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Temporal Variation of Methanogenic Microbial Community in Palm Oil Mill Effluent (POME) Anaerobic Digester

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Palm Oil Mill Effluents (POME) serve as suitable substrates for methane gas production through anaerobic digestion. This process relies on a complex microbial community that plays a critical role in ensuring stable anaerobic digester operation and efficient biogas production. Among these microorganisms, methanogenic archaea are pivotal in methane generation by utilizing diverse substrates under anoxic conditions. However, the knowledge of the microbial communities, particularly those involved in methane production in POME anaerobic sludge at different time

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intervals, remains limited. This study aims to uncover temporal variations in microbial communities, including diversity, composition, and structure, within POME anaerobic sludge, specifically focusing on the methanogenic archaea community. The temporal dynamics of microbial communities in the eighteen POME anaerobic sludge samples collected from a palm oil mill were investigated through 16S rRNA amplicon sequencing. The results reveal consistent microbial community diversity in POME anaerobic sludge over the study periods. Then, the sequencing also showed that *Bacillota* (26.9 ± 3.3%), *Bacteroidota* (20.2 ± 5.3%), and *Chloroflexota* (15.0% ± 6.3%) were the dominant bacterial phyla in POME anaerobic sludge across different time frames. Concurrently, *Halobacteriota* (5.9 ± 2.8%), *Methanobacteriota* (2.5 ± 0.6%), and *Nanoarchaeota* (2.3 ± 1.2%) were the primary archaeal phyla identified in anaerobic sludge at various time intervals. Furthermore, amplicon sequencing revealed the presence of two methanogenic archaea genera, *Methanothrix* and *Methanobacterium*, associated with acetoclastic and hydrogenotrophic methanogenesis, respectively. These findings suggest that acetoclastic and hydrogenotrophic methanogenesis pathways are the primary contributors to methane production in the POME anaerobic digestion process.

Keywords: Amplicon sequencing; microbial diversity; methane, methanogens; POME.

1. INTRODUCTION

Palm oil mill effluent (POME) is a viscous, brownish liquid waste resulting from palm oil extraction. It is primarily generated through three main sources: the sterilization of fresh fruit bunches, the pressing of empty fruit bunches, and the clarification of extracted crude palm oil [1-3]. In brief, one ton of crude palm oil can generate approximately 2.5-3.5 tons of POME [1,4,5]. Malaysia generates roughly 45 to 67.5 million tons of Palm Oil Mill Effluent (POME) annually through the processing of 90 million tons of fresh fruit bunches [6]. POME typically exhibits several notable characteristics, including elevated discharge temperature, acidity, high levels of biochemical oxygen demand (BOD), and chemical oxygen demand (COD), as well as a substantial organic content, including carbohydrates and lipids [2,4,7-12]. Effective treatment of POME is essential before its discharge into water bodies, as untreated POME can result in harmful consequences and environmental pollution [6].

To date, anaerobic digestion (AD) has been widely employed for treating POME due to its capacity for generating methane gas (CH_4), which can be harnessed for energy production [1,2,4,8,11,13,14]. Generally, each ton of POME introduced into the AD system has the potential to yield 28 m^3 of biogas as output [1,15,16]. AD involves a succession of synchronized processes in which bacterial and archaeal communities perform the biotransformation of organic matter into biogas. These processes encompass hydrolysis, acidogenesis, acetogenesis, and methanogenesis [17-19]. Methanogenesis represents the final phase in AD, where all the accessible intermediates like hydrogen, acetate, and carbon dioxide are utilized by methanogen to produce methane gas. Methanogens are categorized into three groups according to their methanogenesis substrates: hydrogenotrophic, acetoclastic, and methylotrophic [20,21]. Hydrogenotrophic methanogens employ hydrogen to reduce carbon dioxide into methane, acetoclastic methanogens break down acetate to produce methane, and methylotrophic methanogens generate methane gas by utilizing methylated compounds [20,21]. Notably, the growth rate of methanogenic archaea is relatively slow, and usually, they are sensitive to operation parameters like pH and temperature [10,22]. Hence, a thorough understanding of the microbial communities present in terms of their behavior, diversity, and taxonomic composition in POME is critical to enhancing AD performance.

Next-generation sequencing, particularly -omics technology, has been exclusively applied in studies of microbial communities in POME [4,23,24]. 16S rRNA gene serves as a commonly used phylogenetic marker in amplicon sequencing to investigate microbial communities in diverse settings, including anaerobic digesters [4,23-25]. A prior study utilizing 16S rRNA amplicon sequencing on POME anaerobic sludge samples from biohydrogen production reactions revealed that 85% of the microbial community in POME sludge comprises bacteria, while 13% belongs to archaea within the phylum *Euryarchaeota* [23]. Additionally, amplicon sequencing detected three Archaeal families: *Methanomicrobiaceae, Methanobacteriaceae,* and *Methanomassiliicoccaceae.* The existence of

Methanomicrobiaceae and *Methanobacteriaceae* indicates the dominance of hydrogenotrophic methanogens in the POME AD process [23]. However, the discovery of microbial diversity from the previous study revealed a dearth of information on the microbial consortium responsible for methane gas production. Therefore, 16S rRNA amplicon sequencing can be a valuable tool for revealing temporal variations in the microbial communities in anaerobic POME sludge.

