Review

Microbial Symbiosis: A Network towards Biomethanation

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Biomethanation through anaerobic digestion (AD) is the most reliable energy harvesting process to achieve waste-to-energy. Microbial communities, including hydrolytic and fermentative bacteria, syntrophic bacteria, and methanogenic archaea, and their interspecies symbioses allow complex metabolisms for the volumetric reduction of organic waste in AD. However, heterogeneity in organic waste induces community shifts in conventional anaerobic digesters treating sewage sludge at wastewater treatment plants globally. Assessing the metabolic roles of individual microbial species in syntrophic communities remains a challenge, but such information has important implications for microbially enhanced energy recovery. This review focuses on the alterations in digester microbiome and intricate interspecies networks during substrate variation, symbiosis among the populations, and their implications for biomethanation to aid stable operation in real-scale digesters.

Impact of Organic Waste in Anaerobic Digestion

AD is considered to be the most reliable cutting-edge waste-to-energy model for converting organic waste to biomethane [1]. AD is a complex metabolic process that occurs under anoxic environments and involves distinctive and consecutive biochemical phases of hydrolysis (see Glossary) and acidogenesis, acetogenesis, interspecies electron transfers (IETs), and methanogenesis (Figure 1, Key Figure) [2,3]. Hydrolytic and fermentative bacteria, syntrophic bacteria, and methanogenic archaea associate via intricate symbiosis to allow the volumetric reduction and conversion of organic matter to gaseous methane during AD [3]. Organic waste including polymers such as polysaccharides, proteins, and lipids and simple molecules including volatile fatty acids (VFAs) and ethanol from domestic, municipal, food processing, animal husbandry, and slaughterhouse units are cheap and abundant sources of substrates for AD [4–6]. The conversion efficiency of these organic resources to biomethane is relatively high (lipids, 94.8%; proteins, 71%; carbohydrates, 50.4%) compared with that of sewage sludge (40–50%) alone during AD and contributes to 69% of the atmospheric CH4 [7–9]. As electron donors, organic waste matter facilitates IET and maintains the electron pool during AD [4,10].

Heterogeneity in organic waste is a bottleneck for its utilization in AD [11]. The performance, efficiency, and productivity of AD depends on several physicochemical factors, such as substrate type and operational conditions [e.g., temperature, pH, trace elements, organic loading rate (OLR), hydraulic residence time (HRT), total solid (TS), volatile solid (VS)]. Changes in these factors significantly influence the microbial community structure and their metabolic processes, including fermentation, acetogenesis, and methanogenesis [12–15]. Microbial community shifts have been widely observed as a functional redundancy due to compositional variations in substrates [16,17]. For example, excessive acidosis or NH4+ accumulation during the AD of carbonaceous and proteinaceous substrates at high OLR and low HRT caused instability in the operational pH [13,14,18–20]. In this review, we comprehensively discuss microbial symbiosis during anaerobic
Key Figure
A Schematic of Interspecies Symbiosis during the Anaerobic Digestion of Organic Wastes for Biomethanation

Figure 1. The red box highlights the symbiotic association among hydrogenogenic exoelectrogenic bacteria and acetoclastic/hydrogenotrophic methanogens. FDH, formate dehydrogenase; FAD, flavin adenine dinucleotide; F_{420}, coenzyme F_{420}; H_{2}ase, hydrogenase; FMD, formyl-MFR dehydrogenase; FTR, formyl-MFR-H_{4}MPT formyl transferase; MCH, methenyl-H_{4}MPT cyclohydrolase; MER, methylene-H_{4}MPT reductase; HMD, methylene-H_{4}MPT dehydrogenase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; ACS, acetyl-CoA synthase; MTR, methyl-H_{4}MPT:HS-CoM methyl transferase; MCR, methyl-CoM reductase [2,28,38,57,112].
bimethanation followed by alteration in microbial communities in AD due to the feeding of various types of organic waste, and the impact of microbial alterations on interspecies networks and biomethanation. Assessing the metabolic roles of the microbiota in syntrophic associations remains a challenge, but this information has significance in microbially enhanced energy recovery technologies. Thus, a study of microbial symbiotic networks among microbial communities and their performance during substrate variations could be beneficial for a precise understanding of AD and its stable performance for continuous biomethanation.

