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**Mesophilic and thermophilic anaerobic digestion of animal manure:
integrated insights from biogas productivity, microbial viability and
enzymatic activity**

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Abstract

Anaerobic digestion (AD) is considered a promising technology for the sustainable management of current large quantities of animal waste. However, the efficiency and underlying mechanisms remain poorly understood, particularly regarding the different characteristics of different types of animal wastes. The present study investigated the methane production, community structure, microbial viability, and enzymatic activity following mesophilic and thermophilic AD of four types of animal waste, including carnivores (lion), herbivores (elephant), and omnivores (hippopotamus and orangutan). Methane yield obtained with thermophilic AD of four animals was higher than mesophilic ones. The methane yields of lion manure under the mesophilic and thermophilic AD were 0.326 L/g VS and 0.391 L/g VS, respectively, higher than that of herbivore and omnivore waste. Bacteria were divided into low-DNA (LDNA) and high-DNA (HDNA) bacteria by flow cytometry based on DNA content. Compared with mesophilic AD, thermophilic AD had higher cells density, which has the same trend with methane production. The results showed that the live LDNA bacteria abundance was increased by 98.6% in orangutan manure AD system after heating up to 55 °C. However, most of the dead bacteria were HDNA, indicating sensitivity of HDNA cells to environmental conditions. For functional enzymes, the activities of dehydrogenase, phosphotransacetylase and protease were enhanced in the thermophilic AD system of all kinds of animal manure. Nevertheless, the acetate kinase activities of herbivorous animal manure and coenzyme F420 activities of omnivorous animal manure had been inhibited at high temperatures.

Keywords: Anaerobic digestion; Flow cytometry; Bacterial viability; High-DNA; Low-DNA; Functional enzymes

1. Introduction

With the increasing concern on solid waste management and greenhouse gas emissions, the large annual amount of animals waste has bestowed many challenges [1, 2]. The amount of waste generated in a zoo is an order of hundreds of tons per year [3]. Improper disposal of animal waste is harmful to the environment, due to the possibility of releasing chemical and biological contaminants, such as nutrients, toxic gas and pathogens [1, 4]. On the contrary, animal waste is recently considered as an alternative resource as the waste is mainly organic rich. Thus, it is necessary to convert animal waste to useful material, appealing for suitable technology.

Solid waste is traditionally disposed by landfilling, dumping and burning. However, these methods are environmentally unsustainable and may also fail to fully utilize the resources of animal waste. Anaerobic digestion (AD) is a promising candidate as it is a cost-effective technology to convert waste biomass to methane (a biofuel) and to make up for the lack of non-renewable energy. Swine manure [5, 6] is among the most frequently used animal waste in AD process for the production of methane. Mesophilic and thermophilic conditions are both suitable for the AD of animal manure, with thermophilic AD generally showing a higher methane yield [7, 8]. However, the AD process could be unstable due to varying reasons, such as organic and pH shock, which lead to a decrease in methane yield. Therefore, different pretreatment strategies have been studied to improve the degradation of complex organic and methane production. which could be divided into physical (eg. ultrasonic/microwave to form radical), chemical (eg. alkaline treatment to destroy or

liquefy cell walls), and biological (eg. enzyme to increase degradation rate) methods [9]. In addition, different additives were explored for the possible enhancement of AD of animal waste. It has been proved that zero-valent iron promoted 22% methane production and 51% digestion time saving by enhancing propionate utilization and direct interspecies electron transfer (DIET) in high solid AD of swine manure[10]. The straw was also mixed with animal manure to optimize the C/N ratio and achieve a high methane production rate [11]. It is well documented that AD is widely employed to convert animal waste to methane [5, 6, 8, 12, 13], which can be a sustainable development way to achieve waste management and energy recovery.

