of metaproteomic data. Because of these limitations, only a small number of studies have used metaproteomics to investigate the biogas-producing microbiomes. Continued improvement in sample preparation, protein separation and identification, a grouping of redundant proteins, and new algorithms for verification of protein identification will help improve metaproteomic studies of biogas-producing microbiomes. As illustrated above, for comprehensive analysis of the microbiomes in AD bioreactors, metaproteomics should be used in concert with other technologies, such as microscopy, cytometry, metataxonomics, metagenomics, metatranscriptomics, and metametabolomics.

## 4.6 Metametabolomics

Metabolism (catabolism for degradation and anabolism for synthesis) is the ensemble of biochemical transformations occurring in cells of all organisms, and it is embodied in the conservation of energy, degradation of substrates, and synthesis of various molecules essential to sustain life. Metabolomics is a comprehensive analysis to identify and quantify all the metabolites in an organism. Metametabolomics is the microbiome-targeted version of

metabolomics used on individual organisms. It collectively analyzes all the metabolites in a particular physiological state of a microbiome. In metametabolomics, metabolites are defined as small molecules (MM $\leq$ 1000 Da; e.g., glucose, cAMP, GMP, glutamate, etc.) transformed in the process of metabolism and are important to or required for the maintenance, growth, and function of individual microorganisms. Broadly speaking, the products (i.e., SCFA) of acidogenesis during AD are also metabolites. Metametabolomics is also reported to be useful in elucidating the intracellular metabolic pathways that produce the metabolites within a microbiome (Sasaki et al., 2014). Different analytical techniques have been used for metametabolomic analyses, such as high-performance liquid chromatography (HPLC) coupled with UV detection, Fourier-transform infrared (FTIR) spectroscopy, liquid or gas chromatography coupled with mass spectrometry (GC-MS and LC-MS, respectively), and nuclear magnetic resonance (NMR). Most metametabolomic studies reported thus far used MS and/or NMR because both can detect a wide range of metabolites with relatively high specificity and reproducibility. NMR and GC-MS-based metametabolomic approaches have been used to identify extracellular metabolites, while GC-quadrupole-MS (GC-Q-MS) and liquid chromatography triple-stage quadrupole MS (LC-QqQ-MS) were used to identify intracellular metabolite profiles (Sasaki et al., 2014). Metametabolomics can provide two types of analysis: targeted analysis and non-targeted discovery-oriented analysis, with the former analyzing a specific class of compounds (e.g., amino acids, fatty acids, lipids, carbohydrates), while the latter capturing a global overview of the metabolic diversity of a microbiome by using technologies that can detect and identify most, if not all, the metabolites.

Only a small number of studies have used metametabolomics hitherto, and nearly all of them used untargeted metametabolomics. The first metametabolomic study on biogas-producing microbiomes investigated the degradation processes of glucose, starch, and cellulose using NMR spectroscopy and microbiome profiles using DGGE (Date et al., 2012). Variations in metabolites were evaluated as peak heights of $^{1}$H NMR signals. DGGE-NMR correlation analysis identified some simple relationships between microbiome profiles and metabolites, including the negative correlation between glucose concentration and several DGGE bands related to *Firmicutes* and *Thermotogae*. It was speculated that bacteria represented by those DGGE bands might use glucose. By analyzing the intracellular metabolites using metametabolomics, Sasaki et al. (2014) compared the central metabolite profiles from glucose in the microbiome of two AD bioreactors

that were operated at neutral (pH 7.5) vs. acidic (pH 5.0) pH. The metametabolomic analysis focused on the identification of the metabolites of the EMP pathway, the pentose phosphate pathways, and the tricarboxylic acid (TCA) cycle. The ratio of coenzymes, ATP, NADH, and NADPH were also determined. It was found that the concentrations of intracellular metabolites of the EMP pathway and the pentose phosphate pathway, except for pyruvate, remained high during stable methanogenesis at the neutral pH, indicating increased carbon flux through the glycolysis pathway during stable methanogenesis. The extracellular acetate concentration temporarily increased, consistent with a higher ATP level, during stable methanogenesis. Intracellular concentrations of the intermediates of the reductive branch of the TCA cycle, including malate, fumarate, and succinate, were higher during deteriorated methanogenesis at the acidic pH. It was surmised that NADH was consumed during acetate production under stable methanogenesis, whereas NADH was used for lactate and succinate production during deteriorated methanogenesis. Also, glutamate production probably led to NADPH consumption. During deteriorated methanogenesis, intracellular pyruvate and acetyl-CoA were accumulated, which was suggested to be the cause of delayed glucose consumption and decreased methane production. In a recent study, metametabolomics, together with metagenomics, was used to examine the impact of operational shocks (42 °C vs. 32 °C, either side of mesophilic 37 °C; and 20% loading of lipids) to biogas production, functional potential of the microbiome, and its metabolism in lab-scale AD bioreactors (Beale et al., 2016). The results showed that increased biogas production correlated with an increase in SCFA when lipids were added. Another study (Yang et al., 2014) compared the metametabolomes between the two stages of a two-staged AD bioreactor fed corn stalk using GC-MS. Increased levels of sugars and sugar alcohols during methanogenesis and fatty acids during acidogenesis were reported. Identification of stage-specific metabolic pathways will help understand the metabolic pathways that are predominant or important in each stage of staged AD.