Symbiosis in Anaerobic Digestion

Hydrolysis is the first phase of AD, where the insoluble organic polymers (e.g., polysaccharides, proteins, lipids) are hydrolyzed to generate soluble or insoluble monomers and oligomers (Figure 1). This phase is also considered the primary rate-limiting step in AD as the rate of hydrolysis determines substrate availability to the subsequent phases. A rapid rate of substrate conversion and subsequent production of VFAs and other toxic byproducts could limit efficient biomethanation [21]. Hydrolytic microorganisms such as Clostridium, Bacillus, Bacteroides, Butyribrio, Fusobacterium, Hydrogenoanaerobacterium, Paraclostridium, Anaerossilbacter, Prevotella, Eubacterium, Micrococcus, and Lachnoclostridium secrete various hydrolytic enzymes, such as cellulase, xylanase, amylase, protease, and lipase [6, 22, 23]. Hydrolysis is immediately followed by acidogenesis, the acid-forming step (Figure 1). Acidogenesis is typically the fastest reaction in the anaerobic conversion of complex organic matter in AD [24]. Sugars, glycerol, and amino acids derived from hydrolysis are utilized as substrates in acidogenesis by fermentative bacteria such as Clostridium, Bacillus, Tissierella, Lactobacillus, Streptococcus, Hydrogenoanaerobacterium, Paraclostridium, Prevotella, Eubacterium, and Lachnoclostridium for acidification [22, 23]. Organic acids, including acetic, propionic, butyric, and other short-chain fatty acids (SCFAs) as well as alcohols (e.g., methanol, ethanol), H\textsubscript{2}, and CO\textsubscript{2} are the products of acidogenesis [3]. Acidogenesis occurs via hydrogenation and dehydrogenation depending on the microbial metabolism [25]. The long-chain fatty acids (LCFAs) (C13–C22) are subsequently oxidized to C2 (i.e., acetate) and hydrogen by aceticogenic bacteria. LCFAs produced during hydrolysis are also oxidized via β-oxidation to acetate and H\textsubscript{2} during aceticogenesis by proton-reducing aceticogenic bacteria belonging to Clostridiaeae, Syntrophomonadaceae, Syntrophaceae, Enterobacteriaceae, and Bacteroidida families. Sequencing and comparative genomics of the members of these families has provided more information on fatty acid degradation pathways and their regulatory mechanisms [25]. Enhanced methane production is often correlated with a high relative abundance of syntrophic acetate oxidizers (SAOs) and syntrophic fatty acid oxidizers (SFAOs), including Clostridium ultunense, Syntrophomonas, and Syntrophus [26, 27]. SFAOs oxidize higher fatty acids (LCFAs) into acetate and SAOs oxidize acetate into H\textsubscript{2} and CO\textsubscript{2}. Acetate, alcohols, H\textsubscript{2}, and CO\textsubscript{2} produced during aceticogenesis and acetogenesis serve as substrates for methanogenesis, where CH\textsubscript{4} is produced (Figure 1) via the oxidation of acetate, and the reduction of carbon dioxide and alcohols through electron/hydrogen uptake by aceticlastic, hydrogenotrophic, and methylotrophic methanogenic archaea, respectively [3, 28–30].

The syntrophic association (Figure 1) among hydrogenogenic exoelectrogenic bacteria and aceticlastic/hydrogenotrophic methanogens facilitates the stable performance of AD by maintaining the acidity and hydrogen pressure inside digesters [4, 23, 31]. Exoelectrogens are acidogenic and aceticogenic bacteria that generate and transfer electrons/hydrogen/formate out of the cells to insoluble metallic electron acceptors or to electrotrophic microorganisms (e.g., methanogens) directly or indirectly by maintaining thermodynamic stability [4, 32, 33]. Oxidation of organic matter and reduction of CO\textsubscript{2} to CH\textsubscript{4} in AD occur through close association among syntrophic partners [2]. Exploitation of hydrogen and formate as electron carriers during the IET
process is a major means of electron exchange among syntrophic microorganisms. For the growth and metabolism of syntrophs via interspecies hydrogen transfer (IHT) and interspecies formate transfer (IFT), hydrogen or formate are consumed rapidly by methanogens as reducing equivalents (Figure 1). Methanogens gain energy from the reduction of CO$_2$ to CH$_4$ by using hydrogen as an electron donor [2,7]. Formate is an important diffusive redox mediator in the methanogenic community due to the higher diffusion rate of IFT than that of hydrogen. By using hydrogen-uptake hydrogenase as an electron carrier, coenzyme F$_{420}$ and oxidized ferredoxin are reduced (Figure 1). Reduced FAD and reduced F$_{420}$ (F$_{420}$H$_2$) then act as direct electron donors in IHT. Formate is oxidized to CO$_2$ by formate dehydrogenase (FDH) and is further reduced to methane [28]. A slight disruption in the rate of H$_2$ consumption by methanogens creates an imbalance in syntrophic metabolism, leading to the accumulation of SCFAs and digester perturbation [34].

**Direct IET (DIET)** has been intensively studied as an alternative to IHT and IFT. In DIET, microbial species establish electrical conduits through conductive proteinaceous filamentous structures (i.e., pili) and extracellular c-type cytochromes (e.g., OmcS, MacA, OmcC, PgcA) located on the outer cell surface (Figure 1) [2,35–37]. DIET can outcompete interspecies H$_2$/formate transfer in syntrophic methanogenic systems due to a higher electron transfer efficiency via electrical conduits, thus minimizing the loss of intermediates. During methanation, methanogens can directly accept electrons through DIET from syntrophic exoelectrogens [7,32,38]. Syntrophic co-culture has a strong correlation with the abundance of genes for outer membrane multiheme c-type cytochromes (e.g., OmcS) and pili. OmcS provides heme-to-heme electron transfer, forming an electron path between surface cytochromes, which are also electrically connected to the periplasm and pili [39]. Conductive nanowires are DIET machineries that also use pili for long-range (several micrometers in length) electron transfer under suitable conditions [39–41]. DIET along the pili has been observed in co-cultures of several exoelectrogenic partners, including *Geobacter* spp., *Pelotomaculum* sp., and *Syntrophus* sp., with electrotrophic methanogens, such as *Methanosaeta*, *Methanosarcina*, *Methanothermobacter*, and *Methanolinea* [33,38,42,43].