Nevertheless, prior studies generally focus on waste from domesticated animals such as swine, cow, and chicken [5, 6, 14]. Zoo animal waste has unique characteristics, and may be different from domestic waste. Specifically, a zoo generally has a full collection of animals, ranging from herbivores to carnivores. These animals have diverse diets, leading to different waste characteristics. For example, horse manure is highly fibrous and hippopotamus manure is slurry containing high water content [12]. The vast characteristic difference could impact AD performance. As such, current AD scheme may not be suitable for the treatment of zoo waste, awaiting further demonstration and verification. Horse manure was shown to be a suitable substrate for AD process [15], in which the biogas yield was linked to enzyme activity in horse manure of different ages [16]. A one-year study showed that horse manure can be a co-substrate for co-digestion with cattle manure in a laboratory-scale [17]. For waste produced by elephant and rhinoceros, mesophilic

digestion performs better than thermophilic conditions [18], an observation different from livestock manure digestion [19]. Therefore, zoo animal waste is a promising feedstock for anaerobic digestion, but warranting further optimization and mechanistic understanding of the scheme of the current digestion system.

Therefore, the present study aimed to investigate the performance of AD of four animals' manure under mesophilic and thermophilic conditions. Different zoo animals including carnivores (lion), herbivores (elephant), and omnivores (hippopotamus and orangutan) were selected. The approach was to evaluate synergistically across methane production, microbial community structure, microbial viability, DNA content of the microbe, and enzymatic activity. Importantly, the existing study usually revealed the functional microorganisms based on the microbial community, which has limitations on reflecting the viable functional metabolic activities in AD process. The microbial viability may provide new integrated insights about evaluating the AD process of animal waste. Besides, the AD process has contributed to the waste management, energy recovery and sustainability in the zoo.

2. Materials and Methods

2.1 Substrates and inoculum

The seed sludge used in this study was collected from Ulu Pandan Water Reclamation Plant (UPWRP) in Singapore which has a large-scale anaerobic digester to dispose waste activated sludge from the domestic sewage treatment. Fresh manure of four animals — elephant, hippopotamus, lion and orangutan — were collected

from Singapore Zoo. Then, these manures were dried at 120°C until the weights were constantly followed by being homogenized separately via the blender. The dried manures were sampled for ultimate analyses. The detailed characteristics of seed sludge and animal manure are listed in Table 1.

2.2 Anaerobic digestion experimental procedure

The experiments were conducted in glass anaerobic digesters (i.e., AD reactors) with a working volume of 0.8 L. The reactors containing the seed sludge were divided into four groups. Four different types of animal manure from elephant, hippopotamus, lion and orangutan (referred to as E, H, L and OU) were added into the reactors. The feedstocks were added in a semi-continuous mode which led to a gradual increase in the organic loading rate (OLR) and a final OLR of 1 g VS/L/d. The AD reactors were initially operated at mesophilic condition (35 °C), maintained by thermostats and heating bars inside the reactor. The reactors of different substance at 35°C are correspond to E35, H35, L35 and OU35. The sludge retention time was 30 d. Characteristics of methane yield and microbial composition were obtained from the steady mesophilic operation when the biogas yield was basically stable with a fluctuation range of 0.05 L CH₄ /g VS for at least three days. Subsequently, the temperature of the above reactors was enhanced to 55 °C (referred to as E55, H55, L55 and OU55). After reaching the thermophilic condition, the feedstocks were added into the AD reactors in a same semi-continuous way as mesophilic operation. The same volume of sludge was discharged to keep a constant amount of activated sludge in AD reactor. The microbial community, enzyme and cell viability were measured after the stable operation of the AD reactors. All the experiments were performed in triplicates.

2.3 Analytical methods

Manure samples were dried at 103–105 °C and burnt to ash at 550°C for analysis of total solid [20] and volatile solid (VS) content. The pH and COD were determined by a pH analyzer (Agilent 3200M, USA) and HACH color meter (DR900, USA), respectively. The elemental composition was analyzed via vario MICRO cube (Elementar, Germany). The concentration of methane was measured by a gas chromatograph (Clarus 580 Arnel, PerkinElmer, USA) equipped with a thermal conductivity detector. Biogas volume was recorded by a gas flow meter (JH-LMF-1, X2 Labwares Private Ltd, Singapore). The methane content was analyzed by a gas chromatograph (Clarus 580 GC/TCD, PerkinElmer, USA) with helium as carrier gas, and the temperature of column was set at 140 °C.