Metametabolomics has the advantage to provide phenotypic information, whereas metataxonomics, metagenomics, and metatranscriptomics primarily provide genotypic information. As illustrated above, metametabolomics can provide definitive information on the metabolism of given nutrients and metabolic pathways. However, it suffers from several limitations. First, it is challenging to efficiently extract most of the metabolites from microbiome samples, especially those with a complex matrix while

preventing biased recovery and possible physical and chemical alteration. The complex matrix of AD bioreactor samples makes the extraction of representative and quantifiable metabolites particularly challenging. Second, the physical and chemical properties of different AD bioreactor samples can vary. Different solvents may have to be tested to achieve efficient extraction from different samples. Third, NMR and MS are the major analytical technologies used in most metametabolomic studies. However, each has its limitations in detecting and identify many of the metabolites. Interested readers are referred to two recent reviews (Chaleckis et al., 2019; Smirnov et al., 2016). The lack of a specialty metabolomic database for biogas-producing microbiomes also hinders the analysis of metametabolomic data obtained from AD bioreactors. Continued advancement and improvement in chemical analysis technologies and new approaches will further improve the capability of metametabolomics. For example, multidimensional NMR was shown to be highly resolving, sensitive, high throughput, and quantitative (Marchand et al., 2017). The combination of both MS and NMR also improved the identification and analysis of new metabolites (Bingol et al., 2015). New and improve bioinformatics algorithms and tools, such as Workflow4Metabolomics (Giacomoni et al., 2015), will also enhance the capability of metametabolomics.

## 5. Summary and future perspectives

Research on the biogas-producing microbiomes in the past many decades has led to the accumulation of information and knowledge on the diversity, composition, activities, and microbial interactions that determine the rate of biogas production and process stability of AD. The early studies using cultivation-based methods and analysis enabled the elucidation of the four major phases of AD (depolymerization of polymeric substances in the feedstock, acidogenesis through fermentation, syntrophic oxidation of fatty acids, and methanogenesis through both the acetoclastic and the hydrogenotrophic methanogenesis pathways). Some species involved in each of the four phases were also identified taxonomically, and biochemical characterization of some of the representative species laid the foundation to understand the entire AD process. Additionally, the phylogenetic, biochemical, and physiological information of the cultured microorganisms present in AD bioreactors seeded many of the public databases with important microorganisms of biogas-producing microbiomes.

The studies conducted using the various molecular biology techniques, primarily cloning and sequencing of phylogenetic marker genes (before the -omics became available) expanded the perspectives on the extent of bacterial and archaeal diversity in biogas-producing microbiomes (Nelson et al., 2011). The use of microbiome fingerprinting also made it possible to rapidly evaluate and examine the microbiome dynamics and responses of individual taxa to changed conditions, including startup, loading rate, change of feedstock, and operating temperature and pH. Owing to FISH, groups of microorganisms of interest, such as methanogens, were localized in anaerobic granules. Coupled with DNA-based analysis, guilds of microorganisms, such as acetate-oxidizing bacteria, were identified. The tremendous diversity at low taxonomic ranks (species and below) provided the early evidence of functional redundancy in biogas-producing microbiomes. qPCR also substantially improves the quantification of individual groups of microorganisms of biogas-producing microbiomes, including pathogens and antibiotic-resistant bacteria.

The omics technologies further advanced the findings made in the molecular biology era. In particular, studies using metataxonomics, primarily sequencing analysis of phylogenetic marker genes, documented the majority of the taxonomic diversity (including the diversity of eukaryotes) of biogas-producing microbiomes, while metagenomics unearthed the majority of the functional diversity (Castellano-Hinojosa et al., 2018). A core microbiome was found among AD bioreactors even though variations are inherent depending on a host of conditions (Peces et al., 2018; Treu et al., 2016). Functional redundancy was clearly documented, and it explains the relative stability of AD when the taxonomic diversity and composition changes (Bertucci et al., 2019; De Vrieze et al., 2017). Community resilience is conferred from the functional redundancy and the high taxonomic diversity in biogas-producing microbiomes (Abendroth et al., 2018; Ferguson et al., 2016). The resilience can help biogas-producing microbiomes to recover from shocks. Metatranscriptomics, metaproteomics, and metametabolomics complemented metataxonomics and metagenomics and provided important information toward understanding the phenotypic traits and features of biogas-producing microbiomes. Metagenomics, metatranscriptomics, and metaproteomics can also analyze the viruses and phages present in AD bioreactors. However, except metataxonomics, metagenomics, and metatranscriptomics, the use of metaproteomics and metametabolomics still faces some technical challenges, and they have been used only a few studies. All the techniques and

technologies have inherent applicability and limitations (Table 1). The most appropriate technologies and technologies should be selected to achieve the research goals.

The ultimate goal of the research on and analysis of biogas-producing microbiomes is to effectively improve AD efficiency and stability rationally. To that end, efforts and progress have been made to correlate bacterial and archaeal populations and their functions to AD process parameters (Shin et al., 2016; Venkiteshwaran et al., 2017). Although such correlations cannot directly identify the drivers or barriers of AD, they can guild future research toward rational monitoring and control of AD. Indeed, several studies found distinct responses of certain bacteria and methanogens to ammonia accumulation (Peng et al., 2018), operation temperature (Kim et al., 2017), and pH (Zhang et al., 2019b). Some of the responsive groups of microorganisms may serve as indicators during AD process monitoring (Sun et al., 2019), while others may be used in bioaugmentation to help restore normal AD process (Onwosi et al., 2019) and help with effective management of dysfunction of AD bioreactors (Onwosi et al., 2019). Future research on biogas-producing microbiomes will further help enhance AD efficiency and stability. Such research will benefit greatly from the continued improvement of the omics technologies, particularly metaproteomic and metametabolomics. Furthermore, standardized methods and analyses are needed to produce data that can be compared used in the development and improvement of anaerobic digestion models.