Syntrophic partners can mediate IET through conductive material-mediated IET (CIET), such as magnetite (Figure 1), granular activated carbon, biochar, graphene, iron oxide nanoparticles, and akaganeite [2,7]. Conductive materials induce syntrophy by improving enzymatic activities through oxidation/reduction, thereby enabling the recovery of microbial cells in response to cytotoxic stress [23,31,44]. Magnetite, the most widely tested conductive material, has been shown to promote CIET among *Geobacter* spp., *Thiobacillus* sp., and *Rhodopseudomonas* sp. During co-culture, magnetite can be charged with Fe$^{3+}$ reducers, such as *Geobacter sulfurreducens*, and discharged with Fe$^{2+}$ oxidizers, such as *Rhodopseudomonas palustris*, and *Thiobacillus* sp.. *Geobacter* showed undisturbed electron transport by using dual IET systems including DIET and CIET, thereby creating a stable syntrophy [39]. Graphene promoted the syntrophic CIET between the acetogenic *Levilinea* and the hydrogenotrophic methanogen *Methanobacterium* and enhanced biomethanation by 25% [45]. When granular activated carbon is used as a conductive material, pili and OmcS become unnecessary for IET [46]. Methanation was increased by 30–45% on the addition of biochar in ethanol digestion; this was associated with the abundance of hydrogenotrophic *Methanobacterium*, indicating electron transfer to hydrogenotrophs for CO$_2$ reduction [47]. The novel crystalline form of the synthetic phenazine neutral red was found to harvest electrons from reduced inorganic and organic microbial sources and deliver them to the membrane-bound heterodisulfide reductase (HdrED) of *Methanosarcina*, thus promoting methanation [48]. Rapid conversion of accumulated VFAs (i.e., acetate, propionate) to methane occurred as a result of biochar-mediated syntrophy among
exoelectrogenic acetogens and hydrogenotrophic methanogens (including *Methanothermobacter* and *Methanolinea*) during the AD of oil [49]. Thus, conductive material-rich environments can aid in the reduction of energy expenditure by exoelectrogenic species that are capable of CIET via the enhancement of their extracellular electron transfer (EET) to methanogens [2,7].

The mechanism by which methanogens acquire electrons directly from their exoelectrogenic partners remains to be discovered. The incorporation of several high-throughput omics approaches can help us identify such targeted genes in methanogens [37]. Only a few methanogens belonging to the order Methanosarcinales possess membrane-bound c-type cytochromes or extracellular hydrogenases (Figure 1). In an H-cell reactor experiment, *Methanosarcina barkeri* demonstrated the capacity to uptake electrons directly from the cathodes in the absence of hydrogenase (F420 dependent (Frh), methanophenazine dependent (Vht), and ferredoxin dependent (Ech)), thereby indicating the hydrogen- and hydrogenase-independent nature of *M. barkeri* in generating syntrophic partnerships [50,51]. The conductive proteinaceous archaellum of hydrogenotrophic *Methanospirillum hungatei* could exchange electrons by reducing the extracellular electron acceptors or engaging in DIET with bacteria; however, electron acceptance from exoelectrogenic partners remains unclear [52]. Methanogens, mostly belonging to the hydrogenotrophs, can reduce CO₂. Reduction of CO₂ occurs by the use of formate/H₂ as an electron donor during hydrogenotrophic methanogenesis, where FDH oxidizes formate and F420-H₂ oxidizes itself to deliver electrons (Figure 1) [53]. Acetate contributes to two-thirds of the produced methane as a major intermediate in AD through acetoclastic methanogenesis, where the key players are archaebacteria belonging to the genera *Methanosaeta* and *Methanosarcina* [34]. Acetoclastic methanogens acquire acetate from their acidogenic/acetogenic partners. Oxidation of the carboxyl group of acetate and the simultaneous reduction to CH₄ occur during acetoclastic methanogenesis (Figure 1) [28,38]. Archaea in the genus *Methanosaeta* are kinetically competitive and superior acetate utilizers (up to 44 mM) due to their lower maximum specific growth rate (μmax) and half saturation concentration (Ks) for growth in acetate than those of the genus *Methanosarcina*, which are quite versatile in their response to high acetate concentrations (14–28 M) [54,55]. Interestingly, acetoclastic methanogens expressed syntrophy with acetogenic and exoelectrogenic syntrophs, signifying their dual capabilities for the oxidation of acetate and the reduction of carbon dioxide via IET [23,38,42]. Thus, the abundance of acetoclastic methanogens (>90%) outcompetes the population of hydrogenotrophs (<0.6%) in AD [34].

**Microbial Communities in Anaerobic Digestion of Organic Waste**

Anaerobic digesters, dealing with wastewater sludge, harbor diversified microbiota to perform various metabolic reactions for the methanation of organic matter (Figure 2). These intricate sets of symbioses impede the investigation of the microbiomes through traditional microbiological techniques [56]. Several researches have tried to identify the core AD microbiota responsible for symbiosis in AD. High-throughput omics approaches such as terminal restriction fragment length polymorphism (TRFLP), 16S rRNA amplicon sequencing, metagenomics, and metatranscriptomics have revealed the microbial ecology and dynamics of anaerobic digesters around the globe in the past decade [57–62]. The investigation of multiple full-scale plants has elucidated the presence of the core microbiome that contributes 28–59% of the total 16S rRNA gene sequences, where influent activated sludge has its contribution [17,63,64]. Dynamicity in the populations of acidogens and acetogens was apparent only in co-digestion with polysaccharides and lipids due to substrate variations, which was insignificant in full-scale digesters treating wastewater sludge only (Seoul anaerobic digester sludge-1 (ADS-1), and Daegu anaerobic digester sludge-2 (ADS-2) in Figure 2), located in two different cities in Korea (Republic of) [23,55]. Studies of 90 full-scale digesters at 51 municipal wastewater treatment plants from five countries have shown ecological diversity in microbiomes during AD due to the
heterogeneity of organic feed instead of their geographic locations [60]. Thus, substrate specificity provided a traceable microbial community to dissect AD.

