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RESEARCH ARTICLE

Impact of innovative nanoadditives on biodigesters microbiome

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Abstract

Nanoparticles (NPs) supplementation to biodigesters improves the digestibility of biowaste and the generation of biogas. This study investigates the impact of innovative nanoadditives on the microbiome of biodigesters. Fresh cow manure was anaerobically incubated in a water bath under mesophilic conditions for 30 days. Three different NPs (zinc ferrite, zinc ferrite with 10% carbon nanotubes and zinc ferrite with 10% C76 fullerene) were separately supplemented to the biodigesters at the beginning of the incubation period. Methane and hydrogen production were monitored daily. Manure samples were collected from the digesters at different time points and the microbial communities inside the biodigesters were investigated via real-time PCR and 16 S rRNA gene amplicon-sequencing. The results indicate that zinc ferrite NPs enhanced biogas production the most. The microbial community was significantly affected by NPs addition in terms of archaeal and bacterial 16 S rRNAgene copy numbers. The three ZF formulations NPs augmented the abundance of members within the hydrogenotrophic methanogenic phyla Methanobacteriaceae. While Methanomassiliicoccacaea were enriched in ZF/C76 supplemented biodigester due to a significant increase in hydrogen partial pressure, probably caused by the enrichment of Spirochaetaceae (genus *Treponema*). Overall, NPs supplementation significantly enriched acetate-producing members within Hungateiclostridiaceae in ZF/CNTs, Dysgonomonadaceae in ZF and Spirochaetaceae ZF/C76 biodigesters.

INTRODUCTION

Anaerobic digestion (AD) is an appealing process that recycles organic waste accumulating over the planet earth into a biofertilizer with the accompanying production of methane (Mansour et al., 2020; Abdallah et al., 2021). Methane can be used for energy provision to help solve the energy deficiency problem (Heyer et al., 2015). In addition, methane oxidation helps minimize methane emissions into the atmosphere (Hassaneen et al., 2020). Methane released into the atmosphere magnifies the problem of global warming; methane is the second most significant greenhouse gas after CO_2 (Abbassi-Guendouz et al., 2012). The AD process is performed by a microbial consortium that mediates different stages of AD; hydrolysis, acidogenesis, acetogenesis and methanogenesis (Ganzoury & Allam, 2015). The syntrophy between biodegrading bacteria and methanogenic archaea is essential for the adequacy of AD and biogas production

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(Langer et al., 2015). The reactions mediated by biodegrading bacteria result in the production of CO_2, H_2, acetate, formate and methanol which are used as a substrate for methanogenesis (Glass et al., 2012). Unfortunately, many functional relationships between microorganisms in the AD process are still incompletely evident (Cai et al., 2016).

The aforementioned AD reactions are mediated by enzymes that require trace metals to act as cofactors (Abdelsalam et al., 2016). Supplementation of biodigesters with trace metals in the form of nanoparticles (NPs) was proven to significantly enhance biogas production (Abdelwahab et al., 2022; Abdelwahab et al., 2021; Sliem et al., 2021). Moreover, the addition of conductive carbon NPs to biodigesters positively impacted the efficiency of AD reactions (Abdallah et al., 2019; Jadhava et al., 2021; Lee & Lee, 2019; Mostafa et al., 2020; Zhu et al., 2021). The supplementation of biodigesters with NPs helps improve the AD process stability and performance on an industrial scale (Abdelwahab et al., 2020). A complete understanding of the mechanisms of interaction between the supplemented NPs and the hydrolysing or methanogenic microorganisms is essential in developing biogas production technology. Nano zero-valent iron (nZVI) added at different dosages to waste-activated sludge (WAS) incubated inside biodigesters enhanced biogas production, which was ascribed to the interference of nZVI with the microbial cell membrane (Wang et al., 2018). This interference led to the liberation of hydrolytic intracellular materials boosting hydrolysis and acidification of organic materials in WAS. The nZVI also stimulated the production of favourable volatile fatty acids (VFAs), such as acetic acid which is easily fermentable. A different interaction mechanism was proposed for the impact of conductive NPs as carbon nanotubes (CNTs) and Fe_3O_4 on the AD process (Zhang, Zhang, & Lu, 2018). The addition of nano-Fe_3O_4 and CNTs to lake sediment incubated under anaerobic conditions increased biodegradation and methane production. This was ascribed to the enhancement of direct interspecies electron transfer (DIET) during methanogenic reactions as a result of the high electric conductivity of the added NPs that promoted the syntrophic degradation of butyrate in lake sediments. However, further investigation is required to further support this interaction between NPs and lake sediment microbiome. When added to anaerobic digesters, granular-activated carbon (GAC) was also proposed to promote methane generation by facilitating the DIET (Yu et al., 2020). This mechanism of methane enhancement was further proved when other pathways for electron transfer were inhibited by applying high hydrogen pressure through digesters. For instance, the performing microbial community analysis reflected the enhanced DIET by increased abundance of specific genera such as *Geobacter* and *Methanosarcina*.

Therefore, investigating the microbial consortium inside biodigesters and how they shift when provoked by AD promoters is essential to ensure the utmost biogas productivity from biodigesters (Plugge, 2017). Building up information from different microbial communities inside various forms of digesters should allow scientists to intentionally manipulate the biodigester microbiota in favour of efficient AD and biogas production. Thus, herein, our work aims to understand and report microbiome alterations caused by innovative NPs supplemented with anaerobic digesters in previous research (Hassaneen et al., 2020). Different combinations of metals and conductive carbon were synthesized in the nanoscale in the form of zinc ferrite (ZF), zinc ferrite combined with 10% CNTs (ZF/CNTs) and zinc ferrite combined with 10% C76 fullerene (ZF/C76). The three additives were added at a specific dosage to anaerobically incubated cow manure and biogas production was monitored for 30 days under mesophilic conditions. Following the incubation periods, samples from biodigesters were collected and microbial communities were investigated. Archaeal and Bacterial communities were characterized via real-time PCR and microbiome (16 S rRNA gene) sequencing.

MATERIALS AND METHODS

Substrate sludge preparation and nanoadditives formulation

Fresh cow manure supplied by the farm of Assiut University, Assiut, Egypt, was transferred to the energy and materials laboratory at the American University in Cairo on the same day and stored at 4°C. A sample from the sludge was withdrawn to determine the pH, volatile solids (VS), total solids (TS) and volatile fatty acids prior to anaerobic incubation. The characteristics of the fresh manure utilized as a substrate are listed in Table S1 in the supplementary material. Three different nanoadditives were formulated; zinc ferrite, zinc ferrite with 10% CNTs and zinc ferrite with 10% C76. Zinc ferrite NPs were synthesized according to Abdullah and colleagues (Abdullah et al., 2018). Zinc ferrite/CNTs and zinc ferrite/C76 NPs were synthesized according to the same procedure except for the initial homogenous mixing of the carbon-containing material, either CNTs or C76 fullerene, with ferric salt at a ratio of 1:10.