## References

Abendroth, C., Hahnke, S., Simeonov, C., Klocke, M., Casani-Miravalls, S., Ramm, P., Burger, C., Luschnig, O., Porcar, M., 2018. Microbial communities involved in biogas production exhibit high resilience to heat shocks. Bioresour. Technol. 249, 1074–1079.

Abram, F., Enright, A.M., Reilly, J.O.'., Botting, C.H., Collins, G., Flaherty, V.O.'., 2011. A metaproteomic approach gives functional insights into anaerobic digestion. J. Appl. Microbiol. 110, 1550–1560.

Acs, N., Bagi, Z., Rakhely, G., Minarovics, J., Nagy, K., Kovacs, K.L., 2015. Bioaugmentation of biogas production by a hydrogen-producing bacterium. Bioresour. Technol. 186, 286–293.

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W., Nielsen, P.H., 2013. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat. Biotechnol. 31, 533–538.

Anderson, I., Ulrich, L.E., Lupa, B., Susanti, D., Porat, I., Hooper, S.D., Lykidis, A., Sieprawska-Lupa, M., Dharmarajan, L., Goltsman, E., Lapidus, A., Saunders, E., Han, C., Land, M., Lucas, S., Mukhopadhyay, B., Whitman, W.B., Woese, C., Bristow, J., Kyrpides, N., 2009. Genomic characterization of methanomicrobiales reveals three classes of methanogens. PLoS One 4, e5797.

Angenent, L.T., Sung, S., Raskin, L., 2004. Formation of granules and *Methanosaeta* fibres in an anaerobic migrating blanket reactor (AMBR). Environ. Microbiol. 6, 315–322.
Ariesyady, H.D., Ito, T., Okabe, S., 2007. Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. Water Res. 41, 1554–1568.
Aßhauer, K.P., Wemheuer, B., Daniel, R., Meinicke, P., 2015. Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. Bioinformatics 31, 2882–2884.
Barker, H.A., 1936. Studies upon the methane-producing bacteria. Arch. Mikrobiol. 7, 420–438.
Barua, S., Dhar, B.R., 2017. Advances towards understanding and engineering direct interspecies electron transfer in anaerobic digestion. Bioresour. Technol. 244, 698–707.
Bastian, M., Heymann, S., Jacomy, M., 2009. Gephi: an open source software for exploring and manipulating networks. In: International AAAI Conference on Weblogs and Social Media.
Beale, D.J., Karpe, A.V., McLeod, J.D., Gondalia, S.V., Muster, T.H., Othman, M.Z., Palombo, E.A., Joshi, D., 2016. An 'omics' approach towards the characterisation of laboratory scale anaerobic digesters treating municipal sewage sludge. Water Res. 88, 346–357.
Bertucci, M., Calusinska, M., Goux, X., Rouland-Lefevre, C., Untereiner, B., Ferrer, P., Gerin, P.A., Delfosse, P., 2019. Carbohydrate hydrolytic potential and redundancy of an anaerobic digestion microbiome exposed to acidosis, as uncovered by metagenomics. Appl. Environ. Microbiol. 85, e00895-19.
Bharagava, R.N., Purchase, D., Saxena, G., Mulla, S.I., 2019. Applications of metagenomics in microbial bioremediation of pollutants: from genomics to environmental cleanup. In: Das, S., Dash, H.R. (Eds.), Microbial Diversity in the Genomic Era. Elsevier Inc, pp. 459–477.
Bingol, K., Bruschweiler-Li, L., Yu, C., Somogyi, A., Zhang, F., Brüschweiler, R., 2015. Metabolomics beyond spectroscopic databases: a combined MS/NMR strategy for the rapid identification of new metabolites in complex mixtures. Anal. Chem. 87, 3864–3870.
Bonnet, R., Suau, A., Dore, J., Gibson, G.R., Collins, M.D., 2002. Differences in rDNA libraries of faecal bacteria derived from 10- and 25-cycle PCRs. Int. J. Syst. Evol. Microbiol. 52, 757–763.
Boone, D.R., Kamagata, Y., 1998. Rejection of the species Methanothrix soehngeniiVPand the genus MethanothrixVPas nomina confusa, and transfer of Methanothrix thermophilaVPto the genus MethanosaetaVPas Methanosaeta thermophila comb. nov. Request for an opinion. Int. J. Syst. Evol. Microbiol. 48, 1079–1080.
Campanaro, S., Treu, L., Kougias, P.G., De Francisci, D., Valle, G., Angelidaki, I., 2016. Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnol. Biofuels 9, 26.
Campanaro, S., Treu, L., Rodriguez-R, L.M., Kovalovszki, A., Ziels, R.M., Maus, I., Zhu, X., Kougias, P.G., Basile, A., Luo, G., Schlüter, A., Konstantinidis, K.T., Angelidaki, I., 2019. The anaerobic digestion microbiome: a collection of 1600 metagenome-assembled genomes shows high species diversity related to methane production. bioRxiv 680553.
Cardinali-Rezende, J., Rojas-Ojeda, P., Nascimento, A.M.A., Sanz, J.L., 2016. Proteolytic bacterial dominance in a full-scale municipal solid waste anaerobic reactor assessed by 454 pyrosequencing technology. Chemosphere 146, 519–525.
Castellano-Hinojosa, A., Armato, C., Pozo, C., González-Martínez, A., González-López, J., 2018. New concepts in anaerobic digestion processes: recent advances and biological aspects. Appl. Microbiol. Biotechnol. 102, 5065–5076.

