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Review article

Current status and future developments of assessing microbiome composition and dynamics in anaerobic digestion systems using metagenomic approaches

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ABSTRACT

The metagenomic approach stands as a powerful technique for examining the composition of microbial communities and their involvement in various anaerobic digestion (AD) systems. Understanding the structure, function, and dynamics of microbial communities becomes pivotal for optimizing the biogas process, enhancing its stability and improving overall performance. Currently, taxonomic profiling of biogas-producing communities relies mainly on high-throughput 16S rRNA sequencing, offering insights into the bacterial and archaeal structures of AD assemblages and their correlations with fed substrates and process parameters. To delve even deeper, shotgun and genome-centric metagenomic approaches are employed to recover individual genomes from the metagenome. This provides a nuanced understanding of collective functionalities, interspecies interactions, and microbial associations with abiotic factors. The application of OMICs in AD systems holds the potential to revolutionize the field, leading to more efficient and sustainable waste management practices particularly through the implementation of precision anaerobic digestion systems. As ongoing research in this area progresses, anticipations are high for further exciting developments in the future. This review serves to explore the current landscape of metagenomic analyses, with focus on advancing our comprehension and critically evaluating biases and recommendations in the analysis of microbial communities in anaerobic

Abbreviations: AD, Anaerobic digestion; NGS, Next-Generation Sequencing; DGGE, Denaturing gradient gel electrophoresis; ARISA, Automated and Ribosomal Intergenic Spacer Analysis; TRFLP, Terminal restriction fragment-length polymorphism; FISH, Fluorescence in situ hybridization method; HTS, High-throughput sequencing; PacBio, Pacific Biosciences; SMRT, Single Molecule Real-Time; ONT, Oxford Nanopore Technology; SOP, Standard operating procedure; PCR, Polymerase Chain Reaction; DIET, Interspecies electron transfert; CBM, Carbohydrate-binding module; mcrA, Methyl-coenzyme M reductase; UASB, Up Flow-Anaerobic Sludge Blanket Reactor; CSTR, Continuous stirred-tank reactor; UPLC-MS/MS, Ultra-performance liquid chromatography-mass spectrometry; ARGs, Antibiotics resistance genes; MGEs, Mobile genetic elements; BP, Biogas production; OTUs, Operational taxonomic units; AAs, Amino acids; OLR, Organic loading rate; AnMBR, Anaerobic Membrane Bioreactor; UBER, Up-flow biocatalyzed electrolysis reactor; MAGs, Metagenome-assembled-genome; CE, Carbohydrate esterase.

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digesters. Its objective is to explore how contemporary metagenomic approaches can be effectively applied to enhance our understanding and contribute to the refinement of the AD process. This marks a substantial stride towards achieving a more comprehensive understanding of anaerobic digestion systems.

1. Introduction

Anaerobic digestion (AD) is a biochemical process with the capacity to convert a diverse range of feedstocks, primarily organic wastes including livestock manure, food wastes, sewage sludge, crop residues, agricultural byproducts, and the organic fraction of municipal solid wastes, into biogas. The growing demand for energy and the imperative to reduce greenhouse gas emissions emphasize the necessity to transition from fossil fuels to renewable energy sources, positioning biogas as a significant player in the evolving energy landscape. Biogas obtained through AD exhibits versatile applications, including combustion for heat production, electricity generation, and as a viable vehicular fuel. The conversion of wastes into biogas via AD is facilitated by a complex community of microorganisms, collectively known as the microbiota, engaging in a series of biological reactions. The intricacies of the AD process often led to the description of anaerobic digesters as "black box" and the AD microbiome as "black matter", primarily due to the complex and heterogeneous ecosystem and the huge diversity of uncharacterized microbes. The efficiency of these processes is influenced by various environmental, microbiological and process parameters [1]. A more thorough understanding of these parameters would provide valuable insights necessary for adjusting production parameters and conditions, ultimately optimizing the process to achieve maximum product yield with minimal production costs.

The increasing accessibility of high-throughput Next-Generation Sequencing (NGS) technologies and the advancement of bioinformatic algorithms have elevated metagenomic analysis to an invaluable method for understanding microbial communities within anaerobic digestion (AD) systems [2]. Thanks to sequencing methods, particularly when applied to metagenomic approaches, scientists have made several exciting discoveries about the microbial populations in anaerobic digestion processes [3]. Metagenomics involves the analysis of genetic material from environmental samples, eliminating the need for the cultivation of individual organisms [4,5]. Furthermore, metagenomics can be used to monitor microbial communities in anaerobic digesters and diagnose issues such as acidification or inhibition. This information can be then applied to develop strategies aimed at improving the stability and efficiency of anaerobic digestion processes [6,7]. Recent findings have unveiled a strong correlation between the structures of taxonomic and functional genes present in anaerobic microorganisms found in biogas-generating digesters [2]. Moreover, it has been revealed that both these gene structures are susceptible to various environmental factors including digester setup, constituents of feedstock, temperature, organic loading rate (OLR), hydraulic retention time (HRT), and levels of free ammonia [8]. In addition, the examination of functional genes using metagenomic investigations and network-based methods enables the estimation of corresponding metabolic pathways. This approach allows for the identification of new metabolic pathways and microbiological mechanisms operating within the biogas digesters [9]. The application of molecular biological methods and DNA sequencing techniques, including NGS, has significantly advanced our understanding of the microbiome in AD systems and the functional roles of different microorganisms in the AD process [10,11]. For example, The study of the AD microbiome has shifted to a new stage involving a genome-centric metagenomic approach [12]. This approach entails the recovery of individual genomes from metagenomic data, enabling a more detailed analysis of genome information for various novel organisms residing in AD microbiomes and facilitate insights into their potential functions and lifestyle. While it holds great promise for studying the AD microbiome, its application is still in its infancy due to the technical challenges associated with metagenomic approaches. Several works have previously discussed the application of metagenomics to study microorganisms involved in AD and biogas production (BP) [9,13,14]. These studies provide general microbiological and bioinformatic workflows and resources for examining microbial ecology in AD [2,15]. The present review summarizes the current and future applications of the metagenomic approaches in the AD process. It is dedicated to delving into the current landscape of metagenomic analyses, focusing on advancing our comprehension and evaluating biases and recommendations in the analysis of microbial communities in anaerobic digesters.

2. Current understanding of the anaerobic digestion process using metagenomic approaches

Anaerobic digestion is a microbe-driven process with significant societal importance, as it plays a dual role in environmental waste management and energy production. AD contributes to reducing our dependence on fossil fuels by generating methane within engineered bioreactors.

Biogas, predominantly composed of CH_4 and CO_2, produced through anaerobic digestion, is facilitated by a complex community of microorganisms, known as the microbiota, present in AD bioreactors. The microbiology of anaerobic transformation of organic waste involves numerous different bacterial species, including acidogenic, acetogenic, and methanogenic bacteria. Methane, a crucial end-product generated during the methanogenesis step of the AD process, is produced by methanogenic Archaea. The production of methane is directly correlated with the composition of the AD microbiome and is under the control of microbial metabolism, which is, in turn, thermodynamically dependent on environmental parameters of the digesters [16,17]. The close connection between these parameters presents unique opportunities to enhance process efficiency, achievable through microbial selection or manipulation.