This study focuses on the methanogenic archaea community within POME anaerobic sludge due to their unique capacity for methane production in anaerobic digesters. The microbial community dynamics of POME anaerobic sludge samples were investigated using 16S rRNA amplicon sequencing at various time points between June and October 2022. Exploring the microbial diversity in POME anaerobic sludge at different intervals can provide a better understanding of how the microbiome composition changes at different intervals and provide valuable insight into the potential methanogenic archaea, which could contribute to the enhancement of biogas production.

2. MATERIALS AND METHODS

2.1 Sample Collection

POME anaerobic sludges were collected from Felda Maokil Palm Oil Mill in Labis, Johor, Malaysia (2.316 N 102.9803 E). Prior to sample collection, the digester outlet valve was opened, allowing the flow for at least two minutes to discard the old sludge in the dead volume. Sampling was conducted monthly from June 2022 to October 2022, and 5 L of samples were collected in triplicate at 5 minutes intervals using a new, tight-fitting capped plastic container. Subsequently, the collected samples were immediately transported to the laboratory at room temperature. The sample container was shaken vigorously to ensure thorough mixing. Then, the samples were transferred into a 50 mL centrifuge tube and immediately preserved at -20°C until DNA extraction [26,27].

2.2 Total Genomic DNA Extraction

50 mL of POME anaerobic sludge samples were centrifuged at 8,000 rpm, at 4°C for 20 minutes. The pelleted anaerobic sludge samples were then transferred to extraction tubes, with approximately 250 mg of sludge pellet used for further DNA extraction. Total DNA was extracted using a Qiagen DNeasy® Powersoil® Pro Kit based on the manufacturer's protocol [28]. The extracted DNA was stored at -20°C until further processing.

2.3 DNA Concentration and Purity

Concentration of the extracted and purified DNA was estimated using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), and the purity was estimated based on the A_{260}/A_{280} and A_{260}/A_{230} ratio. Ultimately, DNA quality and integrity were analyzed on 1%(w/v) agarose gel electrophoresis in 1× TAE buffer.

2.4 16S rRNA Amplicons Library Preparation, and Sequencing

The 16S rRNA amplicon libraries were generated from the total genomic DNA template via polymerase chain reaction (PCR). Bacterial and archaeal 16S rRNA V4-V5 hypervariable region was amplified from the gDNA template using universal primers 515F-GTGYCAGCMGCCGCGGTAA and 926R-CCGYCAATTYMTTTRAGTTT [29]. An additional four bases of the inline barcode were introduced at the 5' end of the primers to enable inline barcoding [30]. Different samples were amplified using different forward and reverse inline primer combinations. PCR was performed using SolarBio PCR mastermix (SolarBio, China) with PCR profiles of: 95°C for 3 minutes followed by 30 cycles of 95°C for 15 s, 50°C for 10 s， and 72°C for 20s. The barcoded amplicons were visualized on a gel, normalized and pooled according to their intensity, and purified with 0.8× vol of SPRI bead. The purified pooled amplicons were subsequently processed with the NEB Ultra II Library preparation kit, including an Illumina adapter and dual-index barcodes. The constructed library was quantified using the Denovix high-sensitivity assay and sequenced on an Illumina NovaSeq 6000 for 2 × 250 paired-end sequencing.

2.5 Bioinformatic Analysis

The raw paired-end reads, and adapters were quality trimmed using fastp v0.21 [31]. Demultiplexing and primer removal of the merged reads via cutadapt v1.18 [32]. The demultiplexed and trimmed reads were imported into QIIME2 v.2022.2 and subsequently denoised with the Divisive Amplicon Denoising Algorithm (DADA2) [33,34]. Further, the amplicon sequence variant (ASV) assignment was performed using the q2-feature-classifer, which

was trained on the latest GTDB release r207 16S rRNA database. This database was trimmed to include only the V4-V5 hypervariable region [35,36]. ASVs with the taxonomic assignment at least to the phylum level were chosen for further analysis. The ASV and taxonomic classification tables were exported using QIIME2 tools into tab-separated values (.tsv format) [37]. Then, the ASV table and taxonomic classification table were manually formatted to generate MicrobiomeAnalyst-compatible input that can be applied further for data visualization [38]. Lastly, the alpha- and beta-diversity estimators were calculated using the QIIME2 plug-ins, and ANOVA was applied to compare the parametric data [39].

2.6 Data Availability

The generated sequencing data from the samples collected at various time intervals has been submitted to the Sequence Read Archive (SRA) of the NCBI database with the accession numbers SAMN33942899 to SAMN33942916.

3. RESULTS AND DISCUSSION

3.1 Microbial Community Diversity and Richness in the POME anaerobic sludge

A total of 835,872 raw 16S rRNA V4-V5 sequences were obtained from 18 POME anaerobic sludge samples. Then, the retrieved reads were classified into 1,568 ASVs. Table 1. summarizes the results of the amplicon sequencing read for all the samples analyzed in this study. The samples were named according to the sampling date and month, and differentiated according to A-F labelling. The assessment of the microbial community within the anaerobic sludge yielded an average of 46,437 ± 5,861 raw reads and 474 ± 30 ASVs reads.