Chaleckis, R., Meister, I., Zhang, P., Wheelock, C.E., 2019. Challenges, progress and promises of metabolite annotation for LC-MS-based metabolomics. Curr. Opin. Biotechnol. 55, 44–50.

Date, Y., Iikura, T., Yamazawa, A., Moriya, S., Kikuchi, J., 2012. Metabolic sequences of anaerobic fermentation on glucose-based feeding substrates based on correlation analyses of microbial and metabolite profiling. J. Proteome Res. 11, 5602–5610.

De Vrieze, J., Regueiro, L., Props, R., Vilchez-Vargas, R., Jáuregui, R., Pieper, D.H., Lema, J.M., Carballa, M., 2016. Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. Biotechnol. Biofuels 9, 244.

De Vrieze, J., Christiaens, M.E.R., Walraedt, D., Devooght, A., Ijaz, U.Z., Boon, N., 2017. Microbial community redundancy in anaerobic digestion drives process recovery after salinity exposure. Water Res. 111, 109–117.

De Vrieze, J., Pinto, A.J., Sloan, W.T., Ijaz, U.Z., 2018a. The active microbial community more accurately reflects the anaerobic digestion process: 16S rRNA (gene) sequencing as a predictive tool. Microbiome 6, 63.

De Vrieze, J., Ijaz, U.Z., Saunders, A.M., Theuerl, S., 2018b. Terminal restriction fragment length polymorphism is an "old school" reliable technique for swift microbial community screening in anaerobic digestion. Sci. Rep. 8, 16818.

DeLong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360–1363.

Dione, N., Khelaifia, S., La Scola, B., Lagier, J.C., Raoult, D., 2016. A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology. Clin. Microbiol. Infect. 22, 53–58.

Douglas, G.M., Maffei, V.J., Zaneveld, J., Yurgel, S.N., Brown, J.R., Taylor, C.M., Huttenhower, C., Langille, M.G.I., 2019. PICRUSt2: an improved and extensible approach for metagenome inference. bioRxiv 672295.

Fang, H.H., 2010. Environmental Anaerobic Technology. Imperial College Press, Covent Garden, London, UK.

Ferguson, R.M.W., Coulon, F., Villa, R., 2016. Organic loading rate: a promising microbial management tool in anaerobic digestion. Water Res. 100, 348–356.

Fischer, M.A., Ulbricht, A., Neulinger, S.C., Refai, S., Wassmann, K., Kunzel, S., Schmitz, R.A., 2019a. Immediate effects of ammonia shock on transcription and composition of a biogas reactor microbiome. Front. Microbiol. 10, 2064.

Fischer, M.A., Gullert, S., Refai, S., Kunzel, S., Deppenmeier, U., Streit, W.R., Schmitz, R.A., 2019b. Long-term investigation of microbial community composition and transcription patterns in a biogas plant undergoing ammonia crisis. J. Microbial. Biotechnol. 12, 305–323.

Franke-Whittle, I.H., Goberna, M., Pfister, V., Insam, H., 2009a. Design and development of the ANAEROCHIP microarray for investigation of methanogenic communities. J. Microbiol. Methods 79, 279–288.

Franke-Whittle, I.H., Goberna, M., Insam, H., 2009b. Design and testing of real-time PCR primers for the quantification of *Methanoculleus*, *Methanosarcina*, *Methanothermobacter*, and a group of uncultured methanogens. Can. J. Microbiol. 55, 611–616.

Franke-Whittle, I.H., Walter, A., Ebner, C., Insam, H., 2014. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. Waste Manag. 34, 2080–2089.

Giacomoni, F., Le Corguille, G., Monsoor, M., Landi, M., Pericard, P., Petera, M., Duperier, C., Tremblay-Franco, M., Martin, J.F., Jacob, D., Goulitquer, S., Thevenot, E.A., Caron, C., 2015. Workflow4Metabolomics: a collaborative research infrastructure for computational metabolomics. Bioinformatics 31, 1493–1495.

Grohmann, A., Fehrmann, S., Vainshtein, Y., Haag, N.L., Wiese, F., Stevens, P., Naegele, H.J., Oechsner, H., Hartsch, T., Sohn, K., Grumaz, C., 2018. Microbiome dynamics and adaptation of expression signatures during methane production failure and process recovery. Bioresour. Technol. 247, 347–356.

Gysi, D.M., Fragoso, T.M., Buskamp, V., Almaas, E., Nowick, K., 2018. Comparing multiple networks using the co-expression differential network analysis (CoDiNA). arXiv*Preprint* arXiv:1802.00828.

Hagen, L.H., Frank, J.A., Zamanzadeh, M., Eijsink, V.G.H., Pope, P.B., Horn, S.J., Arntzen, M.O., 2017. Quantitative metaproteomics highlight the metabolic contributions of uncultured phylotypes in a thermophilic anaerobic digester. Appl. Environ. Microbiol. 83, e01955-16.

Hanreich, A., Schimpf, U., Zakrzewski, M., Schluter, A., Benndorf, D., Heyer, R., Rapp, E., Puhler, A., Reichl, U., Klocke, M., 2013. Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation. Syst. Appl. Microbiol. 36, 330–338.