Nanoadditives characterization

The size, morphology and elemental composition of the NPs were analysed using x-ray diffraction analysis 'XRD' and high-resolution transmission electron microscopy 'HRTEM'.

Anaerobic incubation and biogas production

Fresh manure was mixed with distilled water at a ratio of 1:1 as a weight percentage prior to anaerobic incubation. This step was performed to optimize total solids content to facilitate the AD (Motte et al., 2013). Anaerobiosis was assured by flushing the whole incubation system with nitrogen gas for 30 min. The temperature inside the biodigesters was maintained at 37°C and biogas production was monitored daily for 30 days. After 30 days, the biogas production from the digesters strongly declined and the experiment was terminated. The volume of the digesters was 500 ml, and they were operated at a working volume of 400 ml in batch mode. A series of 12 biodigesters were connected in parallel to represent four study cases presented in triplicates. These four cases were the control biodigester (A), and the three other digester cases were supplemented with 500 mg/L of zinc ferrite NP (B), zinc ferrite with 10% CNTs NP (C), or zinc ferrite with 10% C76 NP (D). A biogas analyser (Biogas5000, Geotech, UK) was utilized to quantify the produced gases daily (Nong et al., 2020).

Manure sampling and DNA extraction

Samples from the sludge were collected at three-time points; before anaerobic incubation, after 20 days of incubation and after 30 days of incubation. The collected samples were aliquoted into volumes of 1 ml in micro-centrifuge tubes and instantly frozen in liquid nitrogen (Hickl et al., 2019). Manure samples were kept at −80°C until the time of DNA extraction. DNA was extracted using Qiamp Fast DNA Stool Mini kit (Qiagen, Germany) according to the manufacturer protocol with few modifications to increase DNA yield. Before DNA extraction, samples were centrifuged for 10 minutes at 8000 rpm at 4 °C and the supernatant was discarded. During extraction, only 750 μl of inhibitEx buffer were added instead of 1 ml and a volume of 300 μl instead of 250 μl of the supernatant after the second centrifugation step was incubated with proteinase K. The DNA yield for each sample is indicated in Table S2.

Real-Time polymerase chain reactions

The estimation of bacterial and archaeal 16 S rRNA abundance was done through real-time PCR. Table 1 represents primer sets selected for archaeal and bacterial amplification. Standard curves were constructed using the pGEM-T Easy Vector System (Promega, United States) cloned with bacterial or archaeal 16 S rRNA genes. Bacterial DNA was extracted from *Escherichia coli,* while the archaeal one was extracted from *Methanosarcina thermophila*. 100-fold serial dilutions were used to cover a range of $1E^{10}$ to $1E^{01}$ gene copies/μl (Tao et al., 2020). Real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (Thermo Fisher Scientific, United States) and applied biosystems 7500 (Thermo Fisher Scientific, United States). The total reaction volume was 10 μl. 0.5 μl of template DNA and 0.5 μl of each primer (10 μM) were added to each reaction. The reaction was set according to the manufacturer's protocol. The annealing temperature for bacterial reactions was 53°C (Stubner, 2004) while for archaeal reactions was 60°C (Lee et al., 2008). All reactions were set in triplicates, and negative control was set in all 96-well plates.

Next-generation Illumina sequencing

DNA extracted from different digesters at two-time points (1 and 3) were selected for microbiome (16 S rRNA) sequencing to understand the variations in the microbial community composition upon NPs supplementation. A set of universal primers amplified the V4 regions of both bacterial and archaeal 16 S rRNA genes; 515 F (5′-GTG CCA GCM GCC GCG GTAA- 3′) and 806 R (5′-GGA CTA CVS GGG TAT CTAAT–3′). The expected amplicon size slightly exceeded 250 bps (Liu et al., 2018). The utilized forward primer had a unique barcode consisting of 6-bps attached to the 5′-end of the primer (Table S3). PCR amplifications were carried out using My Taq Red mix (Bioline, United States). PCR cycles were preceded by initial denaturation at 94°C for 7 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 30 min. QIAquick PCR Purification Kit (Qiagen, Germany) was used

TABLE 1 Sequence of primers used in conventional PCR reactions.

Primer	Sequence	Expected product size	Reference
519F	CAGC(AC)GCCGCGGTAAN(AT)C	388 bp	(Stubner, 2004)
907R	CCGTCAATTC(AC)TTT(AG)AGTT		
787F	ATTAGATACCCSBGTAGTCC	273 bp	(Lee et al., 2008)
1059R	GCCATGCACCWCCTCT		

to purify all resulting amplicons. Amplicons were pooled at equal concentrations and sent to 'Novogen, Singapore' for Illumina paired-end library preparation and sequencing on the Illumina Miseq-PE250 platform.

Bioinformatic and statistical data analysis

Data analysis was done using QIIME 2 software with the DADA2 plugin (Bolyen et al., 2019). Raw data were received as multiplexed forward and reverse sequences with quality scores. Paired ends were merged utilizing VSEARCH software (Gaspar, 2018). Then, data were imported to QIIME 2 and reads were demultiplexed. Several reads processing steps were done such as trimming of sequences longer than 250 bp, Chimeric sequences filtering, amplicon sequence variants counting and denoising (Callahan et al., 2016). Microbial community richness and evenness were investigated qualitatively through the determination of Shannon index and Pielou's Evenness index respectively (Ferguson et al., 2018). Categorical analysis of microbial diversity between the samples based on their content of NPs was done by assessing the beta diversity through PERMANOVA. Finally, sequences were blasted against the SILVA database (138_99), for the 16 S rRNA gene V4 (512–806 bp) region identification. After taxonomic classification, subsampling was carried out using the q2-srs plugin to normalize all samples to 16,000 reads for further statistical testing (https://www.mdpi.com/2076-3417/11/23/11473).

All tests were performed in triplicates except for the microbiome sequence of the seed digester and digester B were presented as duplicates, and results were represented as mean. One-way ANOVA was used to show if there is a significant difference in means between the seeds A, B, C and D for methane emission, hydrogen partial pressure, Shannon diversity index and Pielou's Evenness. Tukey's and Dunnett's tests were used for multiple comparison testing to assess the significant differences in mean (Dunnett, 1955; Tukey, 1977). *T*-test was used to compare the significant difference in 16 S rRNA gene copy number between NPs supplemented digesters, control, and the digester seed. Results of $p > 0.05$ were reported as statistically significant.

Difference between taxa among different treatment was calculated using a two-way ANOVA followed by multiple comparison test (fisher test) that was corrected for false discovery rate using the two-stage step-up method of Benjamini, Krieger and Yekutieli. Corrected *p*-values are indicated as *q*-values (Benjamini et al., 2006; Benjamini & Hochberg, 1995; Benjamini & Yekutieli, 2001). Multiple test comparison was used to compare all (30 days incubation) digesters to the Seed digester, and all NPs supplemented digesters with the control digester (30 days incubation). For microbial community taxonomic multiple test comparison, a *q*-value of $q > 0.1$ was considered significant. All statistical tests were calculated using GraphPad prism 9.