Several alpha diversity indices, such as Chao-1 richness, Shannon, and Simpson diversity, were used to measure the microbial community diversity, evenness, and richness within the POME anaerobic sludge [40]. Table 2 shows the biodiversity indices of each sample investigated in this study, and the boxplots in Fig. 1, Fig. 2, and Fig. 3 show comparable diversity in anaerobic sludge according to the time interval. The average Shannon and Simpson indices were 7.227 ± 0.147 and 0.982 ± 0.005, respectively. Meanwhile, the Chao-1 richness average is 598.975 ± 64.472. Based on the indices, the alpha diversity of the microbial community in anaerobic sludge did not vary significantly across the study periods [39]. From here, on average, it indicates that the microbial community diversity in the anaerobic digester has similar richness and evenness.

Table 1. Summary of amplicon sequencing read outputs for all samples.

Sample	Number of Raw Reads	Average Raw Reads	Number of ASVs	Average ASVs
All Samples	835,872	46,437 ± 5861	1,568	474 ± 30
A_JUNE_02	133,564	44,521 ± 9155	734	489 ± 51
B_JUNE_30	133,143	44,381 ± 1565	722	477 ± 8
C_JULY_28	137,123	45,707 ± 4860	693	466 ± 36
D_AUGUST_25	155,794	51,931 ± 1073	714	467 ± 21
E_SEPTEMBER_26	140,427	46,809 ± 6900	730	475 ± 17
F_OCTOBER_25	135,821	45,273 ± 8557	725	472 ± 35

Table 2. Microbial diversity indices in anaerobic sludge for each time interval

Sample	Shannon	Simpson	Chao-1
All Samples	7.227 ± 0.147	0.982 ± 0.005	598.975 ± 64.472
A_JUNE_02	7.380 ± 0.141	0.983 ± 0.006	595.827 ± 105.539
B_JUNE_30	7.223 ± 0.100	0.980 ± 0.000	612.873 ± 7.870
C_JULY_28	7.323 ± 0.117	0.987 ± 0.006	559.000 ± 71.399
D_AUGUST_25	7.150 ± 0.123	0.983 ± 0.006	598.950 ± 60.573
E_SEPTEMBER_26	7.223 ± 0.107	0.983 ± 0.006	609.693 ± 56.372
F_OCTOBER_25	7.063 ± 0.127	0.977 ± 0.006	617.507 ± 93.795

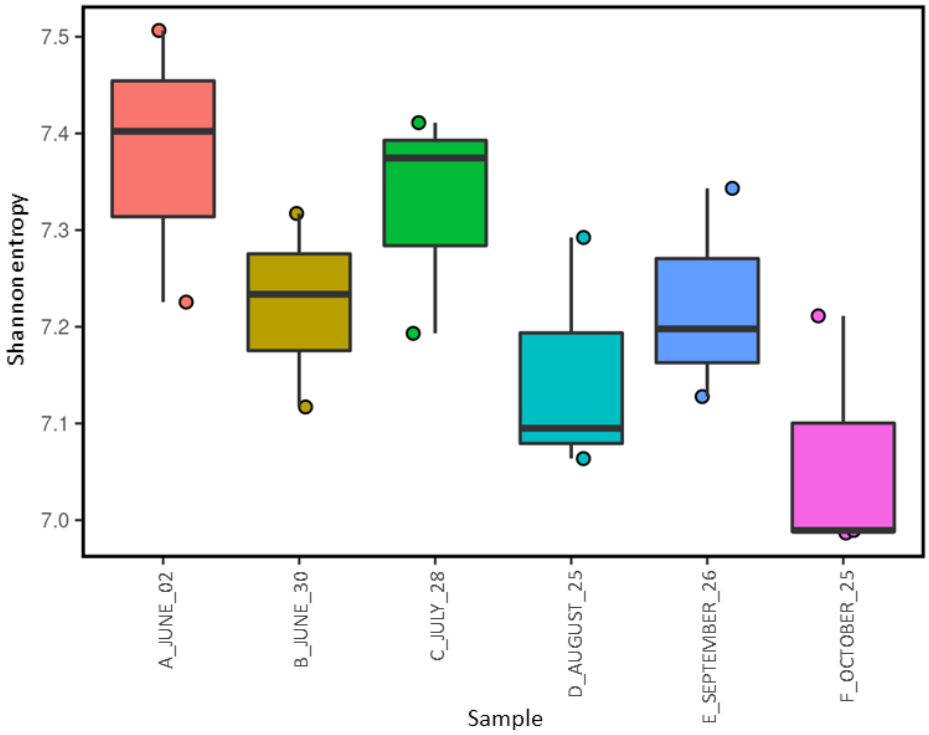

Fig. 1. Boxplot showing the microbial diversity in the anaerobic sludge at different time intervals based on Shannon index