Hao, L., Lu, F., Mazeas, L., Desmond-Le Quemener, E., Madigou, C., Guenne, A., Shao, L., Bouchez, T., He, P., 2015. Stable isotope probing of acetate fed anaerobic batch incubations shows a partial resistance of acetoclastic methanogenesis catalyzed by Methanosarcina to sudden increase of ammonia level. Water Res. 69, 90–99.

Hassa, J., Maus, I., Off, S., Puhler, A., Scherer, P., Klocke, M., Schluter, A., 2018. Metagenome, metatranscriptome, and metaproteome approaches unraveled compositions and functional relationships of microbial communities residing in biogas plants. Appl. Microbiol. Biotechnol. 102, 5045–5063.

Heyer, R., Kohrs, F., Reichl, U., Benndorf, D., 2015. Metaproteomics of complex microbial communities in biogas plants. J. Microbial. Biotechnol. 8, 749–763.

Heyer, R., Schallert, K., Siewert, C., Kohrs, F., Greve, J., Maus, I., Klang, J., Klocke, M., Heiermann, M., Hoffmann, M., Püttker, S., Calusinska, M., Zoun, R., Saake, G., Benndorf, D., Reichl, U., 2019. Metaproteome analysis reveals that syntrophy, competition, and phage-host interaction shape microbial communities in biogas plants. Microbiome 7, 1–17.

Ho, D.P., Jensen, P.D., Batstone, D.J., 2013. Methanosarcinaceae and acetate-oxidizing pathways dominate in high-rate thermophilic anaerobic digestion of waste-activated sludge. Appl. Environ. Microbiol. 79, 6491–6500.

Hori, T., Akuzawa, M., Haruta, S., Ueno, Y., Ogata, A., Ishii, M., Igarashi, Y., 2014. Involvement of a novel fermentative bacterium in acidification in a thermophilic anaerobic digester. FEMS Microbiol. Lett. 361, 62–67.

Iwai, S., Weinmaier, T., Schmidt, B.L., Albertson, D.G., Poloso, N.J., Dabbagh, K., DeSantis, T.Z., 2016. Piphillin: improved prediction of metagenomic content by direct inference from human microbiomes. PLoS One 11, e0166104.

Jia, Y., Ng, S.K., Lu, H., Cai, M., Lee, P.K.H., 2018. Genome-centric metatranscriptomes and ecological roles of the active microbial populations during cellulosic biomass anaerobic digestion. Biotechnol. Biofuels 11, 117.

Jia, Y., Leung, M.H.Y., Tong, X., Wilkins, D., Lee, P.K.H., 2019. Rare taxa exhibit disproportionate cell-level metabolic activity in enriched anaerobic digestion microbial communities. mSystems 4, e00208–e00218.

Jun, S.R., Robeson, M.S., Hauser, L.J., Schadt, C.W., Gorin, A.A., 2015. PanFP: pangenome-based functional profiles for microbial communities. BMC. Res. Notes 8, 479.

Kampmann, K., Ratering, S., Kramer, I., Schmidt, M., Zerr, W., Schnell, S., 2012. Unexpected stability of Bacteroidetes and Firmicutes communities in laboratory biogas reactors fed with different defined substrates. Appl. Environ. Microbiol. 78, 2106–2119.