RESULTS AND DISCUSSION

Nanoadditives characterization

The XRD spectra for zinc ferrite showed characteristic peaks of Zinc ferrite$_2O_4$ (ICDD card # 04–015-7052). For zinc ferrite with 10% CNTs and zinc ferrite with 10% C76 fullerene, the same peaks appeared but were slightly shifted, see Figure S1. The HRTEM showed the plain ZF NPs to have particle size ≤9 nm, while ZF/CNTs and ZF/C76 fullerene to be 50 nm and 19 nm respectively. The morphology of the formed NPs is depicted in Figure S2.

Biogas production

The influence of NPs supplementation on methane production after 10 and 30 days of incubation was investigated. Methane production was not significantly different in the ZF/CNTs and ZF/C76 supplemented digesters compared to seed digester after the first 10 days (Figure 1). Nonetheless, methane production was significantly higher in ZF supplemented biodigesters (B10) compared to seeddigesters (A10). After 30 days of incubation, the volume of methane generated from ZF-containing biodigesters (B30) was the highest (190.58%) compared to the control digesters (A30). At the same time point, the accumulative volumes of methane produced from carbon-containing digesters (C30 and D30) were 155.65% and 134.06%, respectively, compared to the control digesters. All digesters incubated for 30 days were significantly higher in methane production from the seed digester. Moreover, Figure S3 shows the accumulative volume of carbon dioxide produced.

Hydrogen production was monitored during the experiment to assess the impact of hydrogen partial pressure alteration on methanogenesis. Hydrogen acts as an electron donor in the interactions between syntrophic organics-degrading bacteria and methanogenic archaea (Shen et al., 2016). NPs supplementation had a statistically insignificant impact on hydrogen accumulation. Hydrogen production in different digesters showed a similar trend to methane production after 10 days of anaerobic incubation. However, after 30 days of incubation hydrogen production was the highest in D30 digesters reaching 337.67% compared to control digesters. Hydrogen production from B30 and C30 digesters was 171.1% and 159.26%, respectively, compared to control digesters. The excessively high hydrogen partial pressure in D30 digesters could be related to the low

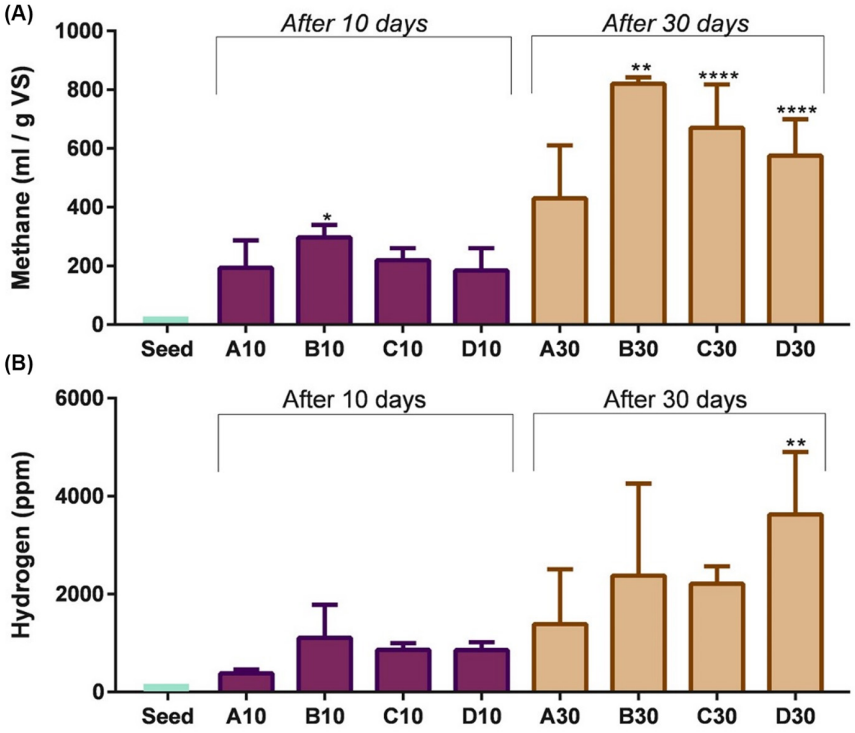

FIGURE 1 Methane (A) and hydrogen (B) produced volumes from different biodigesters at the time points targeted for microbial community analysis. A, B, C and D depicts control biodigester, $ZnFe^{3+}$ (ZF), ZF/CNTs and ZF/C76 supplemented biodigester respectively. 10 and 30 following digester letter indicates the incubation days. Asterisks indicate significance of the results compared to control digester a (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$).

methane content in these digesters. Excessive hydrogen could interfere with hydrolytic and acetogenic reactions leading to increased production of VFAs (Zhu et al., 2020). Increased VFAs decrease the pH of the sludge beyond the pH preferred for AD (Cioabla et al., 2012).

Biodigester microbiome quantification

Analysis of bacterial and archaeal 16 S rRNA gene copy numbers via qPCR assessed the archaeal and bacterial communities' absolute abundance in response to NPs addition inside the biodigesters. Microbial communities were quantified before incubation, after 10 and 30 days of AD (Figure 2). In general, a relatively higher archaeal level was observed in all the samples. This is likely due to the higher affinity of the utilized archaeal 16 S rRNA gene primer pair when compared to the bacterial primer. After 10 days of anaerobic incubation at 40 °C, the bacterial population increased by 3.69 folds in the control digesters (A10), compared to the seed manure population. Following 10 days of incubation, bacterial community absolute abundance was not significantly affected by ZF (B10) when compared to the control. In contrast, the bacterial community's absolute abundance significantly decreased (2.48- and 27.36-fold decrease) by the supplementation of ZF/CNTs (C10) and ZF/C76 (D10) NPs respectively. Following 30 days postanaerobic incubation, all reactors containing NPs showed a significant increase in bacterial 16 S rRNA gene copy number. 16 S rRNA gene copy number was the highest in ZF/CNTs-containing digesters (C30) followed by ZF (B30) and ZF/C76 (D30) containing digesters, with an increase in 14.69, 2.05 and 1.46 folds compared to control digesters (A30) respectively.

The archaeal 16 S rRNA gene copy numbers were lower in the ZF-containing digesters (B10), ZF/CNTs-containing digesters (C10) and ZF/C76-containing digesters (D10) by ~5.6, 5.6 and 15 folds when compared to the control digesters (A10) after 10 days. After 30 days of incubation, ZF/CNTs and ZF supplementation caused a 2.5- and 2.45-fold increase in archaeal 16 S rRNA gene copies respectively.