Microbiota in the Digestion of Polysaccharides

Polysaccharides are an integral part of organic waste (e.g., food waste, fruit waste), comprising about 60% of the total mass [65]. Conversion of polysaccharidic waste to biomethane depends on the abundance and metabolic activity of the syntrophic non-methanogenic bacteria and their syntrophy with methanogens due to their versatile nature. An increase in the abundance of the acidogenic saccharolytic genera, including Prevotella, Eubacterium, and Lachnolactobacterium of the phyla Bacteroidetes and Firmicutes was observed in co-digestion with polysaccharidic waste. This increases the total population of acidogens to 60% from the normal abundance of 35–38% in wastewater sludge digesters (Figure 2). AD of ryegrass increased the population of Bacteroidia, Betaproteobacteria, Clostridia, Gammaproteobacteria, Methanomicrobia, and Negativicutes in mesophilic semicontinuous reactors to up to 93% of the total operational taxonomic units (OTUs), resulting in the acidification of the reactors [67]. At a high paper waste concentration in thermophilic co-digestion, there was an increase in the population of cellulose-degrading bacteria, Defluvium tunisiensis, and a reduction in the syntrophic proportion [68]. Saccharolytic or cellulolytic activities allowed these acidogens to thrive in polysaccharidic waste co-digestion for hydrolysis and acidogenesis (Figure 2). Acidogens exhibited syntrophy with acetogens to facilitate the intermediate stages of biomethanation (Figure 2 and Figure S1A in the

Figure 2. Various Metabolic Reactions (i.e., Hydrolysis, Acidogenesis, Acetogenesis, Methanogenesis) and the Most Abundant (>0.3%) Genera during the Anaerobic Digestion of Organic Wastes. (A–H) are the products of different metabolic reactions. Anaerobic digester sludge (ADS)-1 and -2 represent the microbiota of two different anaerobic digesters located in Seoul, Korea (Republic of) and Daegu, Korea (Republic of), respectively. Both digesters are used to digest municipal wastewater sludge. ‘Polysaccharide’ and ‘lipid’ represent the microbiota of the digesters when the polysaccharidic and lipidic wastes are digested [23,55,70].
supplemental information online). ADS-1 and ADS-2 exhibited insignificant differences in the abundance of acidogens, as heterogeneity in microbial communities is dependent on the constituents of substrates rather than on the geographic location [6,23,60].

A positive association was observed between propionate-oxidizing acetogens of the phylum Chloroflexi (i.e., Anaerolinea, Bellilinea, Levilinea, and Longilinea) and acetoclastic methanogens such as Methanosaeta and Methanosarcina in the AD of polysaccharidic waste (Figures 2 and 3); this accelerated the methanogenic activity by seven times [23]. The higher affinity of Methanosaeta for acetate produced via propionate oxidation allowed them to be competitively prevalent to create a syntrophic association. Filamentous acetogens belonging to Anaerolinea thermolimosa, Levilinea saccharolytica, and Leptolinea tardivitalis were found to promote polysaccharide fermentation by syntrophically associating with acetoclastic/hydrogenotrophic methanogens [3,69]. Nevertheless, the relative abundance of acetogens in the polysaccharide co-digestion was close to the population in ADS-1 and ADS-2 (Figures 2 and 3) as the utilization of polysaccharide-containing wastes follows a similar trend of hydrolysis and acidogenesis, followed by oxidation of VFAs in acetogenesis [70]. The archaea belonging to Methanosaeta remain dominant in the core communities of ADS-1, ADS-2, and polysaccharidic co-digestion (Figure 2), contributing two-thirds of the methane production through the oxidation of acetic acid [34]. Six of eight clusters from the microbiomes of 90 full-scale digesters showed the global prevalence of Methanosaeta [60]. The hydrogenotrophic methanogen Methanolinea showed syntrophy with hydrogenogenic syntrophs including Clostridium, Pelotomaculum, Syntrophomonas, and Thermomarinilinea (Figure 3 and Figure S1A) due to their higher affinity for electrons/hydrogen for uptake during digestion [71]. Acetoclastic methanogens also exhibited a syntrophic association with exoelectrogens such as Bythopirellula and Mariniphaga (Figure 3 and Figure S1A) to facilitate efficient digestion through IETs [23,34]. Thus, syntrophic associations make acetoclastic methanogenesis the most prevalent methanation pathway for the stable performance of anaerobic digesters.