Khelaifia, S., Lagier, J.C., Nkamga, V.D., Guilhot, E., Drancourt, M., Raoult, D., 2016. Aerobic culture of methanogenic archaea without an external source of hydrogen. Eur. J. Clin. Microbiol. Infect. Dis. 35, 985–991.
Kim, T.G., Jeong, S.Y., Cho, K.S., 2015. Development of droplet digital PCR assays for methanogenic taxa and examination of methanogen communities in full-scale anaerobic digesters. Appl. Microbiol. Biotechnol. 99, 445–458.
Kim, M.-S., Kim, D.-H., Yun, Y.-M., 2017. Effect of operation temperature on anaerobic digestion of food waste: performance and microbial analysis. Fuel 209, 598–605.
Kirkegaard, R.H., McIlroy, S.J., Kristensen, J.M., Nierychlo, M., Karst, S.M., Dueholm, M.S., Albertsen, M., Nielsen, P.H., 2017. The impact of immigration on microbial community composition in full-scale anaerobic digesters. Sci. Rep. 7, 9343.
Kohrs, F., Heyer, R., Magnussen, A., Benndorf, D., Muth, T., Behne, A., Rapp, E., Kausmann, R., Heiermann, M., Klocke, M., Reichl, U., 2014. Sample prefractionation with liquid isoelectric focusing enables in depth microbial metaproteome analysis of mesophilic and thermophilic biogas plants. Anaerobe 29, 59–67.
Lagier, J.C., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C., Bittar, F., Fournous, G., Gimenez, G., Maraninchi, M., Trape, J.F., Koonin, E.V., La Scola, B., Raoult, D., 2012. Microbial culturomics: paradigm shift in the human gut microbiome study. Clin. Microbiol. Infect. 18, 1185–1193.
Langer, S.G., Ahmed, S., Einfalt, D., Bengelsdorf, F.R., Kazda, M., 2015. Functionally redundant but dissimilar microbial communities within biogas reactors treating maize silage in co-fermentation with sugar beet silage. J. Microbial. Biotechnol. 8, 828–836.
Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G., Huttenhower, C., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821.
Li, Y.F., Wei, S., Yu, Z., 2013. Feedstocks affect the diversity and distribution of propionate CoA-transferase genes (pct) in anaerobic digesters. Microb. Ecol. 66, 351–362.
Li, Y.F., Nelson, M.C., Chen, P.H., Graf, J., Li, Y., Yu, Z., 2015. Comparison of the microbial communities in solid-state anaerobic digestion (SS-AD) reactors operated at mesophilic and thermophilic temperatures. Appl. Microbiol. Biotechnol. 99, 969–980.
Li, Y., Zhang, Y., Yang, Y., Quan, X., Zhao, Z., 2017. Potentially direct interspecies electron transfer of methanogenesis for syntrophic metabolism under sulfate-reducing conditions with stainless steel. Bioresour. Technol. 234, 303–309.
Limam, R.D., Chouari, R., Mazeas, L., Wu, T.D., Li, T., Grossin-Debattista, J., Guerquin-Kern, J.L., Saidi, M., Landoulsi, A., Sghir, A., Bouchez, T., 2014. Members of the uncultured bacterial candidate division WWE1 are implicated in anaerobic digestion of cellulose. Microbiology 3, 157–167.
Lu, X., Rao, S., Shen, Z., Lee, P.K.H., 2013. Substrate induced emergence of different active bacterial and archaeal assemblages during biomethane production. Bioresour. Technol. 148, 517–524.
Lü, F., Bize, A., Guillot, A., Monnet, V., Madigou, C., Chapleur, O., Mazéas, L., He, P., Bouchez, T., 2014. Metaproteomics of cellulose methanisation under thermophilic conditions reveals a surprisingly high proteolytic activity. ISME J. 8, 88–102.
Lv, W., Schanbacher, F.L., Yu, Z., 2010. Putting microbes to work in sequence: recent advances in temperature-phased anaerobic digestion processes. Bioresour. Technol. 101, 9409–9414.
Manor, O., Borenstein, E., 2017. Systematic characterization and analysis of the taxonomic drivers of functional shifts in the human microbiome. Cell Host Microbe 21, 254–267.
Marchand, J., Martineau, E., Guitton, Y., Dervilly-Pinel, G., Giraudeau, P., 2017. Multidimensional NMR approaches towards highly resolved, sensitive and high-throughput quantitative metabolomics. Curr. Opin. Biotechnol. 43, 49–55.

Maus, I., Koeck, D.E., Cibis, K.G., Hahnke, S., Kim, Y.S., Langer, T., Kreubel, J., Erhard, M., Bremges, A., Off, S., Stolze, Y., Jaenicke, S., Goesmann, A., Sczyrba, A., Scherer, P., Konig, H., Schwarz, W.H., Zverlov, V.V., Liebl, W., Puhler, A., Schluter, A., Klocke, M., 2016. Unraveling the microbiome of a thermophilic biogas plant by metagenome and metatranscriptome analysis complemented by characterization of bacterial and archaeal isolates. Biotechnol. Biofuels 9, 171.

Maus, I., Bremges, A., Stolze, Y., Hahnke, S., Cibis, K.G., Koeck, D.E., Kim, Y.S., Kreubel, J., Hassa, J., Wibberg, D., Weimann, A., Off, S., Stantscheff, R., Zverlov, V.V., Schwarz, W.H., Konig, H., Liebl, W., Scherer, P., McHardy, A.C., Sczyrba, A., Klocke, M., Puhler, A., Schluter, A., 2017. Genomics and prevalence of bacterial and archaeal isolates from biogas-producing microbiomes. Biotechnol. Biofuels 10, 264.

Maynaud, G., Pourcher, A.M., Ziebal, C., Cuny, A., Druilhe, C., Steyer, J.P., Wéry, N., 2016. Persistence and potential viable but non-culturable state of pathogenic bacteria during storage of digestates from agricultural biogas plants. Front. Microbiol. 7, 1469.

McIlroy, S.J., Kirkegaard, R.H., Dueholm, M.S., Fernando, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. Culture-independent analyses reveal novel Anaerolineaceae as abundant primary fermenters in anaerobic digesters treating waste activated sludge. Front. Microbiol. 8, 1134.

Mosbaek, F., Kjeldal, H., Mulat, D.G., Albertsen, M., Ward, A.J., Feilberg, A., Nielsen, J.L., 2016. Identification of syntrophic acetate-oxidizing bacteria in anaerobic digesters by combined protein-based stable isotope probing and metagenomics. ISME J. 10, 2405–2418.

Muth, T., Behne, A., Heyer, R., Kohrs, F., Benndorf, D., Hoffmann, M., Lehtevä, M., Reichl, U., Martens, L., Rapp, E., 2015. The MetaProteomeAnalyzer: a powerful open-source software suite for metaproteomics data analysis and interpretation. J. Proteome Res. 14, 1557–1565.

Nelson, M.C., 2011. An integrated investigation of the microbial communities underpinning biogas production in anaerobic digestion systems. In: Environmental Sciences Graduate Program. The Ohio State University, Columbus.

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739.

Nilsson, R.H., Larsson, K.-H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., Saar, I., Kõljalg, U., Abarenkov, K., 2018. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. 47, D259–D264.

Onwosi, C.O., Eke, I.E., Igbokwe, V.C., Odimba, J.N., Ndukwe, J.K., Chukwu, K.O., Aliyu, G.O., Nwagu, T.N., 2019. Towards effective management of digester dysfunction during anaerobic treatment processes. Renew. Sustain. Energy Rev. 116, 109424.