Biodigester microbiome identification

Microbial community diversity

The count of denoised and non-chimeric sequences in the different biodigesters ranged from 16,444 to 99,488 reads/sample. Microbial community diversity assessment using 16 S rRNA was performed in the samples incubated for 30 days only. The 16 S rRNA bacterial relative abundance was higher than archaea. The lowest bacterial relative abundance was observed in the A30 digesters (85.7%) and the highest was observed in the D30 digesters (98.37%). Microbial (both bacterial and archaeal) diversity and evenness were highest in the seed manure as indicated by Shannon index and Pielou evenness tests (Figure 3). Both evenness and diversity decreased upon anaerobic incubation in the control digesters. Supplementation of digesters with NPs enhanced both microbial evenness and diversity by trend. This agrees with previous findings that show

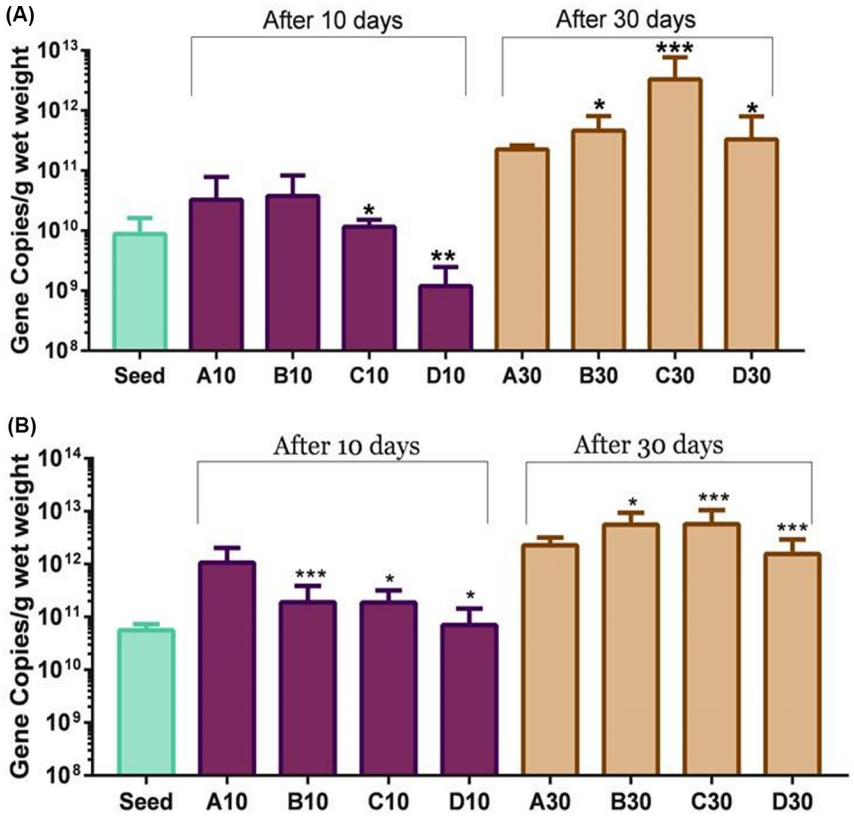

FIGURE 2 Number of bacterial (A) and archaeal (B) 16S rRNA gene copies in DNA samples from biodigesters after 10 and 30 days of incubation. A, B, C and D depicts control biodigester, $ZnFe^{3+}$ (ZF), supplemented with ZF/CNTs and ZF/C76 respectively. 10 and 30 following digester letter indicates the incubation days. Asterisks indicates significant difference compared to control digester a (*$p < 0.05$, **$p < 0.005$ and ***$p < 0.001$).

that higher microbial evenness was correlated with better AD reactions due to better allocation of microbial groups performing different stages of AD (Werner et al., 2012). Moreover, lower community evenness results in lower digester adaptability to sudden changes in pH, volatile fatty acids accumulation, etc. (Ferguson et al., 2018), and higher community diversity enhances the AD stability (Ali Shah et al., 2014). Hence, it could be hypothesized that NPs supplementation in this study stabilized the AD process in the digesters.

Microbial community transformation

Supplementation of NPs had a considerable impact on microbial consortium composition in the different biodigesters that could be seen in the quantitative analysis of the communities, mainly after 30 days of incubation. Hence, microbial communities' compositions were analysed before the incubation (seed) and after 30 days of anaerobic incubation (Figure 4 and Figure S4). The most dominant bacterial phyla in the seed manure community were Firmicutes, Bacteroidetes and Proteobacteria constituting 83% of the total bacterial community. After 30 days of AD, Proteobacteria abundance decreased significantly from 17.5% to 2.1%, 2%, 2.4% and 2.8% in C30, D30, A30 and B30 respectively. In contrast, Spirochaetota abundance increased significantly after anaerobic incubation in all digesters except C30, reaching 15.9%, 7.2% and 6.3% in D30, A30 and B30 respectively. In addition, NPs supplementation promoted the multiplication of both Firmicutes in C30 and Bacteroidetes in D30 which are known to be correlated with stable AD performance (Chen et al., 2016). Archaeal communities were dominated by Euryarchaeota constituting 71.4% of the total archaea community in the seed manure samples. After 30 days of AD, the hydrogenotrophic Crenarchaeota were enriched in all digesters (relative abundance between 11 and 32%). Even though methane production was historically associated with the Euryarchaeota, methane production after 30 days of incubation could be correlated with the enrichment of Crenarchaeota (Evans et al., 2019).

The bacterial community in the seed manure was characterized by high biodiversity as more than 37.7% of 16S rRNA were assigned to bacterial families with a relative abundance of <1%, and 22.4% were assigned to bacterial families with a relative abundance of <5% (Figure 5 and Figure S5). Approximately 39.8% of the total bacterial community was assigned to four families including Lachnospiraceae, Clostridiaceae, Rikenellaceae and Moraxellaceae. Similarly, cow manure bacterial community were reported to be dominated by the hydrolytic Ruminococcaceae, the acetogenic Bacteroidaceae and Clostridiaceae, together with the acidogenic Rikenellaceae and Lachnospiraceae (Ozbayram et al., 2018).