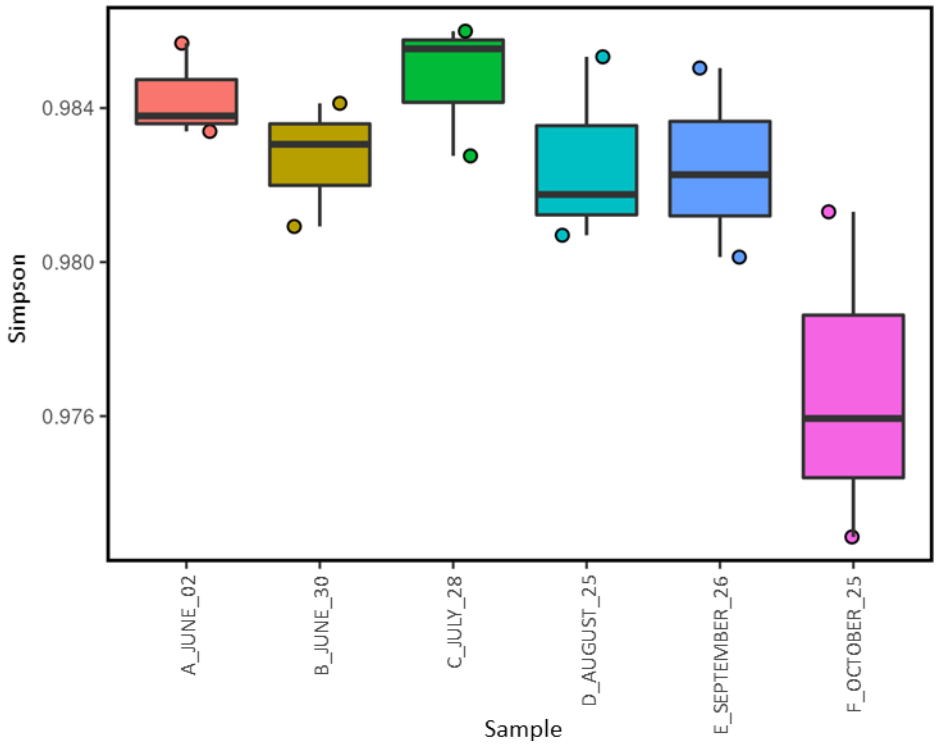

Fig. 2. Boxplot showing the microbial diversity in the anaerobic sludge at different time intervals based on Simpson index

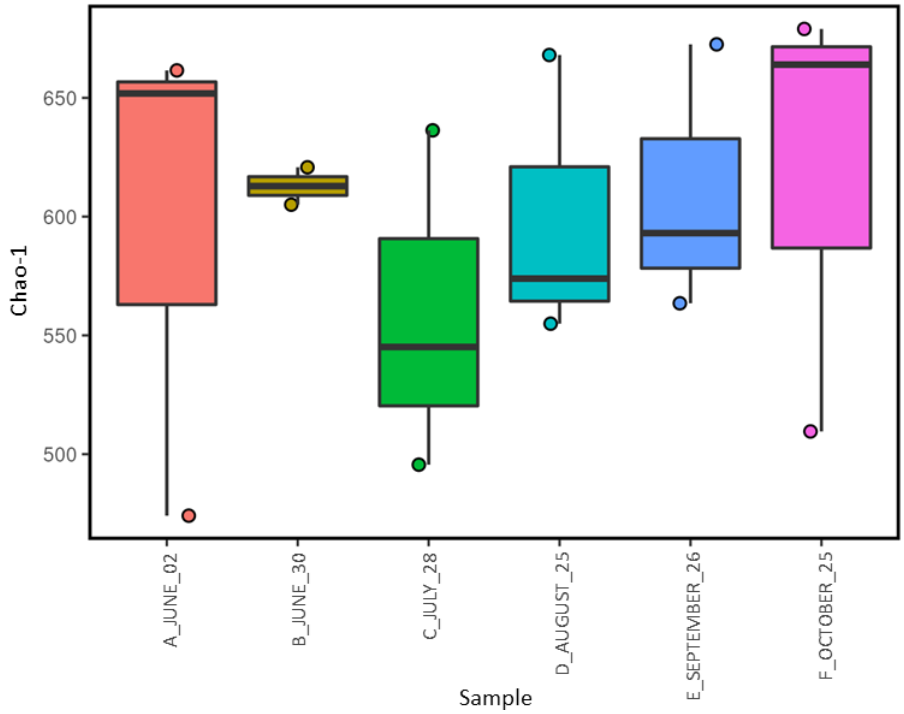

Fig. 3. Boxplot showing the similar microbial diversity in anaerobic sludge at different time intervals based on Chao-1 index

The microbial community structure in the anaerobic sludge at different time intervals: 2nd June, 30th June, 28th July, 25th August, 26th September, and 25th October, was also evaluated based on the beta diversity index. This study assessed beta diversity using principal coordinate analysis (PCoA) to delineate the variation between the samples. Fig. 4 shows the PCoA plot for all the anaerobic sludge samples, revealing that the samples could be categorized into six distinct groups based on the sampling data. Principal components 1 and 2 explained 39.16% and 20.05% of the total sample variability, respectively. The sample replicates