Microbiota in the Digestion of Lipids

Lipids such as fat, oil, and grease are constituents of wastewater discharged from municipal services, food processing and dairy industries, slaughterhouses, and oil refineries and comprise LCFAs (saturated and unsaturated) and glycerol [72,73]. Lipids are one of the most abundant organic C sources, whose utilization in AD is reported to enhance biomethane yields by 250–350% due to their higher convertibility (94.8%) to biogas than other sources. The utilization of LCFAs in AD occurs through several cycles of β-oxidation that yield acetic acid and hydrogen as substrates for methanation. Hydrogenogenic syntrophs such as acetogens or sulfate-reducing bacteria oxidize LCFAs, outcompeting the fermentative acidogens [3,74]. The population of acetogens is increased significantly in digesters with lipids, where they account for more than half of the total microbial population (Figure 2). Nevertheless, degradation of saturated LCFAs (e.g., palmitic and stearic acids) is non-spontaneous and the thermodynamic feasibility of their β-oxidation requires a low hydrogen partial pressure (1 Pa) and low acetate concentration (8 or 9 mmol l⁻¹), which are accomplished through syntrophic coupling among acetogens and methanogens [75,76]. A hydrogen partial pressure of <2 Pa favors methanogenic activity [77]. Sharing of the released chemical energy from the partial degradation of LCFAs among the syntrophic partners aids the thermodynamic feasibility for process stabilization [78].

The syntrophic association of the acetogenic oleate (unsaturated LCA) oxidizer Syntrophomonas (43%) and the dominant acetoclastic Methanosaeta (>40% of total archaea) drives rapid oleate conversion to methane through simultaneous acetate consumption during pulse-fed co-digestion [79]. The absence of adequate methanogenic populations limits the complete...
Figure 3. Syntrophic Interactions among Syntrophs and Methanogens during the Anaerobic Digestion of Various Organic Wastes [Including Anaerobic Digester Sludge (ADS)-1, ADS-2, Polysaccharides, and Lipids]. Syntrophic genera include Aminivibrio (A1), Anaerolinea (A2), Bythopirellula (B2), Cloacimonas (C1), Clostridium (C2), Geobacter (G1), Levilinea (L1), Longilinea (L2), Mariniphaga (M1), Moorella (M12), Pelotomaculum (P1), Sedimentibacter (S1), Smithella (S2), Sphaerochaeta (S3), Sporosarcina (S4), Syntrophomonas (S5), Syntrophorhabdus (S6), Syntrophus (S7), and Thermomarinilinea (T1). Methanobacterium (M2), Methanobrevibacter (M3), Methanoculleus (M4), Methanolinea (M5), Methanomassilicoccus (M6), Methanomethylovorans (M7), Methanosphaerula (M10), and Methanospirillum (M11) are hydrogenotrophic methanogens. Methanosaeta (M8) and Methanosarcina (M9) are acetoclastic methanogens [23,56].
degradation of palmitate during the acidogenic fermentation of lipids, thus influencing the population of syntrophic acetogens, whereas unsaturated LCFA (including oleic acid, γ-linolenic acid, and linoleic acid) show complete degradation [6]. An increase in the abundance of the acetogenic LCFA oxidizers Sporosarcina (41%), and Syntrophomonas (11%) was prominent during the co-digestion of lipidic waste, with a shift in the methanogenic population towards Methanosarcina (95% of methanogens) (Figure 2), resulting in improved methanation through acetate oxidation [55]. A positive correlation among dominant acetogens and Methanosarcina accelerated the conversion of lipidic waste to methane (Figure 3 and Figure S1B in the supplemental information online). Similarly, the presence of an acetogenic family, the Syntrophaceae (e.g., Syntrophus) facilitated LCFA degradation in a psychrophilic environment (10°C), and improved methane yields (79–85%) from LCFA-containing synthetic dairy wastewater. Syntrophy among LCFA-degrading acetogens and acetoclastic Methanosaeta improved the maximum methane production rate by 4.7 times with 10 days of digestion, indicating the dominance of Methanosaeta in psychrophilic environments over other archaea [80]. Thus, anaerobic digestion of lipidic waste requires close syntrophy among LCFA-oxidizing syntrophs and methanogenic archaea.

Effects of Microbial Alterations on Interspecies Networking and Biomethanation

The stability of AD and consequent biogas yield are highly dependent on the characteristic resistance, resilience, and functional redundancy of the microbial community [16]. The predominant microbial community shifts (Table 1) have been widely observed as functional changes in the types and composition of substrates (rate-limiting step), TS [81], OLR [18], a decline in operating pH due to high acetate and other VFA concentrations typically in the AD of organic-C-abundant substrates [27,82], NH₄⁺ accumulation mostly in AD of proteinaceous substrates [83], changes in temperature [84], HRT [20], type of inoculum or seed sludge [85], and the number of cycles in a reactor [86]. This suggests that the right kind of population diversity among the hydrolytic, acidogenic, and acetogenic bacteria along with methanogenic archaea is required to ensure the stability and success of overall methanation [3].