Clostridiaceae and Moraxellaceae abundance in all digesters significantly decreased. In contrast,

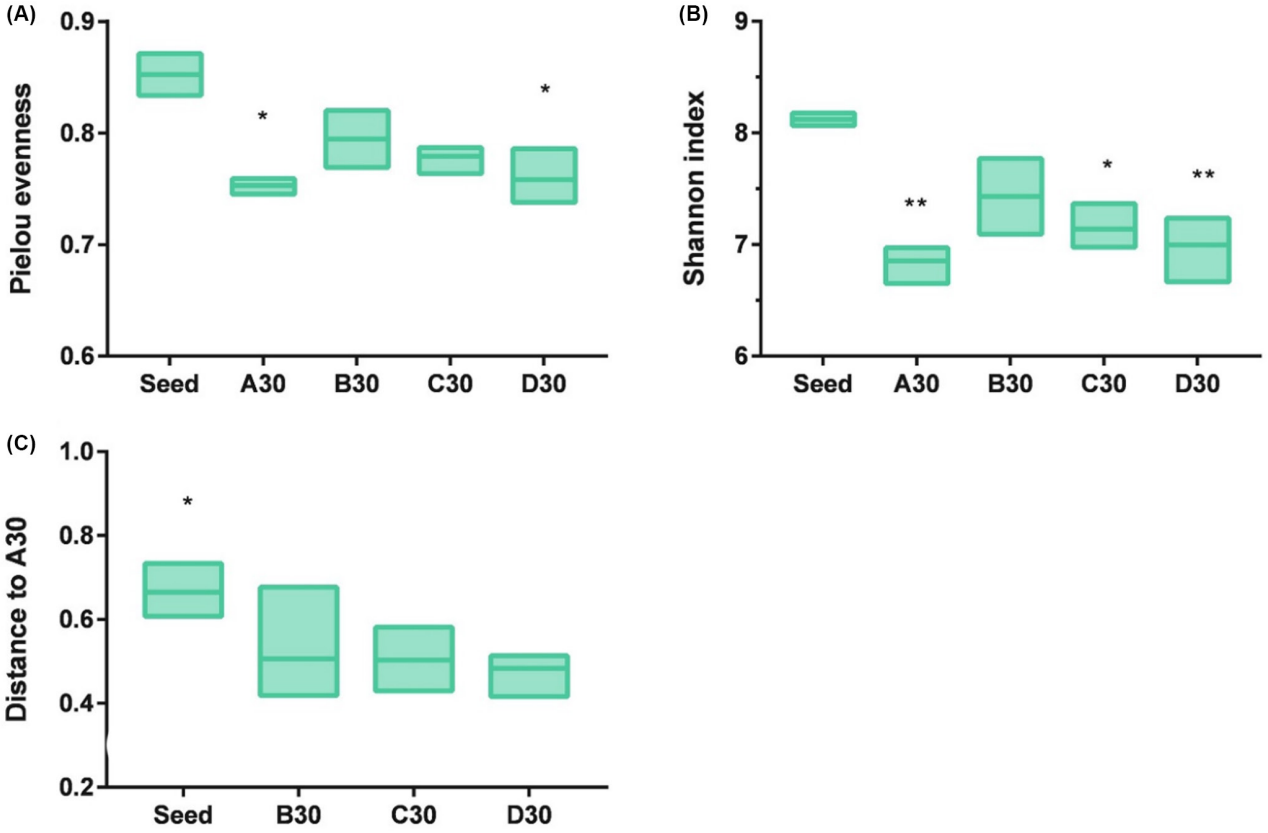

FIGURE 3 Alpha diversity of biodigesters microbiome represented by boxplots for (A) evenness, (B) phylogenetic diversity of microbial community. A, B, C and D depicts control biodigester, $ZnFe^{3+}$ (ZF), supplemented with ZF/CNTs and ZF/C76 respectively. (C) beta diversity representing the diversity between digesters compared to control digesters. The mean values are depicted by the horizontal line. Asterisks represent significant difference compared to the seed digester (*$p < 0.05$, **$p < 0.005$).

the abundance of Ruminococcaceae, unclassified Fibrobacterales and Dysgonomonadaceae significantly increased after 30 days of incubations. NPs supplementation manipulated bacterial community composition notably. Zinc ferrite (B30) addition to biodigesters significantly augmented the acetogenic family Dysgonomonadaceae (Figure 5) when compared to the control digester (A30), likely due to increased hydrogen production (Figure 1). However, zinc ferrite (B30) inhibited the proliferation of hydrolytic families such as unclassified Fibrobacterales and Marinilabiliacaea by trend. The inhibition may also be due to increased hydrogen partial pressure in the B30 digesters (Cazier et al., 2015).

The enrichment of the cellulose-degrading acetate-producing Hungateiclostridiaceae-genus HN-HF0106 and Lachnospiraceae-genus *Herbinix*) was evident in ZF/CNTs biodigester (C30) (Figure 5, Table S4). Previous studies have demonstrated the abundance of Hungateiclostridiaceae; HN-HF0106 (Table S4) in thermophilic environment (Zhu et al., 2021). It is likely that the carbon nanotube (CNTs) prompted thermal conductivity in the biodigester. Similarly, the enrichment of the cellulose-degrading *Herbinix* could be due to the thermal conductivity of the CNTs, since *Herbinix* is a thermophile (Koeck et al., 2021). However, further studies are required to support the relationship between the thermal conductivity of carbon nanotubes and biodigester community structure.

Spirochaetaceae-genus Treponema (Table S4) was enriched in D30. This agrees with the notable increase in hydrogen production observed in this sample (Figure 1b) (Bengelsdorf et al., 2018).

The shifts in the bacterial community after 30 days of AD are in agreement with the reported abundance of Spirochaetaceae, Chloroflexi and Fibrobacterales following AD. While a discrepancy with our data is noted in the reported decrease in Lachnospiraceae and Ruminococcaceae during the AD process (Dong et al., 2019).

Regarding archaeal families, Methanobacteriaceae was the predominant family prior to anaerobic incubation with a relative abundance of 76.7% of the total archaeal community. However, after anaerobic incubation, an enrichment of Bathyarchaeia, Methanosarcina and Methanomassiliicoccacaea was evident in the control digesters (A30). This shift in the archaeal community following anaerobic incubation was previously described for cow manure that was originally mainly dominated by Methanobacterium;