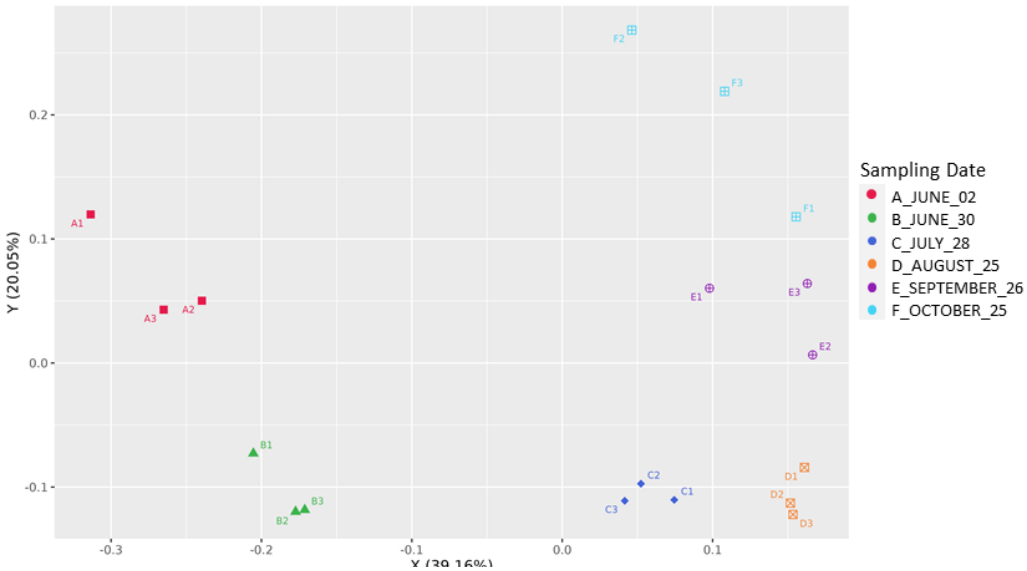

Fig. 4. Principal coordinate analysis (PCoA) plot for all the anaerobic sludge samples according to different time intervals. The colours represent the sampling date

also clustered together based on the time intervals, suggesting a similar microbial community structure between the replicates. The separation of the groups indicated the variation of the microbial community structure if compared to different time intervals.

3.2 Microbial Community Composition in POME Anaerobic Sludge

The POME anaerobic sludge was found to have a consistent microbial community across different time intervals. Specifically, the sequences were classified as a combination of bacterial and archaeal phyla, totaling 48 phyla. The relative abundance of the taxa at the phylum level is depicted in Fig. 5.

Taxonomic classification at the phylum level revealed that the phylum *Firmicutes* (currently known as *Bacillota*) (26.9 ± 3.3%) were the dominant bacterial phylum in the anaerobic sludge, followed by *Bacteroidota* (20.2 ± 5.3%) and *Chloroflexota* (15.0% ± 6.3%) (Fig. 5.). Meanwhile, the major archaeal phyla found in the anaerobic sludge were *Halobacteriota* (5.9 ± 2.8%), *Methanobacteriota* (2.5 ± 0.6%) and *Nanoarchaeota* (2.3 ± 1.2%) (Fig. 5.). The bacterial phyla *Bacillota, Bacteroidota*, and *Chloroflexota* are commonly found in biogas reactor systems, particularly during the acidogenesis stage of anaerobic digestion [4,17,24,41,42]. Furthermore, *Halobacteria* and *Methanobacteriota* are the methanogenic archaea mainly involved in biogas production [23,41,43,44]. This study investigated the methanogenic archaeal community further, owing to their distinct ability to generate methane in anaerobic digesters.