Various molecular fingerprinting techniques have been employed to investigate the genetic structure of microbial communities in numerous anaerobic digesters. Approaches include denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel

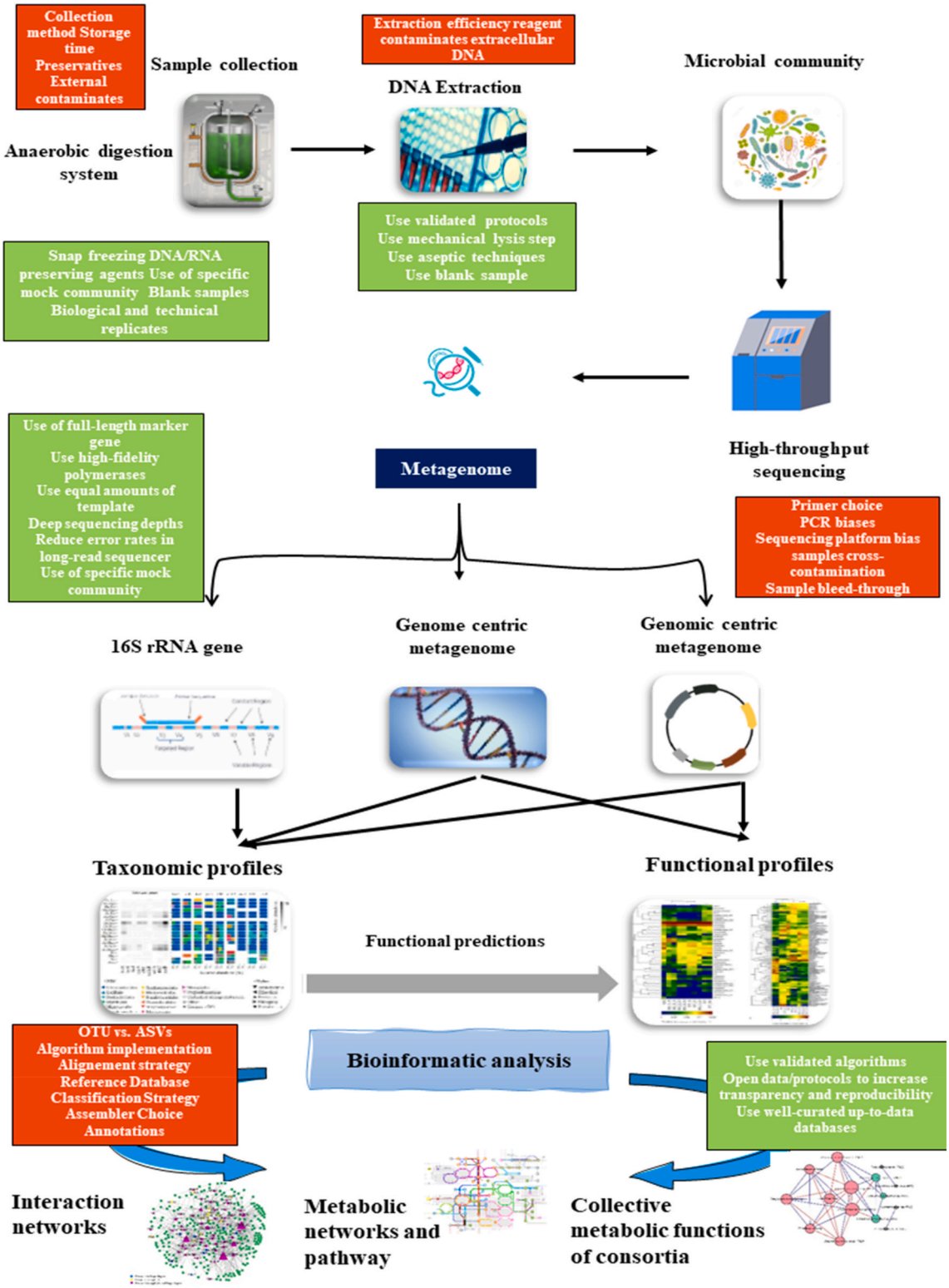

Fig. 1. Overview of integrated metagenomic approaches in microbial and functional interpretation of AD processes. Red boxes represent the various areas that can result in the introduction of bias and green boxes represent some recommended best practices during sequenced-based metagenomic studies.

electrophoresis TGGE [18,19], Automated Ribosomal Intergenic Spacer Analysis (ARISA) [20,21], Terminal restriction fragment-length polymorphism technique (T-RFLP) [22,23], and Fluorescence in situ hybridization method (FISH) [24]. With the emergence of sequencing technologies, DNA sequencing techniques have been successfully applied in the field of environmental engineering [9]. High-throughput sequencing technologies (HTS), also known as next-generation sequencing (NGS) technologies, represent the latest advancements in DNA sequencing methods. These technologies enable rapid and large-scale analysis of genetic material with higher speed and efficiency compared to traditional sequencing methods [4,25]. The revolutionary impact of these technologies extends to genomics, empowering researchers to analyze DNA and RNA sequences on a massive scale. This has led to breakthroughs in various fields, including personalized medicine, agriculture, and evolutionary biology. High-throughput sequencing, has been utilized for the comprehensive analysis of the entire DNA from complex samples [26], derived from AD systems. This application enables the monitoring of the diversity and dynamics of various microbial communities. Recent advances in sequencing technologies and bioinformatics have fundamentally transformed the landscape of DNA and RNA analysis. Illumina® Sequencing as an example, where its exceptional methodology, utilizing reversible terminators, enables the simultaneous sequencing of multiple DNA fragments [27]. Another groundbreaking technology is Ion Torrent® Sequencing, which leverages semiconductor technology to detect pH changes during DNA synthesis [28]. Pacific Biosciences® (PacBio) Single Molecule Real-Time (SMRT) sequencing technology is also noteworthy, extensively probing complex genomic regions and producing longer reads [29]. Among the latest sequencing technologies that have improved DNA and RNA sequencing, we highlight the Oxford Nanopore Technology [30]. Its sequencing approach allows for real-time analysis, portability, and the capability for long-read sequencing. Oxford Nanopore Technology has found application across various research fields, including genomics, transcriptomics, and metagenomics [31]. It has empowered scientists to sequence DNA and RNA from various sources, including viruses, bacteria, and human cells, with high accuracy and speed [5,32].

2.1. Anaerobic digestion process technology and metagenomic approaches

Anaerobic digestion is a process technology involving the decomposition of organic matter by microorganisms in the absence of oxygen to produce biogas. Biogas is a mixture of methane (CH_4) and carbon dioxide (CO_2) alongside small amounts of other gases like hydrogen sulfide (H_2S), nitrogen (N_2), and traces of water vapor [33]. This process can be used for different organic waste treatment, including agricultural residues, food waste, industrial wastes, and, sewage sludge. AD has the potential to provide renewable energy in the form of biogas [34]. Metagenomics is the exploration of genetic material derived from entire microbial communities, facilitating the analysis of the genomes of the most abundant microbial species without the need for individual isolation and cultivation [28]. In the pre-next-generation sequencing (NGS) era, metagenomics studies usually relied on DNA cloning and Sanger sequencing. However, the progress and cost reductions in NGS technologies have led to the widespread adoption of massively parallel metagenomic sequencing, replacing the traditional low-throughput approaches as the primary method for studying diverse microbial communities [35]. In the context of anaerobic digestion, metagenomics can be used to study the microbial communities responsible for organic matter degradation and Biogas Production [2]. Indeed, it provides insights into the diversity of microorganisms present in different types of digesters, their metabolic capabilities, functions, and how they interact to achieve the digestion process [36,37]. Metagenomic analysis can also identify key microorganisms responsible for biogas production, as well as potential inhibitors that may impact the efficiency of the digestion process [38]. This information can be used to optimize the design and operation of anaerobic digesters, improving their overall performance [39]. However, depending on the metagenomic approach used, such as amplicon sequencing, shotgun metagenomics, or genome-centric metagenomics (Fig. 1), different biases may affect specific steps of the microbiome investigation process, especially when dealing with complex and heterogeneous samples from anaerobic digesters [40].