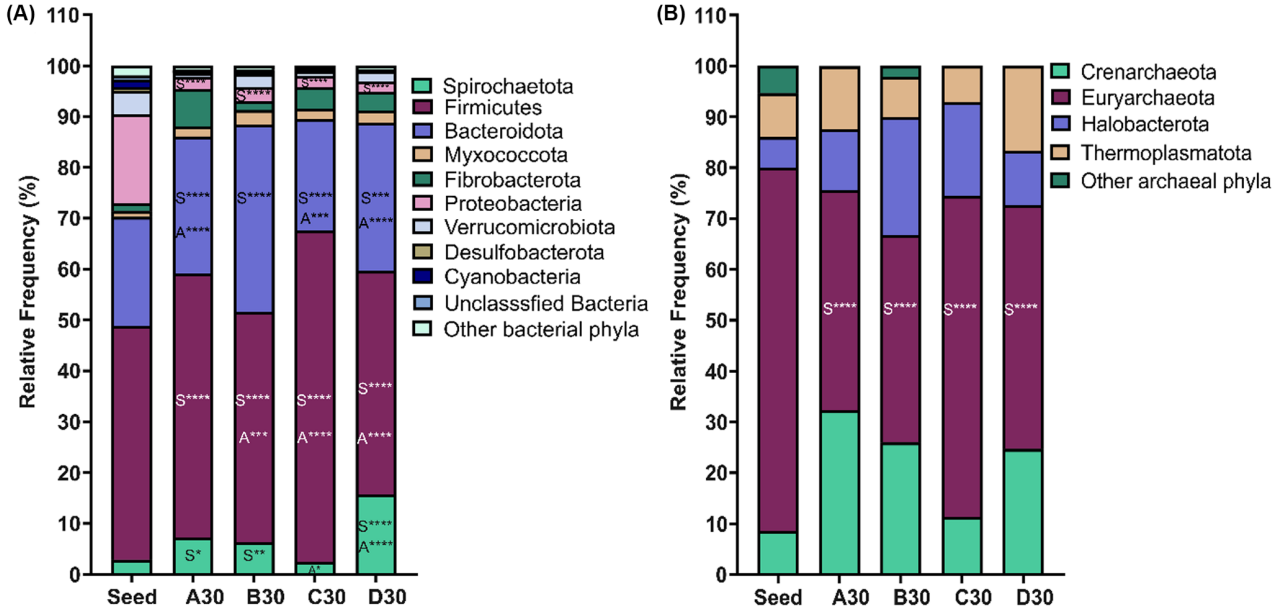

FIGURE 4 Bacterial (A) and archaeal (B) community structure at phylum level. The graph shows the mean values of replicas. A, B, C and D depicts control biodigester, $ZnFe^{3+}$ (ZF), supplemented with ZF/CNTs and ZF/C76 respectively. Multiple-test comparison between seed digesters and 30 days incubated digesters were indicated by an 'S' followed by the p-value, while multiple-test comparison between the control digester (A30) and the NPs supplemented digesters (B30, C30 and D30) were indicated by an 'a' followed by the *p*-value. Asterisks represent the *p*-value (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

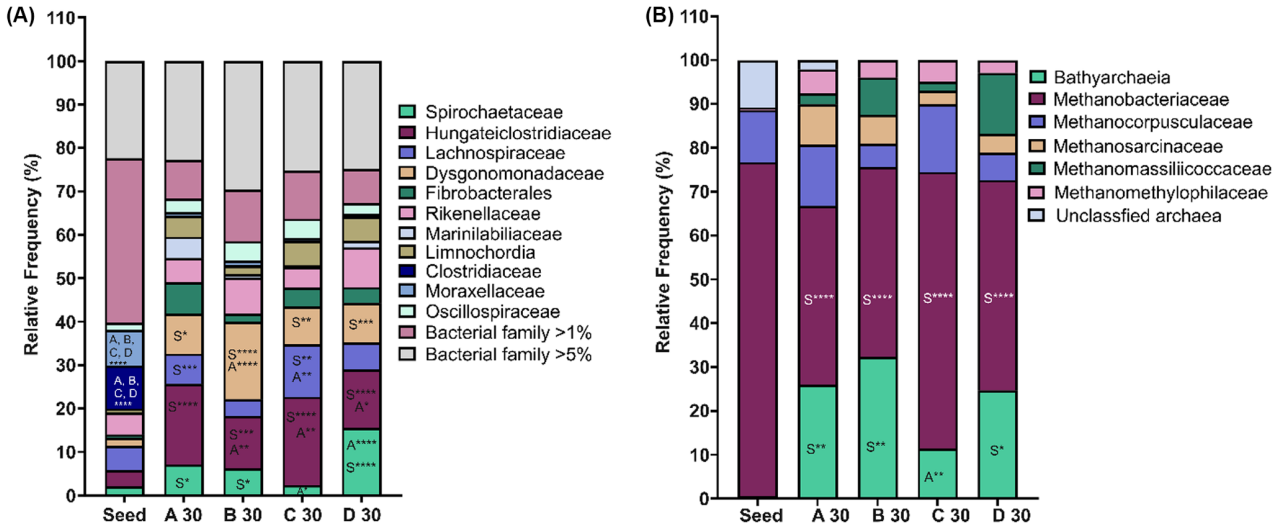

FIGURE 5 Bacterial (A) and archaeal (B) community structure at family level. The graph shows the mean values of replicas. A, B, C and D depicts control biodigester, $ZnFe^{3+}$ (ZF), supplemented with ZF/CNTs and ZF/C76 respectively. Multiple-test comparison between seed digesters and 30 days incubated digesters were indicated by an 'S' followed by the p-value, while multiple-test comparison between the blank digester (A30) and the NPs supplemented digesters (B30, C30 and D30) were indicated by an 'a' followed by the p-value. The relative abundance difference of Clostridiaceae and Moraxellaceae between seed digester and the NPs digester are indicated on the seed bar as 'A, B, C, D' followed by *p*-value asterisks represent the *p*-value (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

however, after anaerobic incubation for 15 months, a 70% increase in Bathyarchaeota and Methanosarcina was noticed. The increased abundance of the biodegrading Firmicutes and Bacteroidetes together with the methanogenic Methanosarcina explains the enhanced biogas production following incubation (Dong et al., 2019).

The addition of NPs to the biodigesters altered the archaeal community in favour of hydrogenotrophic methanogens compared to control digesters. All NPs enhanced (by trend) the abundance of Methanobacteriaceae that perform hydrogenotrophic methanogenesis (Liu, 2010). This can be attributed to the fact that metallic and active-carbon-containing NPs stimulate DIET in the favour

of hydrogenotrophs by mediating electron transfer between hydrogenotrophic archaea and bacteria (Jadhav et al., 2021). This was also accompanied by a significant decrease in Bathyarchaeota relative abundance in digester C30. Bathyarchaeota possesses diverse modes of metabolism as they can act as hydrolytic, acetogenic and hydrogenotrophic methanoges (He et al., 2016). The relative abundance of Methanomassiliicoccacaea increased to 13.8% in D30 digesters compared to 8.4%, 2.6% and 2.1% in A30, B30 and C30 digesters respectively. This was probably driven by the abundance of H_2 in the digester (D30) since Methanomassiliicoccacaea is a hydrogen utilizing methylotrophic methanogen (Kurth et al., 2020).

CONCLUSIONS

The supplementation of biodigesters with NPs containing zinc ferrite, and conductive carbon materials enhanced the productivity of biogas by altering both bacterial and archaeal communities. ZF/CNTs NPs increased bacterial 16S rRNA gene copy numbers the most. Spontaneously, in blank digesters, the process of methanogenesis was shifted towards the methylotrophic methanogenesis. However, the addition of NPs to the biodigesters altered the archaeal community in favour of hydrogenotrophic methanogenesis (Methanobacteriaceae) in all digesters, and the hydrogen utilizing methylotrophic methanogen in digester D30. The increase in the relative abundance of the methanogenic Methanomassiliicoccacaea was directly proportional to the hydrogen partial pressure in D30 digester.