2.4 Microbial community analysis

The extraction kit (MO BIO Laboratories, Inc. Carlsbad, USA) was used to extract DNA from the samples according to the manufacturer's instructions. The purity of extracted DNA was checked by analyzing its absorbance at 260 and 280 nm with a NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, USA). Sequencing of metagenomic DNA was determined by Illumina Miseq™ sequencer (Illumina Inc., USA). The V3 and V4 regions of bacterial 16S rRNA genes were amplified using a set of bacterial primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). The amplified PCR products were purified, quantified and then sequenced on Illumina Hiseq 2000 (Sangon Biotech Shanghai Co., Ltd., People's Republic of China). The initial sequencing consequences were treated by Trimmomatic to obtain the pure and effective sequencing data according to the principles as follows: 1) groomed paired-end sequences of reads; 2) eliminated sequences including ambiguities ("Ns"); 3)

eliminated reads that were shorter than 35 nt. 4) eliminated low-quality sequences such as a sequence that the quality value was lower than 20; 5) eliminated sequences quality that the tail was <20 bases using sliding window protocol[21]. The operational taxonomic unit (OUTs) was defined at 97% similarity level using Ribosomal Database (<http://rdp.cme.msu.edu/>). Subsequently, the differences between microbial communities were analyzed by non-metric multidimensional scaling (NMDS).

2.4 Flow cytometry analysis

The viability assessment of sludge was performed following previous reports with flow cytometry [22, 23]. Briefly, sludge sample was disaggregated by a mechanical bender on ice for 3 min. The process was repeated once for a better homogenization. After that, the homogenized sample was diluted (1/500, V/V) with 0.22 μm filtered phosphate-buffered saline solution (Thermo Fisher Scientific Inc., USA) and vortexed. The sample was then filtered by a 60 μm filter to avoid clogging of the instrument [24, 25]. The filtered sample was then further diluted (1/100, V/V) and subjected to two different staining processes—total bacterial staining and viability staining.

For total bacterial population, the sample was stained with 1.5 μl of 3.34 mM SYTO 9 (Thermo Fisher Scientific Inc., USA) and incubated for 15 min at room temperature in the dark. The discriminator was set on green fluorescence (525 ± 40 nm). The green versus red fluorescence (585 ± 42 nm) cytograms revealed the presence of total bacteria with high and low DNA contents (Supporting Information). Accordingly, the abundance of bacteria with high and low DNA contents (i.e.,

$C(HDNA_{tot})$ and $C(LDNA_{tot})$ can be calculated [26].

For viability measurement, the sample was stained with 1.5 μ l of 3.34 mM SYTO 9 and 1.5 μ l of 30 mM propidium iodide (Thermo Fisher Scientific Inc., USA). The staining mixture was incubated for 15 min at room temperature in the dark. The discriminator was set on green fluorescence. The green versus red fluorescence cytograms distinguished live bacteria with high and low DNA contents and dead bacteria (Supporting Information) and the corresponding abundance (i.e., $C(HDNA_{live})$, $C(LDNA_{live})$ and $C(dead)$) can be calculated.

Beads of 1 μ m diameter (Thermo Fisher Scientific Inc., USA) were added to the samples as an internal standard and for the calibration of the acquisition settings. The concentration of bacteria (C_{bac} , cells per ml) in original sludge sample was calculated with the following formula:

$$C_{bac} = \frac{N_{cell}}{N_{beads}} \times C_{beads} \times D$$

where, N_{cell} and N_{beads} denote the number of cells and beads recorded by flow cytometry, respectively; C_{beads} is the concentration of beads (number per ml) added into the sample; D is the dilution factor. The contribution of HDNA and LDNA to the total dead bacteria was calculated.

2.5 Enzymatic activity analysis

The Enzyme-linked immuno-sorbent assay (ELISA) method was used to test the enzyme activity. The qualitative or quantitative analysis was carried out based on the intensity of the color reaction (Qi et al. 2021). In this study, five specifying antibody substrates were used: purified coenzyme F420(CoF420) antibody, dehydrogenase (DHO) antibody, phosphate acetyltransferase (PTA) antibody, pyruvate ferredoxin

oxidoreductase (POR) antibody, and adenylate kinase [27] antibody. The tested enzyme was labeled with horseradish peroxidase (HRP) and combined the antibody to form an antibody-antigen-enzyme-labeled antibody complex. The 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate was used for detecting the optical density value of samples at a wavelength of 450 nm. The activity concentration of the enzyme in the sample was calculated by a standard curve.