2.2. Workflow biases and recommendations in analyzing microbial communities in anaerobic digesters using sequencing metagenomic approaches

Metagenomics allows for the comprehensive study of microbial communities and their functional potential by analyzing DNA materials extracted directly from in situ samples [41]. Several reviews have previously discussed the application of various metagenomic approaches, their detailed workflow, and bioinformatic analysis for studying the microbiome that drives anaerobic digestion processes [9,10,14,42]. The main steps in metagenomic analysis, as presented in Fig. 1, involve processing the samples to extract genetic material using different methods, such as bead beating, enzymatic lysis, or mechanical disruption [43]. The next step involves library preparation, followed by the generation of reads on NGS platforms such as Roche 454, Illumina, Ion Torrent, PacBio, or Oxford Nanopore [5,27]. After library preparation and read generation, the subsequent step in metagenomic analysis is quality control, which involves trimming adapters and low-quality reads and removing chimeras to minimize errors in downstream analyses caused by artifacts [44]. Following quality control, the data undergoes several steps including assembly, annotation, and taxonomic classification [45]. Assembly involves piecing together the short reads generated by the sequencing into longer contigs [46], while annotation involves identifying the genes and their functions in the assembled contigs. Taxonomic classification assigns the assembled contigs to different taxonomic groups based on their similarity to known reference sequences [47]. These steps ensure that the sequencing data is of high quality and accuracy, enabling researchers to study the functional potential and taxonomic composition of the microbial community. Metagenomic approaches that rely on HTS platforms still face numerous biases, limitations, and challenges. One reason for inconsistent results across studies is the high level of random and systemic bias introduced throughout sequence-based metagenomics studies. As a result, the obtained results from different studies may not align with their previous findings [48]. Throughout a typical metagenomic study, biases are introduced at various stages starting with sample collection and continuing throughout the

Table 1
Examples of monitoring studies for AD systems applying metagenomic approach.

AD system/Reactor type	Feedstock/	Temperature	Objective	Sequencing platform	Dominant bacterial taxa	Dominant archaeal taxa	References
Full-scale anaerobic digesters	Municipal wastewater treatment	__	Explore the microbial communities and functions in anaerobic digestion sludge (ADS) from two wastewater treatment plants based on a metagenomic view.	Illumina high-throughput sequencing	*Proteobacteria (9.52–13.50 %); Bacteroidetes (7.18 %– 10.65 %); Firmicutes (7.53 %–9.46 %).*	*Methanosaeta (3.48%) and Methanosarcina (3.24%)*	[56]
Upflow anaerobic sludge blanket (UASB)	Industrial wastewater (purified terephthalic acid (PTA)	__	Identification of critical microbial interactions at macro- and micro-level eco- system's ecology.	MiSeqReagent kitv3and MiSeqsystem(Illumina)	*Syntrophorhabdus; Syntrophus.*	*Methanosaeta; Methanolinea; Methanobacterium; Methanoregula; Methanomassiliicoccus; and Methanomethylovorans.*	[65]
Laboratory scale/Batch mode	Cattle manure	Mesophilic temperature (35%).	Impacts of biochar on the environmental risk of antibiotic resistance genes and mobile genetic elements during anaerobic digestion of cattle farm wastewater.	Illumina HiSeq PE250 platform	*Firmicutes. (23.3%–62.1%) Proteobacteria (4.9–5.1%).*	*Methanosarcina; Methanoculleus; and Methanobrevibacter.*	[15]
Batch mode biomethanation	Rice straw	Thermophilic temperature (55°C)	Biomethanization of rice straw in a thermophilic anaerobic bioreactor under optimized conditions, using meta-omics for analyzing the microbiome involved in methane production.	Illumina sequencing platform/Ion Torrent PGM sequencer	*Clostridia; Actinobacteria; Bacilli; Bacteroidia.*	*Methanosaeta; Methanosarcina; Methanobacteria; and Methanomicrobia.*	[70]
Full-scale anaerobic digesters	Municipal wastewater treatment plant	__	Examination of short-term enrichments degrading single Amino Acid degraders using metagenomics and meta transcriptomics.	Illumina HiSeq-2500 1 TB platform/Binning metagenomics	*Bacteroidetes (79.2% – 88.6%); Syntrobacterales (15%).*	*Methanofastidiosa (2.7%); Methanoculleus (1.3%); Methanosaeta (0.5%).*	[71]
Full-scale anaerobic digesters	20 different type of wastewater from different reactors	__	Application of the metagenomic approach to samples from 20 different reactors to estimate the taxa performance for different AD types and compare two technologies of sequencing.	Illumina Sequencing/ MinIONSequencer, Oxford Nanopore./16S ribosomal RNA	*Firmicutes (5%–10%; Bacteroidetes (17%–70%).*	_____	[5]
Anaerobic membrane bioreactors (AnMBRs) equipped with different membrane pore size (0.4 or 0.05)	Domestic wastewater	25°C	Effects of hydraulic retention time HRT and process stability performance on the bacterial community in anaerobic digestion.	Illumina HiSeq PE250 platform/16S rRNA gene amplicon sequencing	*Anaerolineae (19%-42%); Bacteroidia (11%-38%); Clostridia (7%-35%).*	*Methanosaeta (23%); Methanobacteria (16%).*	[7]
Mesophilic continuously stirred tank reactor (CSTR).	Microcrystalline cellulose, whey protein, olive oil, and chicken manure as inoculum	__	The performance of anaerobic co-digestion of organic components at different loading levels was investigated in terms of methane yield, metabolic transformation, and microbial response.	Illumina Miseq sequencing	*Firmicutes; Bacteroidota; Synergistota; Caldatribacteriota and; Chloroflexi.*	*Methanosaeta (55.05%); Methanobacterium (55.32%).*	[38]
Laboratory scale/Batch mode	Cow manure and corn straw	__	Enhancement of methane production from mixed anaerobic digestion by adding hydrochar and biogas slurry reflux, to provide attachment for microbial growth, and abundant surface functional groups.	High throughput 16SrRNA gene pyrophosphate sequencing	*Bacteroidetes. (6.35 % – 56.62%) Firmicutes; (23.52% –28.12%); Proteobacteria. 13.45% – 44.22%).*	*Methanobacterium; (66.22%); Methanobrevibacter (13.22%).*	[72]
Semi-continuous anaerobic co-digestion (AcoD)	Pig manure and corn straw	Mesophilic temperature (37%).	Identify the effects of biochar addition on internal environmental changes, gas production characteristics, and the structure and dynamics of microbial populations, under recirculating biogas sludge conditions.	Illumina high-throughput sequencing	*Firmicutes; (53.7 %-56.9 %); Bacteroidota; (29.1 %– 32.1 %); Proteobacteria (2.0 %-8.8 %); Actinobacteriota (2.4 %-4.6%).*	*Methanosarcina (39.0 %); Methanobacterium (22.2 %); Methanosarcina (16.1 %); Methanobrevibacter (12.2 %).*	[73]

entire experiment, as reviewed by Nearing, Comeau and Langille (2021) [49] (Fig. 1). Standardization and quality assurance are important for minimizing these biases and improving the consistency of microbiota measurements in metagenomic studies. Ongoing efforts in this area include the development of standardized protocols for sample collection, DNA extraction, library preparation, and data analysis. Additionally, the use of mock communities as controls helps assess the accuracy and reproducibility of different sequencing methods and bioinformatics pipelines. The adoption of best practices for quality control and data normalization can further minimize the impact of technical variations on the results. On the other hand, the AD microbiome is considered as a "black matter", due to the complex and heterogeneous ecosystem, and the diverse microbial community that includes bacteria, archaea, fungi, and viruses. Many of these microorganisms cannot be cultivated [50]. Using high-throughput sequencing HTS, several researchers have gained new insights into the spatial distribution of uncultivated microbes [51]. In a recent study, genome-centered metagenomics was employed to explore the metabolic properties of planktonic bacteria firmly attached to plant biomass [52]. The findings from this study have enhanced our understanding of diverse metabolic strategies involved in polysaccharide degradation among various *Bacteroidetes* and *Clostridiales* species, especially considering that 85% of them are uncultivated [52]. With HTS, the authors identified a potential mechanism for biomass decomposition in uncultivated *Bacteroidetes* species, relying on a cluster of genes responsible for cellulose degradation, disaccharide cleavage into glucose, and subsequent transport to the cytoplasm. To address existing scientific research challenges, Bin Yang et al. (2022) employed HTS technology in their study [53]. They focused on analyzing the microbial community structure within anaerobic digestion systems, initially identifying CH_4-producing strains and their proportions to gain insights into the individual strains' roles in CH_4 production within anaerobic digestion systems. Furthermore, the technology was used to explore abiotic factors, bacterial communities, and archaeal communities within low-temperature anaerobic digestion systems. Therefore, we suggest adopting the HTS standard operating procedure (SOP) developed by the International Human Microbiome Consortium to improve the quality and consistency of amplicon sequencing and metagenomic studies of AD [54]. Additionally, the development of ecosystem-specific references and databases that adapt methodologies and analyses to the unique characteristics of the ecosystem under study is an emerging tool for improving the accuracy and reproducibility of sequence-based metagenomics studies of AD [55].