AUTHOR CONTRIBUTIONS

Fatma Y Hassaneen: Formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal). **Rehab Z Abdallah:** Formal analysis (equal); investigation (equal); methodology (equal); writing – review and editing (equal). **Muhammed S Abdallah:** Formal analysis (equal); methodology (equal). **Nashaat Ahmed:** Formal analysis (equal); methodology (equal). **Shereen M. M. Abd Elaziz:** Supervision (equal). **Mohamed A. El-Mokhtar:** Supervision (equal). **Mohamed S. Badary:** Supervision (equal). **Rania Siam:** Formal analysis (equal); writing – review and editing (equal). **Nageh K Allam:** Conceptualization (equal); formal analysis (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Abbassi-Guendouz, A., Brockmann, D., Trably, E., Dumas, C., Delgenès, J.P., Steyer, J.P. et al. (2012) Total solids content drives high solid anaerobic digestion via mass transfer limitation. *Bioresource Technology*, 111, 55–61.

Abdallah, M.S., Hassaneen, F.Y., Faisal, Y., Mansour, M.S., Ibrahim, A.M., Abo-Elfadl, S. et al. (2019) Effect of Ni-ferrite and Ni-Co-ferrite nanostructures on biogas production from anaerobic digestion. *Fuel*, 254, 115673.

Abdallah, M.S., Mansour, M.S. & Allam, N.K. (2021) Mapping the stability of free-jet biogas flames under partially premixed combustion. *Energy*, 220, 119749.

Abdelsalam, E., Samer, M., Attia, Y.A., Abdel-Hadi, M.A., Hassan, H.E. & Badr, Y. (2016) Comparison of nanoparticles effects on biogas and methane production from anaerobic digestion of cattle dung slurry. *Renewable Energy*, 87, 592–598.

Abdelwahab, T.A.M., Mohanty, M.K., Sahoo, P.K. & Behera, D. (2020) Application of nanoparticles for biogas production: Current status and perspectives. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, 1–13. Available from: https://doi.org/10.1080/1556703620201767730

Abdelwahab, T.A.M., Mohanty, M.K., Sahoo, P.K. & Behera, D. (2022) Impact of iron nanoparticles on biogas production and effluent chemical composition from anaerobic digestion of cattle manure. *Biomass Convers Biorefinery*, 12, 5583–5595.

Abdelwahab, T.A.M., Mohanty, M.K., Sahoo, P.K. & Behera, D. (2021) Metal nanoparticle mixtures to improve the biogas yield of cattle manure. *Biomass Convers Biorefinery*, 2021, 1–12.

Abdullah, I.H., Ahmed, N., Mohamed, M.A., Ragab, F.M.A., Abdel-Wareth, M.T.A. & Allam, N.K. (2018) An engineered nanocomposite for sensitive and selective detection of mercury in environmental water samples. *Analytical Methods*, 10, 2526–2535.

Ali Shah, F., Mahmood, Q., Maroof Shah, M., Pervez, A. & Ahmad Asad, S. (2014) Microbial ecology of anaerobic digesters: The key players of anaerobiosis. *Scientific World Journal*, 2014, 183752.

Bengelsdorf, F.R., Beck, M.H., CatarinaErz, S.H., Karl, M.M., PeterRiegler, S.W., AnjaPoehlein, D.W.-B. et al. (2018) Chapter four - bacterial anaerobic synthesis gas (syngas) and $CO_2 + H_2$ fermentation. *Advances in Applied Microbiology*, 103, 143–221.

Benjamini, Y. & Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B (Methodological)*, 57, 289–300.

Benjamini, Y., Krieger, A.M. & Yekutieli, D. (2006) Adaptive linear step-up procedures that control the false discovery rate. *Biometrika*, 93, 491–507.

Benjamini, Y. & Yekutieli, D. (2001) The control of the false discovery rate in multiple testing under dependency. *Annals of Statistics*, 29, 1165–1188.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A. et al. (2019) Reproducible, interactive, scalable