2.6 Data analysis and statistical analysis

The data were analyzed using a one-way analysis of variance (ANOVA). A Student's *t*-test was employed to compare the differences between groups.

3. Results and discussion

3.1 Methane production

The operation temperature of the AD reactors was increased from 35°C to 55 °C, and the methane yields were determined to indicate the AD performance (Fig. 1A). The methane content of lion manure under the mesophilic steady-state was 0.326 L/g VS, higher than the values obtained with manure from other three animals. It has been proved that the presence of specific enzymes in carnivore manure digestive systems could degrade proteins protein and lipid, further the microbial community and enzymes in carnivore manure strengthened the degradation of complex carbohydrates in AD reactors [28]. After elevating the temperature to 55 °C, an increase in methane production was observed, corresponding to higher COD contents under thermophilic AD conditions in comparison to mesophilic ambience (Fig. 1B). The methane yield of elephant and hippopotamus manure stabilized to be 0.25-0.28 L/g VS with enhancement of 148% and 213%, respectively. It was noted that the reactor feeding lion manure as substance had the highest methane yield of 0.391 L/g VS during

thermophilic AD. The results showed that the manure of lion, a carnivorous animal, has higher methane generation potential than omnivores and herbivores. Due to dietary differences, herbivore manure contains a lot of fibrous material, with 50–80% intact lignocellulosic material. However, the cellulose is generally to be fully converted during AD process and remains in the fermentation residue, resulting in low methane production [29]. In addition, the pH profile during AD of manure from four groups of animals was similar under mesophilic (6.9-7.5) and thermophilic (7.0-7.2) conditions. This indicates that the AD reactors performed under stable conditions.

3.2 Microbial community diversity and abundance

Rarefaction analysis showed that all the curves reached their asymptotes, suggesting the sufficient read depth (Supporting Information). This is further supported by the good's coverage of 1 (Supporting Information). Alpha diversity analysis based on operational taxonomic units (OTUs) was performed to assess the microbial diversity in different samples (Supporting Information). The results of the Abundance-based Coverage Estimator and Chao1 (Supporting Information) indices showed that the microbial abundance in thermophilic AD systems was higher than mesophilic AD systems. The Shannon diversity index of lion, elephant and orangutan samples slightly decreased with increment in temperature. This result may be explained by the dynamic changes in microbial composition with the changes of temperature. In addition, not all bacteria in the AD systems exhibited activity and function [19].

3.3 Bacterial community composition analysis

The bacteria at the phylum level were detected to investigate the selective enrichment of bacterial community. Fig. 2(a) showed that the *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the dominant phyla during the AD of animal manure. In AD system, *Bacteroidetes* primarily degraded protein [32], while *Firmicutes* played an active role in cellulose and hemicellulose degradation [33]. A previous study has proposed that the ratio of relative abundance of *Firmicutes* to *Bacteroidetes* can be a critical indicator to evaluate the performance of AD process, including organic loading rate, organic acid concentration, and methane yield [34]. The results showed a large increase of *Firmicutes* count at a higher temperature compared to mesophilic AD system, which was corresponded with an increase in biogas yield. *Proteobacteria*, has been proved that it was capable of inducing cell lysis and subsequently, the release of intracellular substances in hydrolysis and acidification processes [35, 36]. Hydrolysis is a rate-limiting stage in AD, which is initiated by exo-enzymes[37]. In this study, most phyla were similar in relative abundance in all manures from different animals, except for *Cloacimonetes* under mesophilic AD, where a higher relative abundance was observed for lion and orangutan manure (10%) than that for elephant and hippopotamus manure (3%). It was worth noting that the AD of lion manure had the highest methane yield of 0.39 L CH₄/g VS at 35 °C. *Cloacimonetes* was found to have the potential to degrade amino acids with H₂ as products and might be syntrophically associated with hydrogenotrophic methanogens [38].