3. Applications of metagenomic approaches for monitoring the dynamics of the microbiome's communities in the anaerobic digestion (AD) process

In the past, microbiological similarities were primarily investigated using PCR-based approach with specific primers for bacteria or archaea. However, this method did not allow for an instant comparison of the AD microbiome. In contrast, metagenomic approaches enable the direct analysis of the abundance and diversity of bacteria and archaea [56]. Emerging applications of metagenomics are providing a new perspective on complex microbial ecosystems, offering new insights for their development. These tools can be applied to the microbiology of methanization processes to describe the functional potential of these complex ecosystems [57]. Statistical analyses of publications conducted on databases such as Scopus and web of science have shown that, despite the large and growing number of studies on anaerobic digestion, the application of metagenomic approaches for understanding and monitoring the dynamics of microorganisms involved in the BP process remains limited [10]. Although the number of citations has increased in recent years, there has been slow growth in the number of published papers on the microbial communities of anaerobic digestion that are studied and analyzed using metagenomics, next-generation sequencing, or high-throughput sequencing approaches. Since 2013, interest in studying these microbial communities using advanced molecular techniques has increased, even though the anaerobic digestion process is often considered a "black box" [13]. The use of these sequencing techniques has demonstrated that studying the dynamics of the microbiome is essential to ensure the proper functioning of the AD process, especially in response to nutritional changes and environmental conditions [13]. Although the AD microbiome is often stable in terms of biogas or methane yield, the same cannot be said for the abundance and diversity of bacterial and archaeal species. Therefore, studying the complex dynamics between these populations is highly interesting [58,59]. Taxonomic profiling of biogas-producing communities using high-throughput 16S rRNA gene amplicon sequencing has provided a high-resolution overview of the bacterial structures of AD assemblages. While 16S rRNA sequencing is a commonly used method for bacterial community analysis, it has limitations in detecting archaea [60]. Methyl-coenzyme M reductase (*mcrA*) targeted sequencing is a technique used to detect and quantify anaerobic archaea [61]. The technique involves designing specific primer combinations that target the alpha sub-unit of the methyl-coenzyme M reductase (*mcrA*) gene of the archaea of interest [62,63]. The *mcrA* is a functional gene that encodes a key enzyme in methanogenesis, and its targeted sequencing can provide insights into the composition and succession of methanogenic archaea in various environments [64,65].

The metagenomic approach in AD has been applied to understand and monitor the diversity and dynamics of different communities using HTS platforms and Third-generation sequencing technologies for analyzing microbial communities in these complex samples. Table 1 summarizes some monitoring studies for AD systems applying metagenomic approaches. For example, in one study, samples from anaerobic digestion sludge from two wastewater treatment plants were used to explore microbial communities and functions based on a metagenomic view [56]. The results were significant as the authors found many phylotypes that could be classified according to the microorganisms that perform typical functions in these ecosystems, such as *Firmicutes*, which are considered fermenting, syntrophic bacteria, and degrading various substrates. Another study revealed the impact of the inoculum on the development of a robust microbial consortium for adequate substrate degradation. The reactors were initially inoculated with anaerobic granular sludge from a brewery wastewater treatment plant. The inoculum and biomass samples showed changes related to community adaptations to urban organic waste, including a higher relative proportion of *Clostridiales*, with *Ruminococcus spp*. and *Syntrophomonas spp*. as recurrent species. Additionally, *Cloacamonas spp*. (*Spirochaetes*) increased by ~22% in the inoculum to over 10% in the reactor biomass [4]. The new community consolidated cellulose degradation, propionate, and amino acid fermentation processes. Bacterial and archaeal 16S rRNA gene copy numbers were quantified using a PCR reaction, and microbial diversity was characterized using the

Illumina MiSeq platform [4]. Using the previously mentioned technique, Kuroda et al. (2016) identified critical microbial interactions at the macro and micro levels of ecosystem ecology by analyzing 300 PTA-degrading granules from a laboratory-scale UASB reactor and two full-scale reactors. In the previous study, the authors showed for the first time that multiple consortia of microorganisms can coexist in different granules within the same bioreactor. Further analysis of the 16S rRNA gene was conducted using Oxford nanopore Technology and was compared to Illumina sequencing data using different taxonomic indices for read classification [5]. To establish a robust protocol for obtaining less fragmented and high-quality DNA, while preserving bacterial and archaeal composition, samples from 20 different biogas/wastewater reactors were studied. This was done to better understand the heterogeneity and community dynamics in the reactors [5]. In another study, Pore et al. (2019) [28] reported the thermophilic biomethanation of rice straw using cattle manure supplemented with *M. thermautotrophicus*, which was done for the first time. The authors applied a metagenomic and a meta-transcriptomic approach, which helped reveal the primary pathways of methane production via syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis [28]. This study was able to correlate major taxa in the reactors with their predicted functionalities based on the enzymes generated by the main bacteria and archaea, [28]. The metagenomic approaches have been applied in different studies to control the efficiency of some types of digesters and improve the methane yield [66–68]. These studies utilized metagenomics DNA sequencing as a tool for taxonomic and functional profiling of microbial communities in conventional and plug flow (PF) anaerobic digesters, including both once-through and recirculating systems. Compared to conventional digesters, plug flow digesters showed higher levels of sulfate-reducing bacteria (*Desulfurivibrio*), and hydrogen-trophic methanogens (*Methanospirillum.spp.*). In contrast, the recirculating anaerobic digesters showed enrichment in denitrifying bacteria and hydrogenotrophic methanogens, suggesting that the PF digester was efficient [68]. Third-generation sequencing technologies have also been used to study resistance genes carried by cultured coliform bacteria [9]. Seven pilot-scale Continuous stirred-tank reactor (CSTR) anaerobic digesters were set up with sludge and fed with thermal pre-hydrolysis under different conditions. High throughput quantitative PCR, UPLC-MS/MS, and Illumina HiSeq sequencing were used to systematically assess the responses of antibiotics resistance genes (ARGs), and mobile genetic elements (MGEs) [69]. The results of this work showed that the abundance of ARGs and MGEs in thermophilic digesters was lower than in mesophilic digesters. This study provided effective indications for controlling the spread of antibiotic resistance and suggested optimal operating conditions for digesters. Ultimately, identifying the species that inhabit a particular ecosystem can help elucidate the predominant metabolic pathways [69]. The results of such studies are significant because they reveal many phylotypes that can be classified as microorganisms performing typical functions in these ecosystems. In current studies, on AD systems, several characteristics of microbial communities have been examined. However, important questions remain regarding the interconnections between the abundance, diversity, structure, functionality, and population dynamics of the anaerobic communities, and how these are influenced by technical operational parameters. These questions require further in-depth studies to improve our ability to study uncultured microbes and fill gaps in our knowledge.