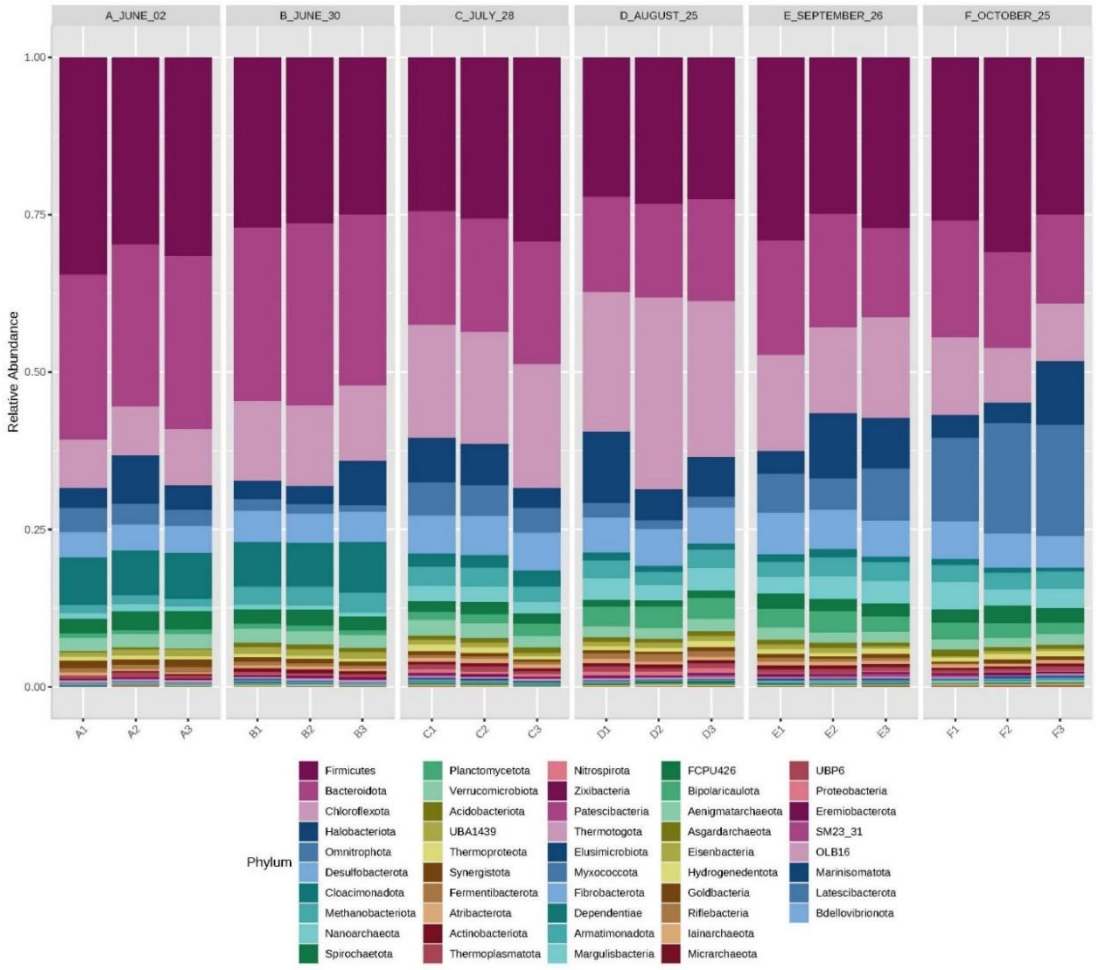

Fig. 5. Bar plot representing the relative abundance of the bacterial and archaeal phyla in anaerobic sludge

Methanogens are microorganisms belonging to the archaea domain that are able to produce methane as a metabolic by-product using various substrates, including hydrogen, carbon dioxide, acetate, and methyl compounds. In this study, at the family level, the detected methanogenic archaea are *Methanotrichaceae, Methanoculleaceae, Methanospirillaceae, Methanoregulaceae* from the phylum *Halobacteriota*; *Methanobacteriaceae* from the phylum *Methanobacteriota*; *Methanomassiliicoccaceae, Methanomethylophilaceae* from the phylum *Thermoplasmatota,* and *Methanomethylicaceae* from the phylum *Thermoproteota*. The percentage of detected methanogenic archaea at the family level to their respective phylum is illustrated in Fig. 6. To further investigate the microbial community in the POME anaerobic sludge, the relative abundance of the bacterial and archaeal members at the family level is presented in Fig. 7.

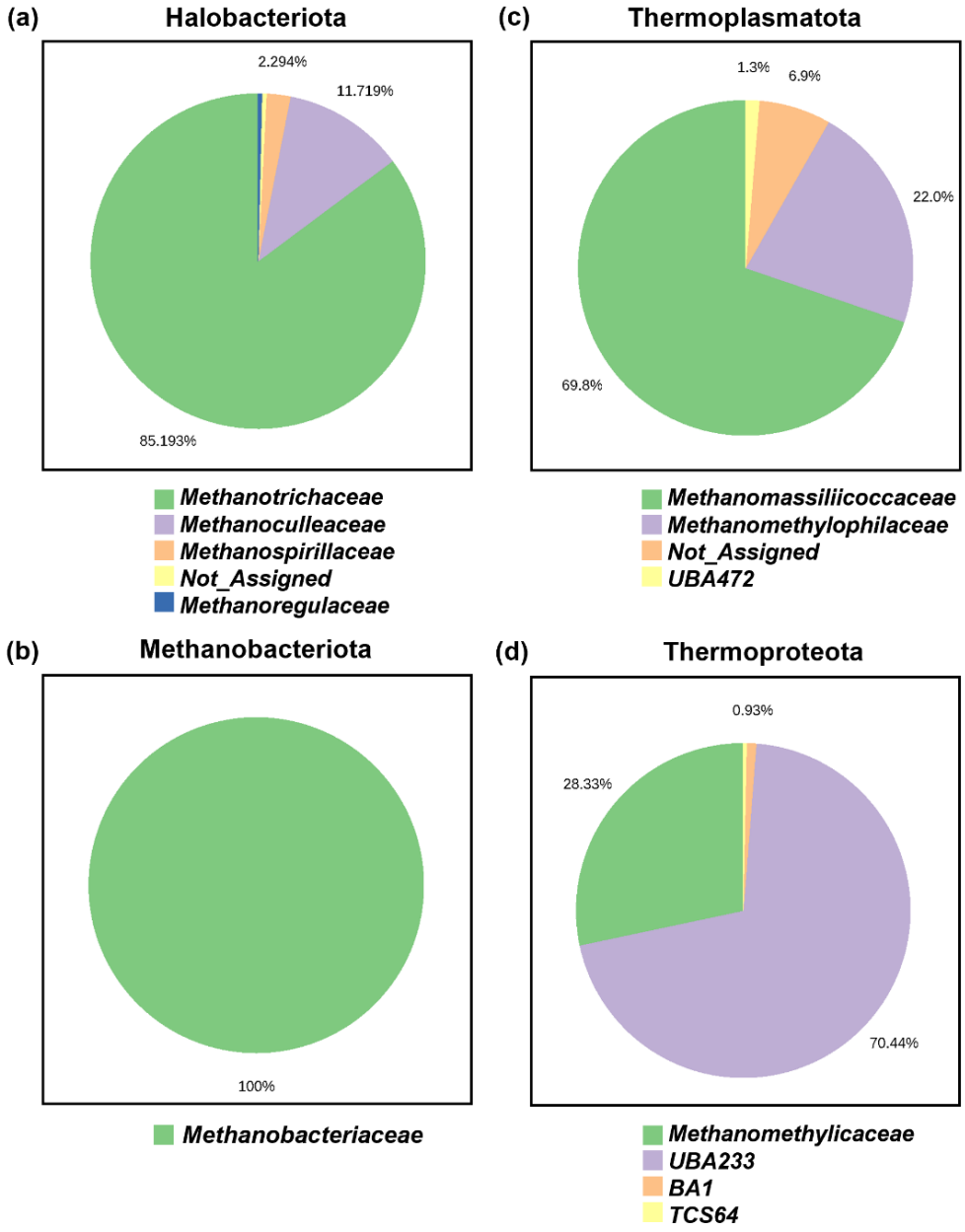

Fig. 6. The detected methanogenic archaea at the family level according to their respective phylum. (a) *Halobacteriota*, (b) *Methanobacteriota*, (c) *Thermoplasmatota*, (d) *Thermoproteota*