Park, T., Yu, Z., 2018. Aerobic cultivation of anaerobic rumen protozoa, *Entodinium caudatum* and *Epidinium caudatum*. J. Microbiol. Methods 152, 186–193.

Patel, G.B., 1984. Characterization and nutritional properties of *Methanothrix concilii* sp. nov., a mesophilic, aceticlastic methanogen. Can. J. Microbiol. 30, 1383–1396.

Peces, M., Astals, S., Jensen, P.D., Clarke, W.P., 2018. Deterministic mechanisms define the long-term anaerobic digestion microbiome and its functionality regardless of the initial microbial community. Water Res. 141, 366–376.

Peng, X., Zhang, S., Li, L., Zhao, X., Ma, Y., Shi, D., 2018. Long-term high-solids anaerobic digestion of food waste: effects of ammonia on process performance and microbial community. Bioresour. Technol. 262, 148–158.

Ruiz-Sanchez, J., Guivernau, M., Fernandez, B., Vila, J., Vinas, M., Riau, V., Prenafeta-Boldu, F.X., 2019. Functional biodiversity and plasticity of methanogenic biomass from a full-scale mesophilic anaerobic digester treating nitrogen-rich agricultural wastes. Sci. Total Environ. 649, 760–769.

Sasaki, D., Sasaki, K., Tsuge, Y., Morita, M., Kondo, A., 2014. Comparison of metabolomic profiles of microbial communities between stable and deteriorated methanogenic processes. Bioresour. Technol. 172, 83–90.

Schlüter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., Mussgnug, J.H., Neuweger, H., Niehaus, K., Pühler, A., Runte, K.J., Szczepanowski, R., Tauch, A., Tilker, A., Viehöver, P., Goesmann, A., 2008. The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. J. Biotechnol. 136, 77–90.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504.

Shaw, G.T., Liu, A.C., Weng, C.Y., Chou, C.Y., Wang, D., 2017. Inferring microbial interactions in thermophilic and mesophilic anaerobic digestion of hog waste. PLoS One 12, e0181395.

Shin, S.G., Koo, T., Lee, J., Han, G., Cho, K., Kim, W., Hwang, S., 2016. Correlations between bacterial populations and process parameters in four full-scale anaerobic digesters treating sewage sludge. Bioresour. Technol. 214, 711–721.

Smirnov, K.S., Maier, T.V., Walker, A., Heinzmann, S.S., Forcisi, S., Martinez, I., Walter, J., Schmitt-Kopplin, P., 2016. Challenges of metabolomics in human gut microbiota research. Int. J. Med. Microbiol. 306, 266–279.

Söhngen, N.L. (1910) Dissertation, Delft, 1906; Rec. tray. chim. Pays-Bas 29, 238, 1910.: Delft University of Technology.

Söhngen, C., Podstawka, A., Bunk, B., Gleim, D., Vetcininova, A., Reimer, L.C., Ebeling, C., Pendarovski, C., Overmann, J., 2016. BacDive—the bacterial diversity metadatabase in 2016. Nucleic Acids Res. 44, D581–D585.

Speece, R.E., Boonyakitsombut, S., Kim, M., Azbar, N., Ursillo, P., 2006. Overview of anaerobic treatment: thermophilic and propionate implications. Water Environ. Res. 78, 460–473.

Stark, L., Giersch, T., Wünschiers, R., 2014. Efficiency of RNA extraction from selected bacteria in the context of biogas production and metatranscriptomics. Anaerobe 29, 85–90.

Stolze, Y., Bremges, A., Rumming, M., Henke, C., Maus, I., Puhler, A., Sczyrba, A., Schluter, A., 2016. Identification and genome reconstruction of abundant distinct taxa in microbiomes from one thermophilic and three mesophilic production-scale biogas plants. Biotechnol. Biofuels 9, 156.

Stolze, Y., Bremges, A., Maus, I., Puhler, A., Sczyrba, A., Schluter, A., 2018. Targeted in situ metatranscriptomics for selected taxa from mesophilic and thermophilic biogas plants. J. Microbial. Biotechnol. 11, 667–679.

Sun, W., Krumins, V., Dong, Y., Gao, P., Ma, C., Hu, M., Li, B., Xia, B., He, Z., Xiong, S., 2018. A combination of stable isotope probing, illumina sequencing, and co-occurrence network to investigate thermophilic acetate- and lactate-utilizing bacteria. Microb. Ecol. 75, 113–122.

Sun, H., Ni, P., Angelidaki, I., Dong, R., Wu, S., 2019. Exploring stability indicators for efficient monitoring of anaerobic digestion of pig manure under perturbations. Waste Manag. 91, 139–146.

Talbot, G., Roy, C.S., Topp, E., Kalmokoff, M.L., Brooks, S.P., Beaulieu, C., Palin, M.F., Masse, D.I., 2010. Spatial distribution of some microbial trophic groups in a plug-flow-type anaerobic bioreactor treating swine manure. Water Sci. Technol. 61, 1147–1155.

Treu, L., Kougias, P.G., Campanaro, S., Bassani, I., Angelidaki, I., 2016. Deeper insight into the structure of the anaerobic digestion microbial community; the biogas microbiome database is expanded with 157 new genomes. Bioresour. Technol. 216, 260–266.

Van Nostrand, J.D., Wu, L., He, Z., Zhou, J., 2010. GeoChips for analysis of microbial functional communities. In: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 4039–4050.