4. Taxonomic classification of AD microbiome

The microbial process is an endergonic operation, meaning that it consumes energy [74,75]. Microorganisms obtain energy

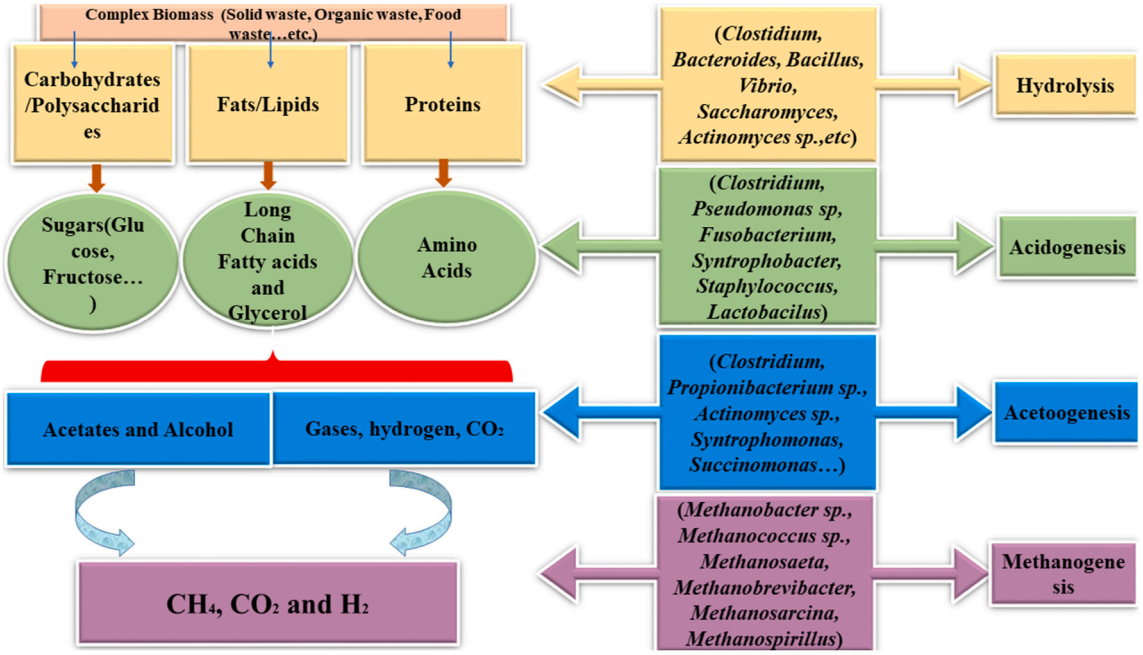

Fig. 2. Description of bacterial communities in organic waste bioconversion via anaerobic digestion phases.

through biochemical redox reactions [76]. Methanogenesis is a microbiological process in which organic compounds are oxidized to generate energy, while reduction reactions lead to produce CH_4 [77,78]. The simplified metabolic pathways describing this transformation process are presented in four distinct phases involving three bacteria types: hydrolytic and fermentative bacteria (hydrolysis

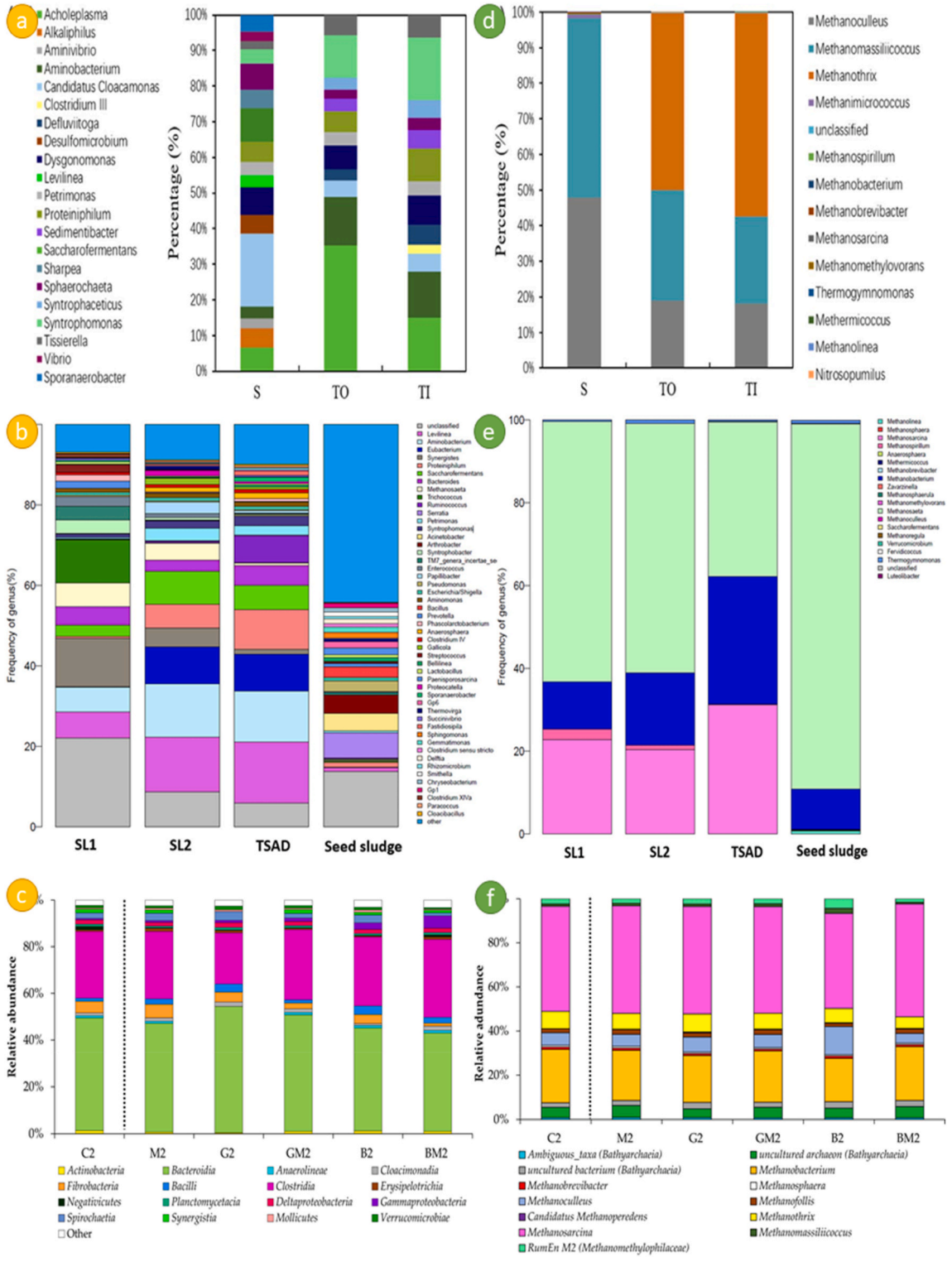

Fig. 3. Taxonomic classification of the dominant bacterial (a, b and c) and, archaeal (d, e and f) groups from anaerobic digesters plants [26,86,87].

and acidogenesis), acetogenic bacteria (acetogenesis), and methanogenic archaea (methanogenesis) (Fig. 2). These three communities must form a balanced ecosystem because reducing equivalents consumed during bacterial anabolism ultimately end up in methane [79]. The hydrolysis stage is carried out by several groups of strict and facultative anaerobic eubacteria, the nature of which depends on the qualitative and quantitative composition of the feed [4]. The main species belong to the genera *Clostridium, Bacillus (Trichococcus), Ruminococcus, Enterobacteroides, Propionibacterium,* and *Butyrivibrio*, [4,80]. In addition, the abundance of several taxa is monitored under different conditions and parameters. During the acetogenic stage, the oxidation of substrates (mainly propionic and butyric acids and ethanol) is coupled with the formation of hydrogen, carbon dioxide, and acetate. This stage involves two main groups of bacteria: homoacetogenic bacteria of the genera *Clostridium, Acetobacterium, Sporomusa, Acetogenium, Aminobacterium, Acetoanaerobium, Pelobacter Butyribacterium* and *Eubacterium,* and sulfate-reducing genera such as *Desulfovibrio, Desulfobacter, Desulfotomaculum, Desulfomonas* [22,68]. It is worth noting that when the partial pressure of hydrogen rises, this oxidation becomes thermodynamically impossible (endergonic reaction). Therefore, the growth of the acetogenic flora and the use of the substrate strictly depends on the elimination of hydrogen from the medium by microorganisms producing methane or even sulfate-reducing bacteria (in the presence of sulfate) [81]. This syntrophic association with hydrogen-methanogenic archaea makes the reactions endergonic. The oxidation of substrates is only possible at low partial hydrogen pressures (10-4 atm). The active bacteria of the methanogenic phase are grouped in a separate group: the Archaea [82]. They have specific characteristics compared to eubacteria and eukaryotes, particularly concerning their coenzymes. The main taxa known for their high methanogenic potential are classified at the genus level as *Methanobacterium, Methanosarcina, Methanosaeta, Methanothermobacter*, *Methanoculleus,* and *Methanobrevibacter* (Fig. 3d, e and f) [66, 83–85]. To date, the most common results show the dominance of bacteria and archaea based on relative abundance [37]. Zhang et al. (2017) designed a three-stage anaerobic digester for food waste and found that *Trichococcus*, *Aminobacterium*, and *Levilinea* were the most dominant populations in the hydrolysis, acidogenesis, and, methanogenesis, respectively [86], as illustrated in (Fig. 3b).