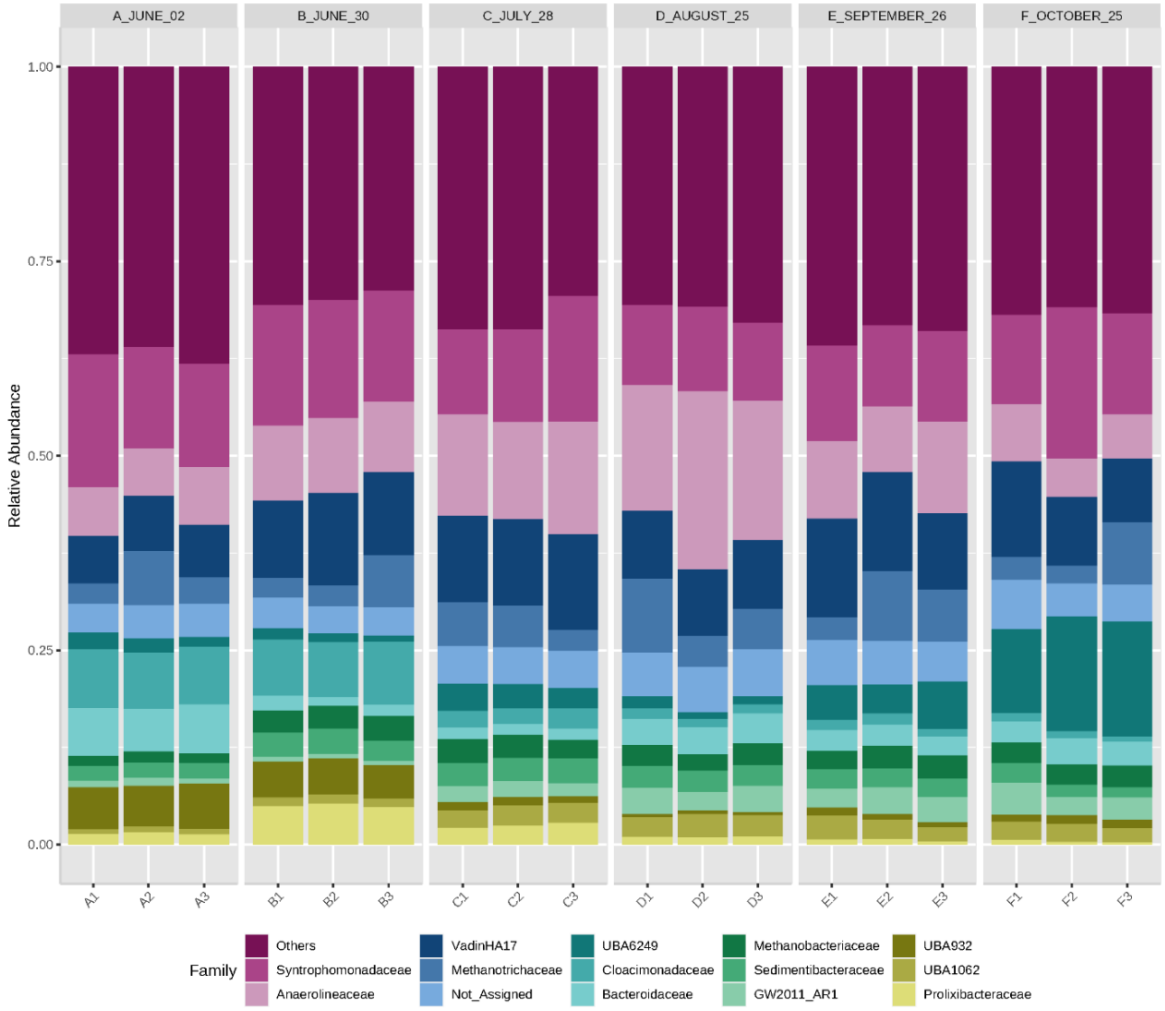

Fig. 7. Bar plot representing the relative abundance of the bacterial and archaeal families in the anaerobic sludge.