Community Structure in the Unstable AD Process

The effects of changes in certain operational conditions on anaerobic microbiota are more severe when a specific substrate is altered (Figure 4). For example, excessive FOG and lignocellulosic substrates with a very high C:N ratio (≥40–47), represent significant challenges associated with the accumulation of VFAs, resulting in a decline in system pH [87]. Acidogenesis and acetogenesis are greatly influenced by the system pH and simultaneous H₂ production [88]. A high loading rate of FOG also contributes to the formation of LCFA and simultaneous conversion to acetic acid and hydrogen by acetogens [27]. Under mesophilic conditions, alterations in the methanogenic archeal community in response to high VFA accumulation are insignificant and the diversity is less affected (Figure 4) due to the presence of methanogens, such as Methanosarcina, Methanoculleus, Methanobacterium, Methanoseta, Methanomicrobia, Methanobrevibacter, and Methanospaera with dual capabilities (hydrogenotrophic and acetoclastic methanogenesis) [27,89]. However, the archeal community was adversely affected by VFA accumulation in thermophilic digestion, as the transition of the methanogenic archeal community from acetoclastic to hydrogenotrophic Methanoculleus sp. and Methanothermobacter sp. has been observed with high VFAs [88]. A similar shift in the methanogenic population to hydrogenotrophic methanogens in the genus Methanothermobacter (4.94 × 10⁶–9.73 × 10⁵ copies g⁻¹ of the sample) was seen...
The decline in pH due to acidogenic VFA production inhibits the microbial metabolism as the undissociated VFAs diffuse into the cell cytoplasm and dissociate into protons and VFA anions. Protons decrease the cytosolic pH and anions increase the osmolarity of the cytoplasm, affecting the integrity of the cell membrane [90]. Nevertheless, inhibition of the methanogenic population due to high VFAs was temporary and methanogens generally recovered after a certain time with a subsequent decrease in VFA concentration [82,88,90].

An effective buffering system plays a crucial role in maintaining the system redox conditions and the stability of the microbial flora in AD. Extremely high C:N ratios can lead to rapid acidification of thermophilic digesters [82].

<table>
<thead>
<tr>
<th>Variable factor</th>
<th>Substrate</th>
<th>Eubacterial community</th>
<th>Methanogenic community</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesophilic (37°C)</td>
<td>Household (80–85%), slaughterhouse, and food industry (15–20%) waste</td>
<td>Bacteroidetes (25–40%), Firmicutes (17–33%), and Cloacimonetes (13–30%)</td>
<td>Methanobacteria, Methanosarcinaceae, and Methanomicrobiales</td>
<td>[82]</td>
</tr>
<tr>
<td>Thermophilic (52°C)</td>
<td>Food waste</td>
<td>Bacteroidetes, Thermotogae, Firmicutes, Actinobacteria, Synergistetes, and Spirochaetes with the inclusion of classes</td>
<td>Methanobacteria, Methanosarcinaceae, and Methanomicrobiales</td>
<td>[82,88,90]</td>
</tr>
<tr>
<td>Mesophilic (35°C)</td>
<td>Food waste</td>
<td>Bacteroidetes, Thermotogae, Firmicutes, Actinobacteria, Synergistetes, and Spirochaetes</td>
<td>Methanosaeta and Methanosarcina</td>
<td>[97]</td>
</tr>
<tr>
<td>Thermophilic (55°C)</td>
<td>Corn stover</td>
<td>Firmicutes (82%)</td>
<td>Methanobacterium (93.4%)</td>
<td>[101]</td>
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<tr>
<td>Low VFA (0.079 g l⁻¹)</td>
<td>Cow manure</td>
<td>–</td>
<td>Methanosaeta, Methanosarcinaceae, Methanobacterium, and Methanoculleus</td>
<td>[82]</td>
</tr>
<tr>
<td>High VFA (17 g l⁻¹)</td>
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<td>Methanothermobacter, Methanoculleus, and Methanobacterium</td>
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</tr>
<tr>
<td>Low VFA (0.1 g l⁻¹)</td>
<td>Kitchen wastes</td>
<td>–</td>
<td>Methanosarcina mazei, Methanobacterium sp., and Methanocorpusculum sp.</td>
<td>[113]</td>
</tr>
<tr>
<td>High VFA (17.9 g l⁻¹)</td>
<td>–</td>
<td>Methanosaeta and Methanoculleus marisnigri</td>
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<tr>
<td>Low TAN (2 g l⁻¹)</td>
<td>Chicken manure</td>
<td>Firmicutes (87%), Bacteroidetes (8.6%), Proteobacteria (1.54%), Spirochaetes (1.4%), Fusobacterium (0.73%), and Actinobacteria (0.73%)</td>
<td>Methanosarcina mazei GO1 (79%), Methanosarcina acetivorans C2A (16%), Methanoa (2%), and Methanoculleus (2%)</td>
<td>[83]</td>
</tr>
<tr>
<td>High TAN (16 g l⁻¹)</td>
<td>Firmicutes (92%), Bacteroidetes (4%), and Proteobacteria (4%)</td>
<td>Methanosarcina (39%), Methanoculleus (30%), and other hydrogenotrophic methanogens</td>
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<tr>
<td>Low [NH₄⁺] (0.5 g l⁻¹)</td>
<td>Sewage</td>
<td>–</td>
<td>Methanosaeta sp. and members of the order Methanomicrobiales</td>
<td>[114]</td>
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<td>High [NH₄⁺] (6 g l⁻¹)</td>
<td>–</td>
<td>Methanobacterium sp. and Methanosarcina sp.</td>
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<tr>
<td>OLR (1.6 g VS l⁻¹ day⁻¹)</td>
<td>Sugar beet pulp</td>
<td>Bacteroidetes (31.6% ± 8.8%), Firmicutes (8.1% ± 3.6%), Chloroflexi, and Synergistetes</td>
<td>Methanobacterium sp. (~50%), Methanosaeta sp., and unknown Methanosprillaceae</td>
<td>[105]</td>
</tr>
<tr>
<td>OLR (4.4 g VS l⁻¹ day⁻¹)</td>
<td>Candidate WWE1 (27.6% ± 3.9), Firmicutes, Bacteroidetes, Synergistetes, and Chloroflexi with dominant families Porphyrimonadaceae, Lachnospiraceae, and Cloacamonaceae</td>
<td>Methanobacterium, Methanosarcinaceae, Methanoculleus sp., and unknown Methanosprillaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLR (2.7 g VS l⁻¹ day⁻¹)</td>
<td>Meadow grass and pig manure</td>
<td>Coprothermobacter proteolyticus, Clostridium thermocellum, and Proteobacteria</td>
<td>Methanosarcina and Methanoculleus</td>
<td>[115]</td>
</tr>
</tbody>
</table>
the system; therefore, the presence of proteinaceous substrates could help to maintain the pH (owing to NH₄⁺ accumulation as a byproduct of protein metabolism) [91]. Due to their high protein content, slaughterhouse and dairy wastes can be considered as potential substrates for co-digestion with carbonaceous waste due to their high methane yields and process stability. A very low C:N ratio due to high protein could lead to the accumulation of high concentrations of NH₄⁺, which exerts inhibitory effects on the anaerobic microbiota [92]. Methanosaeta, a vigorous utilizer of acetate, tends to decrease at higher NH₄⁺-N concentrations (b5g l⁻¹) primarily due to the inhibitory effects of NH₄⁺ on intracellular ion exchange (Figure 4). Methanosarcina outcompeted Methanosaeta at higher concentrations (>7g l⁻¹) by forming clusters of cells that create an NH₄⁺ gradient from the bulk solution to the surface and to the inner part of the microbial cluster, resulting in a decreased NH₄⁺ concentration at the inner core of the aggregate [93,94]. Moreover, Methanosarcina is metabolically more versatile as it can generate CH₄ from H₂/CO₂, acetate, and methanol, thus explaining the three-times-higher specific growth rate compared with Methanosaeta [55,95]. Niu et al. reported an increase in acetoclastic Methanosarcina acetivorans (17% of the total methanogenic population in the steady state to 72% in the recovered state), whereas another acetoclast, Methanosaeta sp., almost disappeared at high total ammonia nitrogen (TAN). A similar trend was observed in the population of Firmicutes during the steady state to recovered state, along with the Bacteroidetes population (flourished to 31% in the recovered state) [83]. In addition to the shift of acetoclastic to hydrogenotrophic methanogenesis, an apparent NH₄⁺-induced shift from acetoclastic methanogenesis to an alternative syntrophic acetate oxidation (SAO) pathway can also be observed. Bacterial phylotypes became more uneven after ammonia addition, but recovered to the initial population once the SAO bacteria helped them obtain a stable performance [96].