4.1. Taxonomic composition of the bacterial community residing in the biogas plant

The use of metagenomics in recent years has led to an explosion in the description of new phyla and species, greatly expanding our understanding of microbial diversity and function in various ecosystems [88]. This approach consists of a collective analysis to identify sequences and their functions in the genomes of microbial communities contained in an environmental sample [89]. AD samples are composed of very diverse microbial communities (bacteria and archaea) that perform several functions [90]. The composition and diversity of this community vary according to different parameters, such as substrate, temperature, pH, and the type of reactor and fermentation used [91,92]. It has been reported that the relative frequencies of the bacterial community in digesters range from 80 to 100%, with a percentage of 0–10% generally occupied by methanogenic archaea [92,93]. The taxonomic profiling of microbial communities can be carried out at different levels, such as phylum, class, order, family, genus, and species. This information can be visualized in a single-species taxonomy diagram at several levels of the entire microbial community [14]. The Sequencing of 16S rRNA gene amplicons has been a reliable method for understanding the taxonomic composition of complex bacterial and archaeal communities at lower cost. Additionally, it has been used to identify correlations among process parameters, community structure, and environmental conditions [94]. Using these approaches, the taxa involved in AD were identified in different digesters (Fig. 3a–f). It has been reported that, in the field of bacteria, the phyla *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria,* and *Chloroflexi* (*Levilinea*) stand out as the most abundant in different types of waste (Fig. 3a, b and c) [9,23,95]. These abundant taxa, belonging to the core microbiome of microbial communities, are the main drivers for starting the BP process [96]. Moreover, this dominance is mainly related to the temperature and the type of substrate used [97]. The abundance of *Firmicutes* and *Bacteroidetes* in digesters is reported to be associated with the abundance of *Proteobacteria* in several studies [46]. These microorganisms are important in the anaerobic digestion process and are usually found in mesophilic digesters, especially those used for wastewater treatment [98]. Most commonly, *alpha, beta, gamma,* and *delta-proteobacteria* are known as bacterial communities that utilize glucose, propionate, butyrate, and acetate for their metabolism [99]. Other phyla such as *Spirochaetes*, *Tenericutes*, *Planctomycetes*, *Thermotogae*, *Acidobacteria,* and *Verrucomicrobia*, have also been detected in digesters, albeit in low percentages [26,59,99,100]. Within the *Firmicutes* phylum, at the class level, *Clostridia,* and *Bacilli* make up about 85% of all *Firmicutes* sequences recovered from digesters [36,101]. Several species belonging to the genus *Clostridium* are primary candidates for hydrolysis as they can degrade complex carbohydrates, including cellulose, hemicellulose, xylan, amylose, and amylopectin [4,102]. Members of the *Clostridia* class are more abundant in thermophilic digesters than in mesophilic digesters. St-Pierre and Wright (2014) performed a metagenomic analysis of the bacterial population in anaerobic digesters using dairy manure as a main substrate. A total of 20,366 sequence reads covering the V1–V2 hypervariable regions of the bacterial 16S rRNA gene were generated, and bacterial diversity was assessed by clustering operational taxonomic units (OTUs) [103]. The results revealed the dominance of *Clostridium alkalicellum*, *Clostridium thermocellum,* and *Clostridium cellobioparum* species, confirming their important role in the degradation of biomass [103,104]. *Clostridia* is a highly diverse class, encompassing various species capable of acetogenesis and fatty acid degradation, as well as cellulose degradation [105,106]. Among the cellulolytic species, members of the *Lachnospiraceae* family are known to ferment complex plant polysaccharides into short-chain fatty acids (butyrate, acetate) and alcohols (ethanol) [76]. These bacteria are considered to be the most abundant taxa in large-scale thermophilic digesters [100]. The current acceleration of genomic approaches has led to the identification of more than 150 species of microorganisms, including *Clostridium bornimense, Herbinix hemicellulosilytica, Herbinix luporum, Herbivorax saccincola, Proteiniphilum saccharofermentans, Petrimonas mucosa, Fermentimonas caenicola,* and *Proteiniborus indolifex,* among others. These bacteria exhibit several genomic characteristics associated with high BP efficiency [59,86,107]. Mesophilic digesters fed with high protein substrates are challenging for bacterial digestion. However, it has been observed that species from the *Bacteroidetes* family are prevalent in these digesters [108]. *Bacteroidetes* is one of the top three phyla that dominate bacterial communities, especially during the hydrolysis phase

(Fig. 2), and in digesters fed with agricultural waste and manure [108,109], suggesting a significant contribution to anaerobic digestion that requires further investigation. At the family level, *Bacteroidaceae*, *Porphyromonadaceae,* and *Rikenellaceae* are among the major families that occupy different functionalities from the populations involved in the methanization process [11,110]. A recent study examined the enrichment of bacteria degrading amino acid (AA) in anaerobic digestion using metagenomics and metatranscriptomics, [71]. Metagenomic assembled bins data related to uncultured *Bacteroidales* collectively represented more than 35% of the sequences. In addition, metatranscriptomics revealed that *Bacteroidales* populations expressed complete metabolic pathways to degrade approximately 17 types of AAs. This suggests that the uncultured *Bacteroidales* were primarily responsible for hydrolyzing proteins and AA, such as *Porphyromonas gingivalis, Acetobacteroides hydrogenigenes*, *Williamwhitmania taraxaci* and *Proteiniphilum saccharofermentans*, indicating their interesting ecological role in natural habitats [71]. *Thermotogae* is a phylum that has become common in thermophilic digester installations, with moderate frequencies ranged from 2% to 19% [39,91]. This phylum is more abundant at 50–60 °C [111].