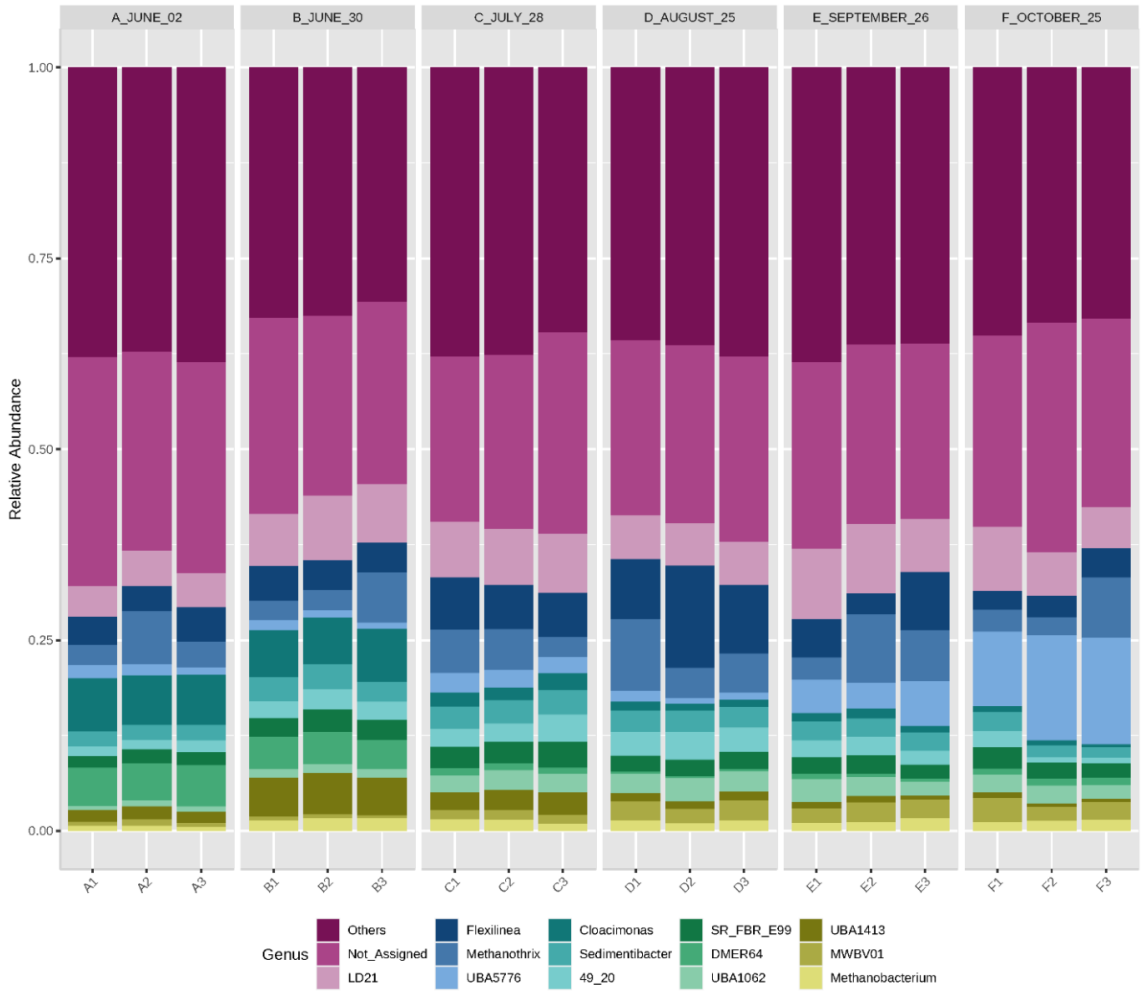

Fig. 8. Bar plot representing the relative abundance of the bacterial and archaeal genus in the anaerobic sludge

As shown in Fig. 7, only two abundant methanogens, *Methanotrichaceae* and *Methanobacteriaceae*, dominated the methanogenic archaeal community, representing a relative abundance of 5.01 ± 2.39% and 2.51 ± 0.62%, respectively. Methanogens, especially *Methanotrichaceae* and *Methanobacteriaceae,* are commonly observed in the previous biogas microbiome study in anaerobic digesters [4,42,45-47]. *Methanotrichaceae* are classified as acetoclastic methanogens capable of converting acetate to methane [47,48]. At the same time, *Methanobacteriaceae* are hydrogenotrophic methanogens mainly involved in reducing carbon dioxide to methane [47,48]. Furthermore, the methanogenic genera *Methanothrix* (4.92 ± 2.36%) and *Methanobacterium* (1.25 ± 0.35%) were discovered in the anaerobic sludge, as shown in Fig. 8. *Methanothrix* belongs to the *Methanotrichaceae* family and is exclusively acetoclastic methanogens. *Methanobacterium* from the *Methanobacteriaceae* family is mainly involved in hydrogenotrophic methanogenesis. Hence, from the methanogenic archaeal community information, the predominant pathways for methanogenesis in the POME anaerobic sludge can be attributed to acetoclastic methanogenesis facilitated by *Methanothrix* and hydrogenotrophic methanogenesis primarily associated with *Methanobacterium*.

4. CONCLUSION

The microbial community in the anaerobic digester based on the anaerobic sludge showed comparable richness and evenness through the monitoring period. However, there is a noticeable variation in the microbial community structure, indicating the temporal variation. The dominant bacterial phyla found include *Bacillota, Bacteroidota,* and *Chloroflexota*. At the same time, the archaeal community is primarily composed of methanogens, with a significant presence of the phyla *Halobacteriota* and *Methanobacteriota*. Methanogenic archaea are the predominant community responsible for methane production in this anaerobic digestion process. Among the identified prevalent methanogens are the acetoclastic *Methanothrix* and hydrogenotrophic *Methanobacterium*. This suggests that both acetoclastic and hydrogenotrophic methanogenesis are involved in methane production in the POME anaerobic digester.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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