Figure 4. Microbial Alterations during Variations in Physiological Parameters. (A) Temperature (M, mesophilic; T, thermophilic); (B) organic loading rate (OLRs); (C) volatile fatty acids (VFAs); (D) total ammonia nitrogen (TAN) [83,89,94,100,107].
The operating temperature in AD is an important parameter that strongly affects microbial community structure, biogas yield, and process stability. Mesophilic (30–40°C) and thermophilic (50–65°C) processes are commonly used in different ADs [97,98]. A transition of operating temperature regime may require a period of adaptation to overcome the imbalance. An increase in temperature of 5–10°C from mesophilic conditions can lead to significant process disturbances due to a decline in the richness and diversity of microbial populations (Figure 4) [99,100]. Thermophilic conditions increase the imbalance between fermentative and methanogenic communities due to the persistent accumulation of VFAs [97]. Firmicutes (30–80%) and Bacteroidetes (10–40%) are the two predominant bacterial phyla commonly observed in steady-state mesophilic anaerobic reactors along with Chloroflexi, whereas members of the Thermotogae, Synergistetes, and Firmicutes predominate in thermophilic AD [98,101,102].

Drastic shifts in bacterial phylum abundances are observed in the mesophilic-to-thermophilic transition [103]. The abundance of Proteobacteria and Bacteroidetes generally decreases under thermophilic conditions with the increase in Thermotogae, Synergistetes, and Firmicutes [84]. By contrast, although the archaeal community is not as abundant as bacteria, they remain functionally stable in the background of changes in operational temperature. In other studies, members of the genus Methanosarcina were dominant under both conditions (accounting for 84.24% and 98.53% in thermophilic AD and mesophilic AD, respectively) [97,102]. Some researchers found that Methanoseta is dominant among the methanogenic community in mesophilic AD, whereas Methanothermobacter, Methanobacterium, and Methanoculleus flourished in thermophilic AD [98,101,103]. Methanosarcina can produce methane as an acetoclast and a hydrogenotroph as well in mesophilic and thermophilic digesters. Studies indicate a clear shift from acetoclasts to hydrogenotrophs during the transition from mesophilic to thermophilic conditions (Figure 4) [103]. As a result, the implications pertaining to VFA inhibition in AD are more severe in the case of thermophilic AD, as can be evidenced by the accumulation of higher amounts of acetate and butyrate [104].