and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, 37, 852–857.
Cai, M., Wilkins, D., Chen, J., Ng, S.K., Lu, H., Jia, Y. et al. (2016) Metagenomic reconstruction of key anaerobic digestion pathways in municipal sludge and industrial wastewater biogas-producing systems. *Frontiers in Microbiology*, 7, 778.
Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. & Holmes, S.P. (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13, 581–583.
Cazier, E., Trably, E., Steyer, J.-P. & Escudié, R. (2015) Biomass hydrolysis inhibition at high hydrogen partial pressure in solid-state anaerobic digestion. *Bioresource Technology*, 190, 106–113.
Chen, S., Cheng, H., Wyckoff, K.N. & He, Q. (2016) Linkages of Firmicutes and Bacteroidetes populations to methanogenic process performance. *Journal of Industrial Microbiology & Biotechnology*, 43, 771–781.
Cioabla, A.E., Ionel, I., Dumitrel, G.A. & Popescu, F. (2012) Comparative study on factors affecting anaerobic digestion of agricultural vegetal residues. *Biotechnology for Biofuels*, 5, 39.
Dong, L., Cao, G., Guo, X., Liu, T., Wu, J. & Ren, N. (2019) Efficient biogas production from cattle manure in a plug flow reactor: A large scale long term study. *Bioresource Technology*, 278, 450–455.
Dunnett, C.W. (1955) A multiple comparison procedure for comparing several treatments with a control. *Journal of the American Statistical Association*, 50, 1096–1121.
Evans, P.N., Boyd, J.A., Leu, A.O., Woodcroft, B.J., Parks, D.H., Hugenholtz, P. et al. (2019) An evolving view of methane metabolism in the archaea. *Nature Reviews. Microbiology*, 17, 219–232.
Ferguson, R.M.W., Coulon, F. & Villa, R. (2018) Understanding microbial ecology can help improve biogas production in AD. *Science of the Total Environment*, 642, 754–763.
Ganzoury, M.A. & Allam, N.K. (2015) Impact of nanotechnology on biogas production: A mini-review. *Renewable and Sustainable Energy Reviews*, 50, 1392–1404.
Gaspar, J.M. (2018) NGmerge: Merging paired-end reads via novel empirically-derived models of sequencing errors. *BMC Bioinformatics*, 19, 536.
Glass, J.B., Orphan, V.J., Gledhill, M. & Stein, L.Y. (2012) Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. *Frontiers in Microbiology*, 3, 61.
Hassaneen, F.Y., Abdallah, M.S., Ahmed, N., Taha, M.M., Abd ElAziz, S.M.M., El-Mokhtar, M.A. et al. (2020) Innovative nanocomposite formulations for enhancing biogas and biofertilizers production from anaerobic digestion of organic waste. *Bioresource Technology*, 309, 123350.
He, Y., Li, M., Perumal, V., Feng, X., Fang, J., Xie, J. et al. (2016) Genomic and enzymatic evidence for acetogenesis among multiple lineages of the archaeal phylum Bathyarchaeota widespread in marine sediments. *Nature Microbiology*, 1, 1–9.
Heyer, R., Kohrs, F., Reichl, U. & Benndorf, D. (2015) Metaproteomics of complex microbial communities in biogas plants. *Microbial Biotechnology*, 8, 749–763.
Hickl, O., Heintz-Buschart, A., Trautwein-Schult, A., Hercog, R., Bork, P., Wilmes, P. et al. (2019) Sample preservation and storage significantly impact taxonomic and functional profiles in metaproteomics studies of the human gut microbiome. *Microorganisms*, 7(9), 367.
Jadhav, P., Nasrullah, M., Zularisam, A.W., Bhuyar, P., Krishnan, S. & Mishra, P. (2021) Direct interspecies electron transfer performance through nanoparticles (NPs) for biogas production in the anaerobic digestion process. *International journal of Environmental Science and Technology*, 2021, 1–13.
Jadhava, P., Muhammad, N., Bhuyar, P., Krishnan, S., Razak, A.S.A., Zularisam, A.W. et al. (2021) A review on the impact of conductive nanoparticles (CNPs) in anaerobic digestion: Applications and limitations. *Environmental Technology and Innovation*, 23, 101526.
Daniela E. Koeck, Regina Rettenmaier, Wolfgang H. Schwarz, Vladimir V. Zverlov & Wolfgang Liebl. (2021). *Bergey's manual of systematics of archaea and bacteria, Herbinix*. New York, NY: John Wiley & Sons, Inc.
Kurth, J.M., Op den Camp, H.J.M. & Welte, C.U. (2020) Several ways one goal—methanogenesis from unconventional substrates. *Applied Microbiology and Biotechnology*, 104, 6839–6854.
Langer, S.G., Ahmed, S., Einfalt, D., Bengelsdorf, F.R. & Kazda, M. (2015) Functionally redundant but dissimilar microbial communities within biogas reactors treating maize silage in co-fermentation with sugar beet silage. *Microbial Biotechnology*, 8, 828–836.
Lee, C., Kim, J., Shin, S.G. & Hwang, S. (2008) Monitoring bacterial and archaeal community shifts in a mesophilic anaerobic batch reactor treating a high-strength organic wastewater. *FEMS Microbiology Ecology*, 65, 544–554.
Lee, Y.J. & Lee, D.J. (2019) Impact of adding metal nanoparticles on anaerobic digestion performance – A review. *Bioresource Technology*, 292, 121926.
Liu, P., Klose, M. & Conrad, R. (2018) Temperature effects on structure and function of the methanogenic microbial communities in two paddy soils and one desert soil. *Soil Biology and Biochemistry*, 124, 236–244.
Liu, Y. (2010) Methanomicrobiales. In: Timmis, K. N. (Ed.) *Handbook of hydrocarbon and lipid microbiology*. Berlin Heidelberg: Springer, pp. 583–593.
Mansour, M.S., Abdallah, M.S., Allam, N.K., Ibrahim, A.M., Khedr, A.M., Al-Bulqini, H.M. et al. (2020) Biogas production enhancement using nanocomposites and its combustion characteristics in a concentric flow slot burner. *Experimental Thermal and Fluid Science*, 113, 110014.
Mostafa, A., Im, S., Song, Y.-C., Kang, S. & Kim, D.-H. (2020) Enhanced anaerobic digestion of long chain fatty acid by adding magnetite and carbon nanotubes. *Microorganisms*, 8, 333.
Motte, J.C., Escudié, R., Bernet, N., Delgenes, J.P., Steyer, J.P. & Dumas, C. (2013) Dynamic effect of total solid content, low substrate/inoculum ratio and particle size on solid-state anaerobic digestion. *Bioresource Technology*, 144, 141–148.
Nong, H.T.T., Unpaprom, Y., Whangchai, K. & Ramaraj, R. (2020) Sustainable valorization of water primrose with cow dung for enhanced biogas production. *Biomass Convers Biorefinery*, 2020, 1–9.
Ozbayram, E.G., Ince, O., Ince, B., Harms, H. & Kleinsteuber, S. (2018) Comparison of rumen and manure microbiomes and implications for the inoculation of anaerobic digesters. *Microorganisms*, 6(1), 15.
Plugge, C.M. (2017) Biogas. *Microbial biotechnology*, 10, 1128–1130.
Shen, L., Zhao, Q., Wu, X., Li, X., Li, Q. & Wang, Y. (2016) Interspecies electron transfer in syntrophic methanogenic consortia: From cultures to bioreactors. *Renewable and Sustainable Energy Reviews*, 54, 1358–1367.
Sliem, M.A., El-Ansary, S., Soliman, W. & Badr, Y. (2021) Enhancing biogas production of cow dung during anaerobic digestion using nanoferrites. *Biomass Convers Biorefinery*, 1, 1–8.
Stubner, S. (2004) Quantification of gram-negative sulphate-reducing bacteria in rice field soil by 16S rRNA gene-targeted real-time PCR. *Journal of Microbiological Methods*, 57, 219–230.
Tao, Y., Ersahin, M.E., Ghasimi, D.S.M., Ozgun, H., Wang, H., Zhang, X. et al. (2020) Biogas productivity of anaerobic digestion process is governed by a core bacterial microbiota. *Chemical Engineering Journal*, 380, 122425.
Tukey, J.W. (1977) Exploratory data analysis (book section). *Exploratory Data Analysis*, 61–100. Available from: https://doi.org/10.1007/978-0-387-32833-1_136

Wang, Y., Wang, D. & Fang, H. (2018) Comparison of enhancement of anaerobic digestion of waste activated sludge through adding nano-zero valent iron and zero valent iron †. *RSC Advances*, 8(48), 27181–27190.

Werner, J.J., Koren, O., Hugenholtz, P., Desantis, T.Z., Walters, W.A., Caporaso, J.G. et al. (2012) Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. *The ISME Journal*, 6, 94–103.

Yu, N., Guo, B., Zhang, Y., Zhang, L., Zhou, Y. & Liu, Y. (2020) Self-fluidized GAC-amended UASB reactor for enhanced methane production. *Chem Eng J*, 420, 127652.

Zhang, W., Zhang, J. & Lu, Y. (2018) Stimulation of carbon nanomaterials on syntrophic oxidation of butyrate in sediment enrichments and a defined coculture. *Sci Rep*, 8(1), 12185.

Zhu, X., Blanco, E., Bhatti, M. & Borrion, A. (2021) Impact of metallic nanoparticles on anaerobic digestion: A systematic review. *Science of the Total Environment*, 757, 143747.

Zhu, X., Zhou, P., Chen, Y., Xiaofeng, L. & Li, D. (2020) The role of endogenous and exogenous hydrogen in the microbiology of biogas production systems. *World Journal of Microbiology and Biotechnology*, 36, 79.

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