4.2. Taxonomic composition of the archaeal community residing in biogas plant

Several studies have reported the presence of archaeal species in biogas digesters as part of the bacterial community. The main classes of methanogens found in AD ecosystems are summarized in Table 1, with different species identified in various substrates. The type of substrate has a direct effect on the presence and dominance of the bacterial community, particularly the methanogenic subcommunity, more than temperature or organic loading rate (OLR) [36]. Methanogenic archaea utilize a relatively limited range of substrates for energy extraction and are generally categorized into four groups based on the catabolic substrates used for methanogenesis. These include (i) hydrogenotrophic methanogenesis which uses H_2/CO_2 and/or C_1 compounds as substrates, (ii) acetotropic (acetoclastic) methanogenesis based on acetate consumption, (iii) methylotrophic methanogenesis utilizing R–CH_3, and (iv) methyl-dependent hydrogenotrophic methanogenesis using H_2 + R–CH_3 [7,100]. The degradation of volatile fatty acids to methane is accomplished through the metabolic activities of hydrogen-producing acetogens and hydrogen-oxidizing methane [112]. Studies have indicated a predominance of the hydrogenotrophic pathway of methanogenic archaea in anaerobic digesters with high organic loading rates [113]. Hydrogenotrophic methanogenesis is carried out by a large fraction of the methanogenic archaea described to date [76,114]. This reaction involves several steps from CO_2 activation to methane release and requires the involvement of several hydrogenotrophic taxa. Among the most represented hydrogenotrophic genera in AD full-scale digesters are *Methanobacterium, Methanobrevibacter, Methylococcaceae, Methanospirillum, Methanogenium, Methanocorspusculum* [19,112]. Some species within these taxa have been adapted to thermophilic temperatures in large-scale plants that use agricultural and animal wastes, which often feature high salt (10–20 g L^{-1}) and ammonia (>2.8 g L^{-1} NH_4) content. This suggests that these archaea are not sensitive to ammonia toxicity, potentially giving them an advantage over other methanogens [115,116]. *Methanobacteria* and *Methanomicrobia* have been consistently identified as major archaeal populations in sewage-treating anaerobic processes such as Anaerobic Membrane Bioreactor (AnMBR), Upflow Anaerobic Sludge Blanket Reactor (UASB), Upflow biocatalyzed electrolysis reactor (UBEF), etc. The third most predominant methanogenic genus is *Methanobrevibacter*, *which* prevails over other genera, especially in the fermentation of animal manure in mesophilic biogas reactors [100,117,118]. The structure of hydrogenotrophic methanogens is dependent on reactor performance, temperature, and hydraulic retention time. Two anaerobic membrane bioreactors (AnMBRs) equipped with different membrane pores, were operated at 25 °C and fed with domestic wastewater [7]. The microbial communities of the two RMBAs were studied by sequencing the 16S rRNA gene amplicons to identify the effects of hydraulic retention time (HRT) on the archaeal community. The results showed a dominance of an acetoclastic methanogen (*Methanosaeta*), and the hydrogenotrophic methanogenic community, namely *Methanobacterium* and *Methanomicrobium*. However, the dominance of these hydrogenotrophic methanogens changed with HRT: the *Methanobacterium* population was higher for longer HRT, while the unclassified *Methanoregulaceae* population was higher for shorter HRT. It appears that the acetotropic methanogenesis pathway is responsible for approximately 65–75% of biologically derived methane emissions [119]. This metabolism is found in *Methanosaeta* and *Methanosarcina* [120]. Several studies have reported that the presence of *Methanosarcinales* is very beneficial for CH_4 production because they are extremely versatile in the metabolic pathway [121]. Indeed, they can use a wide variety of substrates to achieve their methanogenesis, which sets them apart from other methanogenic archaea. They can use hydrogenotrophic, acetoclastic, and/or methylotrophic methanogenesis for their metabolism [122]. Nevertheless, *Methanosaeta* is always dependent on the presence of acetate as a substrate, which means it's abundant in thermophilic reactors [123].

5. Microbial community dynamics in response to anaerobic digestion operating conditions

Metagenomic tools and analyses employed to study AD microbiome have enhanced our understanding of the evolution and functions of microbiome composition over time in response to operational changes. Additionally, the development of deep sequencing of Metagenome-assembled-genomes (MAGs) has transformed the view of the microbiome into an integration of individual populations with specific functions [9]. Biogas is composed of (50–70%) methane, (30–50%) carbon dioxide (CO_2), and trace amounts of nitrogen, ammonia, and hydrogen sulfide. Only methane is utilized, after the purification of the other gases, and increasing its purity could lead to an increase in the demand for biogas as a stable source of renewable energy [108]. Recently, species characterization involved in biomethanation was carried out using genome-centric metagenomic through MAGs reconstruction and metabolic potential analysis to understand the competitive and syntrophic relationship between different taxa [38]. For example, this metagenomic approach has defined the importance of H_2 in the digester, as this compound is rapidly produced and consumed by different microbial taxa throughout the various processes of organic matter degradation, particularly in ecosystems with low organic matter inputs [124]. Its

concentration in this environment is relatively low, leading to competition between hydrogenotrophic methanogenic archaea, sulfate-reducing bacteria, and homoacetogenic bacteria. Methylotrophic methanogenesis is a dismutation reaction found in all *Methanosarcinales*. These archaea use a relatively limited range of methylated substrates for their metabolism [110]. Methanogenic methylotrophic archaea do not oxidize H_2 for their methanogenesis, which is why they do not compete with sulfate-reducing bacteria for H_2 [76]. These findings have prompted researchers to conduct trials for the optimization of BP using metagenomic sequencing techniques. Zhu et al. (2020) reported that the addition of H_2 in an anaerobic digester is a promising technology for increasing the calorific value of biogas [125]. Metagenomic sequencing was used to further identify the microbial community and functional information of methanogens during stages that exhibited significant differences based on reactor performance, using the Illumina MiSeq platform. One dominant metabolic pathway responsible for the increase of CH_4 was identified with the addition of H_2 in the digester. Archaea can compete with each other, and they can also form syntrophic relationships. The most frequent syntrophic reactions in AD reactors include the syntrophic oxidation of acetate and syntrophic interactions with anaerobic hydrogen-producing bacteria. These bacteria produce acetate by oxidizing products of acidogenesis (volatile fatty acids, ethanol, glycerol, and lactate) [81,126]. The production of acetate leads to a co-production of H_2, which accumulates in the medium, causing disbalance in the environment. Moreover, the acetogenesis and cell growth are inhibited when the partial pressure of H_2 reaches or exceeds 10 Pa ($PH_2 \geq 10$ Pa; i.e. ~ 10-4 atm) [127,128]. To overcome this phenomenon, anaerobic hydrogen-producing bacteria develop syntrophic associations with hydrogenotrophic methanogenic archaea through their metabolism, which maintain a PH_2 constantly below 10 Pa [129,130]. The interaction between these microorganisms is known as syntrophy because hydrogenotrophic methanogens regulate the PH_2 levels, which are necessary for the growth of anaerobic bacteria that produce hydrogen. This syntrophic relationship enables the conversion of several substrates such as ethanol, butyrate, propionate, and benzoate, which are converted to acetate and methane [131].

6. Microbial functionality exploration for AD microbiome through metagenomic approaches

The optimization of the AD process requires a better understanding of the functions of microbial communities and their functional response to process perturbations [132]. Omics technologies have replaced most molecular biology tools in studies of biogas-producing microbiomes [133,134]. Metagenomics can be used to predict the functions of bacteria present in biogas plants by analyzing the genes involved in various metabolic pathways important for anaerobic digestion, such as organic matter decomposition,

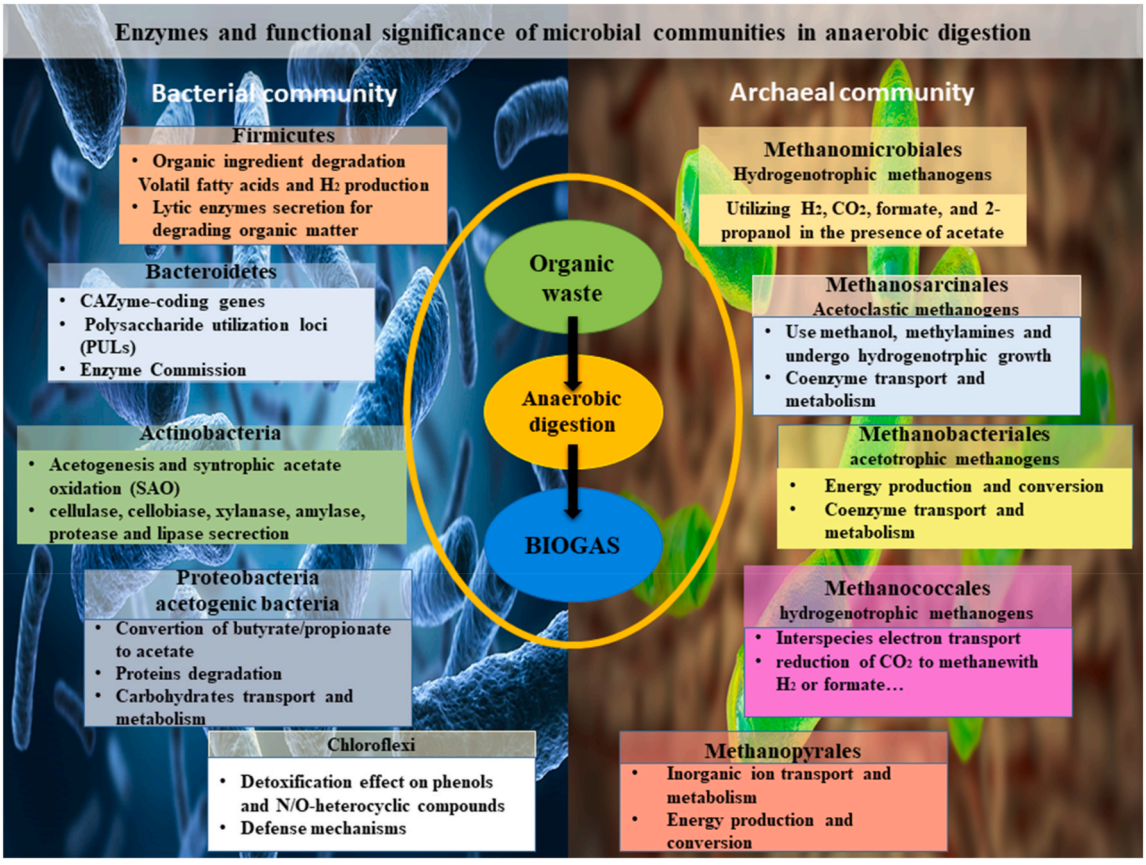

Fig. 4. A diagrammatical representation of the key microbial community involved in anaerobic digestion sludges along with their function and enzymes in anaerobic digestion.