The biodiversity of the anaerobic microbiome is influenced not only by environmental factors (e.g., temperature, pH) but also by the OLR (Box 1). Firmicutes, mostly represented by Bacillus and Clostridium, were the predominant bacteria at low OLR [105]. Other bacterial communities from the phyla Gammaproteobacteria, Actinobacteria, Bacteroidetes, and Deferrribacteres were the most abundant at high OLR [106]. In particular, an increase in the abundance of Firmicutes such as Clostridium and Bacteroidetes such as Proteiniphilum can be observed in reactors fed with high OLRs as these organisms are involved in the production and accumulation of high amounts of acetate, propionate, and butyrate, leading to process failures (Figure 4). Compared with the bacterial community, the archaeal community remains stable up to a certain level of increment in the OLR. This is because archaeal communities are less diverse than bacterial communities, and both acetoclastic and hydrogenotrophic methanogens remain stable when the OLR is increased [107]. High OLRs of polysaccharidic and lipidic substrates tend to acidosis and VFA accumulation, which has been observed to shift acetoclastic methanogenesis to hydrogenotrophic, whereas Bacteroidetes dominated the bacterial populations [105]. Changes in TS cause significant alterations in the bacterial community with increasing TS. The abundance of different genera of Chloroflexi and Firmicutes is decreased following an increase in TS, whereas the members of Bacteroidetes are increased. The selective enrichment of Bacteroidetes at high TS content seems to be consistent with the high protein-input rate, as they are capable of degrading various proteins. Further, a shift from hydrogenotrophic Methanobacteriales and Methanomicrobiales to the acetoclastic methanogenic order Methanosarcinales was observed [81]. By contrast, Liu et al. stated that acetoclastic methanogens mainly dominated by Methanosarcina were decreased as the TS increased, whereas the relative abundance
of hydrogenotrophic and methylotrophic methanogens was increased due to their high free-ammonia tolerance (>0.2 g l\(^{-1}\) in mesophilic, >0.37 g l\(^{-1}\) in thermophilic) in an increased-TS environment [108].

Studies also suggest that different members of the phyla Actinobacteria, Bacteroidetes, and Firmicutes show clear dynamics through different HRTs. Actinobacteria, which play a crucial role in cellulose hydrolysis and degradation, flourish at high HRT and are depleted at low HRT [20]. Although high OLR and low HRT in their respective threshold levels can achieve a high biogas production rate and thus improve process efficiency, an even higher OLR with lower HRT is often associated with concerns of process failure. In a deteriorative state, high OLR and low HRT contributing to high VFA concentrations cause an increase in \(H_2\) partial pressure, which is indicative of hydrogenotrophs with a lower \(H_2\) consumption rate [109]. The composition of anaerobic microbial flora in a particular AD process is highly dependent on the arrival of anaerobic microorganisms from newly fed substrates, which also act as a source of inoculum [16]. Although archaean communities have not been reported to be steered by the addition of substrates, bacterial communities are indeed influenced by their inoculum [110].

**Concluding Remarks**

Enhancement of biomethanation in AD has been evaluated through the utilization of heterogeneous organic waste in the past decades. The performance of AD is solely dependent on the intricate microbial networks existing among hydrolytic and fermentative bacteria, syntrophic bacteria, and methanogenic archaebacteria that aid the mineralization of complex organic substances. Regardless of physicochemical factors, alteration in the microbiota is apparent in anaerobic digestion of heterogeneous substrates.

**Outstanding Questions**

- What are the specific networks for interspecies microbial syntrophy in anaerobic digestion?
- How do methanogens create syntrophic partnership with exoelectrogenic or acidogenic/acetogenic partners?
- Does substrate specificity influence the digester microbiota towards biomethanation? If yes, how does it work?
- Is there any core microbiome for the digestion of heterogeneous substrates?
- How do microbial alterations affect interspecies networking and biomethanation on substrate variation in commercial anaerobic digesters?
- How do deterministic factors influence the microbiota of anaerobic digesters?
digester due to the feeding of heterogenic organic waste. This causes diversification in the digester microbiota and impacts the intricate interspecies networks and their performance, leading to digester perturbation. High-throughput omics approaches have assisted in the dissection of the microbial diversity and its possible influence on metabolic alterations during the AD of organic waste, which was once thought to be limited by changes in physicochemical factors such as temperature, pH, trace elements, OLR, TS, VS, and HRT. The quantification of microbial dynamics has been recommended as a standard method to evaluate the metabolic shifts, microbial syntrophy, and overall digester performance during substrate alterations. Although there are limited transcriptomic and proteomic data, this review comprehensively discusses the existing literature to generate an understanding about the subtle microbial dynamics and networks at the laboratory scale and real scale in anaerobic digesters used to treat various types of organic waste (see Outstanding Questions). A combination of multomics approaches (e.g., 16S rRNA amplicon sequencing, metagenomics, metatranscriptomics, proteomics) could open a wide range of possibilities for the determination of diverse metabolic activities with information regarding the respective microbiota involved during feed alterations, reactor failure, and stabilization. Furthermore, stable performance of anaerobic digesters with optimum biomethanation requires the involvement of microbiota acclimatized to specific substrates to perform the preeminent metabolism. This could be an important operational strategy to maintain microbial networks for the promotion of stable digester performance. Therefore, the application of multomics approaches and substrate-specific microbiota could be beneficial in facilitating improved biomethanation from various organic wastes in real-scale anaerobic digesters.

Authors’ Contributions
All authors researched the data for this review, made substantial contributions to discussions of the content, wrote the article, and reviewed and/or edited the manuscript prior to submission.

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