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., Tyson, G.W., 2014. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr. Opin. Biotechnol. 27, 55–64.

Vasconcelos, E.A.F., Santaella, S.T., Viana, M.B., Dos Santos, A.B., Pinheiro, G.C., Leitao, R.C., 2019. Composition and ecology of bacterial and archaeal communities in anaerobic reactor fed with residual glycerol. Anaerobe 59, 145–153.

Venkiteshwaran, K., Milferstedt, K., Hamelin, J., Fujimoto, M., Johnson, M., Zitomer, D.H., 2017. Correlating methane production to microbiota in anaerobic digesters fed synthetic wastewater. Water Res. 110, 161–169.

Walker, A.W., Martin, J.C., Scott, P., Parkhill, J., Flint, H.J., Scott, K.P., 2015. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome 3, 26.

Wang, T., Zhang, D., Dai, L., Dong, B., Dai, X., 2018. Magnetite triggering enhanced direct interspecies electron transfer: a scavenger for the blockage of electron transfer in anaerobic digestion of high-solids sewage sludge. Environ. Sci. Technol. 52, 7160–7169.

Wei, Y., Zhou, H., Zhang, J., Zhang, L., Geng, A., Liu, F., Zhao, G., Wang, S., Zhou, Z., Yan, X., 2015. Insight into dominant cellulolytic bacteria from two biogas digesters and their glycoside hydrolase genes. PLoS One 10, e0129921.

Weinrich, S., Koch, S., Bonk, F., Popp, D., Benndorf, D., Klamt, S., Centler, F., 2019. Augmenting biogas process modeling by resolving intracellular metabolic activity. Front. Microbiol. 10, 1095.

Wilkens, C., Busk, P.K., Pilgaard, B., Zhang, W.J., Nielsen, K.L., Nielsen, P.H., Lange, L., 2017. Diversity of microbial carbohydrate-active enzymes in Danish anaerobic digesters fed with wastewater treatment sludge. Biotechnol. Biofuels 10, 158.

Witarsa, F., Lansing, S., Yarwood, S., Gonzalez Mateu, M., 2016. Incubation of innovative methanogenic communities to seed anaerobic digesters. Appl. Microbiol. Biotechnol. 100, 9795–9806.

Xia, Y., Yang, C., Zhang, T., 2018. Microbial effects of part-stream low-frequency ultrasonic pretreatment on sludge anaerobic digestion as revealed by high-throughput sequencing-based metagenomics and metatranscriptomics. Biotechnol. Biofuels 11, 47.

Yamada, T., Sekiguchi, Y., Imachi, H., Kamagata, Y., Ohashi, A., Harada, H., 2005. Diversity, localization, and physiological properties of filamentous microbes belonging to Chloroflexi subphylum I in mesophilic and thermophilic methanogenic sludge granules. Appl. Environ. Microbiol. 71, 7493–7503.

Yang, D., Fan, X., Shi, X., Lian, S., Qiao, J., Guo, R., 2014. Metabolomics reveals stage-specific metabolic pathways of microbial communities in two-stage anaerobic fermentation of corn-stalk. Biotechnol. Lett. 36, 1461–1468.

Yergeau, E., Masson, L., Elias, M., Xiang, S., Madey, E., Huang, H., Brooks, B., Beaudette, L.A., 2016. Comparison of methods to identify pathogens and associated virulence functional genes in biosolids from two different wastewater treatment facilities in Canada. PLoS One 11, e0153554.

Zhang, C., Yuan, Q., Lu, Y., 2014. Inhibitory effects of ammonia on methanogen mcrA transcripts in anaerobic digester sludge. FEMS Microbiol. Ecol. 87, 368–377.

Zhang, J., Gao, Q., Zhang, Q., Wang, T., Yue, H., Wu, L., Shi, J., Qin, Z., Zhou, J., Zuo, J., Yang, Y., 2017. Bacteriophage-prokaryote dynamics and interaction within anaerobic digestion processes across time and space. Microbiome 5, 57.

Zhang, Q., Wang, M., Ma, X., Gao, Q., Wang, T., Shi, X., Zhou, J., Zuo, J., Yang, Y., 2019a. High variations of methanogenic microorganisms drive full-scale anaerobic digestion process. Environ. Int. 126, 543–551.

Zhang, L., Ban, Q., Li, J., Wan, C., 2019b. Functional bacterial and archaeal dynamics dictated by pH stress during sugar refinery wastewater in a UASB. Bioresour. Technol. 288, 121464.

Zhao, Q., Liu, Y., 2019. Is anaerobic digestion a reliable barrier for deactivation of pathogens in biosludge? Sci. Total Environ. 668, 893–902.

Ziels, R.M., Svensson, B.H., Sundberg, C., Larsson, M., Karlsson, A., Yekta, S.S., 2018a. Microbial rRNA gene expression and co-occurrence profiles associate with biokinetics and elemental composition in full-scale anaerobic digesters. J. Microbial. Biotechnol. 11, 694–709.

Ziels, R.M., Sousa, D.Z., Stensel, H.D., Beck, D.A.C., 2018b. DNA-SIP based genome-centric metagenomics identifies key long-chain fatty acid-degrading populations in anaerobic digesters with different feeding frequencies. ISME J. 12, 112–123.

Ziels, R.M., Nobu, M.K., Sousa, D.Z., 2019. Elucidating syntrophic butyrate-degrading populations in anaerobic digesters using stable-isotope-informed genome-resolved metagenomics. mSystems 4, e00159-19.