methane production, and nutrient removal [2]. Researchers can identify the bacteria present in the community and make predictions about their functions by comparing the genetic material in the sample to databases of known bacterial genomes and metabolic pathways. For instance, if a particular gene involved in methane production is found in the sample, it indicates that the bacteria carrying that gene are actively producing methane in the anaerobic digestion plant [36]. Metagenomics was first used to characterize anaerobic microbial consortia in a BP process treating cellulosic plant biomass [135]. A total community DNA sample from the reactor was sequenced using a pyrosequencing system. This study allowed the authors to identify the functionality of clostridia in AD systems [136]. However, genetic profiling analyses of AD consortia were not sufficient to identify the full range of functional relationships between complex microbial communities and were limited to the interpretation of dominant genomes and their functionality [7,9]. Under mesophilic conditions, abundant carbohydrate metabolism genes were associated with groups of genera commonly found in biogas digesters such as *Clostridium*, *Fusobacterium, Propionibacterium*, *Tepidanaerobacter* and the archaeal genus *Methanoculleus* [105]. In a recent genome-centric functional analysis, 68 MAGs were recovered, with 32 of them showing substantial enrichment [12]. The study highlighted the dominance of *Firmicutes* spp. in the microbial community, particularly hydrolytic/fermentative and syntrophic bacteria (Fig. 4). *Clostridia* sp. Bin_24, *Clostridiales* sp. Bin_36, and *Firmicutes* sp. Bin_42, all belonging to the Clostridia class, were identified as major contributors [12]. These MAGs exhibited a variety of genes encoding enzymes crucial for stepwise hydrolyses, such as glycoside hydrolases (GH), carbohydrate esterase (CE), and carbohydrate-binding module (CBM), which play a vital role in the breakdown of organic macromolecules. Another study focused on the mechanisms of biological nitrogen removal during anaerobic digestion of pig manure and investigated the impacts of biogas circulation and activated carbon (AC) addition [137]. The findings suggested that biogas circulation could enhance mass transfer, induce air infiltration, and enrich bacteria and functional genes related to nitrification and denitrification. Understanding the functions of bacterial species involved in organic matter degradation and biogas production valuable insights for optimizing the anaerobic digestion process. In the mentioned study, the relative abundances of *Desulfobacterota* in RAC and RCirc-AC were 17.1% and, 67.0% higher compared to the control reactor (RC), respectively. *Desulfobacterota*, recognized for its capability to degrade fatty acids and engage in direct interspecies electron transfer (DIET) [3], suggests that enriching RAC and RCirc-AC with AC could enhance overall AD performance by facilitating the DIET process. Metagenomic analyses serve as a valuable tool to validate the combined strategies, studying their synergistic effects on the enrichment of nitrifying and denitrifying bacteria and functional genes. Moreover such approach can significantly reduce total ammonia nitrogen by 23.6%. The prediction of functionalities within *Firmicutes*, *Bacteroidetes*, and *Synergistetes* has contributed to a better understanding and enhancement of AD systems efficiency. For example, a recent study investigating the impact of different substrate-to-inoculum (S/I) ratios on kinetic parameters, microbial communities, and metabolic pathways during anaerobic digestion, employed metagenomics [138]. By enriching the digester with *Bacteroides* and *Synergistetes*, researchers established a synergistic relationship with hydrogenotrophic methanogens, creating a unique ecological environment. This enrichment led to a higher prevalence of genes encoding key enzymes in methanogenesis, facilitating amino acid metabolism and providing a nutrient-rich substrate for methanogens. The presence of cofactors and vitamins further supported catalytic reactions of cellular enzymes, accelerating the anaerobic digestion process. These findings underscore the crucial role of these pathways in enhancing AD efficiency. In summary, the functional roles of individual microbes in AD are closely tied to catabolic pathways, challenging the conventional four-step concept of AD. Substrate-specific systems, analyzed through a genome-centric metagenomic approach, have provided a traceable microbial community to dissect the AD process, expanding our knowledge beyond the current understanding.

7. Current status, limitations, and prospects of the metagenomic approaches

Metagenomic approaches have revolutionized our understanding of microbial diversity, functionality, and interactions, with applications spanning biotechnology, medicine, ecology, agriculture, and environment [2]. However, metagenomic approaches still have several limitations that need to be addressed [139]. One major challenge is the high cost and complexity associated with sequencing and analyzing vast amounts of data. Additionally, metagenomic data often lack completeness, making the identification of functional genes and pathways challenging, due to the lack of genome references and the presence of highly diverse and unknown microorganisms [5]. Contamination during sample collection and processing is another limitation that can impact the accuracy and interpretation of the results [9]. Despite, these limitations, the prospects for metagenomic approaches are promising. The continuous development of new sequencing technologies and bioinformatics tools has significantly enhanced the sensitivity, accuracy, and speed of metagenomic analysis [14]. Integrating other -omics approaches, such as metatranscriptomics, metaproteomics, and metabolomics, can provide a more comprehensive understanding of microbial communities and their interactions in AD systems [71,87,140,141]. Metagenomic approaches are increasingly applied to study complex microbial ecosystems, such as those in the biogas plant, unraveling their roles in various contexts. In conclusion, metagenomic approaches have yielded significant insights into microbial diversity and functionality. However further advancements are necessary to fully exploit their potential [89,95]. Ongoing developments in sequencing technologies, bioinformatics, and multi-omics integration should contribute to advancing metagenomic approaches as valuable tools for understanding microbial ecosystems and their applications across diverse fields.

8. Conclusion and future perspectives

Anaerobic digestion is a widely employed process for converting organic waste into methane gas, and its efficiency is influenced by the digester microbiome, which is affected by various factors such as substrates and operational conditions. Metagenomic has emerged as a powerful tool for studying the microbial communities involved in AD processes, offering several advantages. Firstly, metagenomics enables the identification of the entire microbial community participating in the process, including non-cultivable

organisms, providing a comprehensive understanding of the metabolic pathways and interactions between different microbial groups. Secondly, metagenomics can overcome limitations in the process, such as the presence of inhibitory compounds or imbalances in the microbial community, by identifying potential solutions for optimizing the AD process. Finally, metagenomics can aid in developing strategies to optimize the AD process, such as the addition of specific microbial groups or modifications to the operating conditions. Recent advances in sequencing technologies have made metagenomics more accessible and effective for studying anaerobic digestion. High-throughput sequencing allows for the rapid identification of microbial community composition and functional genes involved in the anaerobic digestion process. This information is crucial for developing more efficient and sustainable AD systems and for precisely monitoring and troubleshooting existing systems.

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Review and/or approval by an ethics committee was not needed for this study.

CRediT authorship contribution statement

Btissam Niya: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Kaoutar Yaakoubi:** Writing – original draft, Investigation. **Fatima Zahra Beraich:** Writing – original draft, Resources. **Moha Arouch:** Writing – original draft, Supervision, Conceptualization. **Issam Meftah Kadmiri:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization.

Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT-3.5 in order to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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