Bacterial communities at the genus level were shown in Fig. 2 (b). The genera *Pseudomonas* and *Sedimentibacter* were predominant species following the unclassified genera, which belong to *Proteobacteria* and *Firmicutes*, respectively. *Pseudomonas* are electrogenic bacteria responsible for metabolism of volatile fatty acids (VFAs) and transferring electrons to methanogenic partners [39]. *Pseudomonas* was enriched (relative abundance: 12.7%) in the OU manure at 35 °C, higher than average values. It seemed that group OU could distinguish from other three groups in acidogenesis and subsequent methanogenesis for methane production. Moreover, the enrichment of *Sedimentibacter* under moderate temperature (i.e., 35 °C) was higher than at high temperature (i.e., 55 °C). *Sedimentibacter* belongs to hydrogen producing and acetic acid producing bacteria. Therefore, the enrichment of dominant genus *Sedimentibacter* under mesophilic AD may help to enhance methanogenesis by accelerating formation of acetic acid and CO₂ [40] and optimizing the degradation of accumulated VFAs [41].

Bacterial analysis showed that the microbial community structure and functional patterns in AD systems varied under different temperatures. It could be explained by the fact that the optimal growth temperatures were varied with different bacteria. NMDS analysis revealed that the compositions of dominant microbial species were only related through temperature regimes, rather than animal species (Fig. 3).

3.4 Abundance and viability of bacteria

Amplicon sequencing with 16S rRNA can provide massive information on the

microbial community structure. However, it cannot differentiate the viability of the cells, leading to the insufficiency to reflect the actual active constituent in the AD process. In the present study, bacterial viability of bacterial population was examined by fluorescence staining and flow cytometry which allowed to distinguish viable cells from membrane-intact and membrane-compromised bacteria (dead) [42]. Results showed that the cell density of total bacteria was consistently higher at 55 °C compared to 35 °C. The viable bacteria are the dominant functional microorganisms, important evidence for anaerobic digestion stability and high methane production efficiency.

Fig. 4 illustrated the abundance of live bacteria with high and low DNA contents (live-HDNA, live-LDNA) and dead bacteria during AD of four different substrates at two different temperatures (35°C, 55°C). The results showed a higher cell density of bacteria in thermophilic AD than mesophilic AD, which is corresponding to an increased methane production at 55°C. The bacteria communities were dominated by live-LDNA bacteria (median percentage: 52.2%) followed by dead bacteria (median percentage: 28.0%). In general, bacteria have been broadly classified into low DNA content (LDNA) bacteria and high DNA content (HDNA) based on different stages of division and various numbers of plasmids (consisting of double-stranded DNA). The nucleic acid content can be reflected the differences of the individual cell fluorescence density signal in FCM [43]. Previous studies found that the HDNA cells were active and the LDNA cells were inactive and dead. The viable HNA cells were proved to have higher ATP-concentration than LNA cells [44], which also appeared to have

higher specific metabolic and growth rates than the LDNA cells [43]. On the contrary, several researchers [27, 42] have demonstrated that the LDNA bacteria could survive in oligotrophic conditions due to their high affinity and binding-protein ability. The LNA bacteria in aquatic environments have been isolated, which are found most of them belong to the β -proteobacteria cluster [45].

Dead bacterial population in four types of animal manure increased in the thermophilic AD system, especially in the orangutan group, which was about three times higher at 55°C. This could be due to the improved bacterial metabolism under high temperature conditions. For elephant manure, no obvious difference was observed for live bacteria of high and low DNA contents between the AD process at 35 °C and at 55 °C. Digestion of hippopotamus manure at 55°C resulted in more abundance of dead bacteria, live-LDNA and live-HDNA when compared to the digestion at 35 °C. In addition, significantly higher abundance of the three types of bacteria was observed at 55 °C than at 35 °C (one-sided *t*-test, $p < 0.05$) during the AD of orangutan manure with the highest total abundance of $2.8 \times 10^9 \pm 2.6 \times 10^8$ cells/ml at 55 °C. The increase of live-LDNA, live-HDNA and dead bacteria indicated that high temperature was beneficial to the AD process of omnivorous animal feces. The AD results of carnivorous animal manure of lion showed high live-LDNA bacteria abundance at high temperature but no differences in live-HDNA bacterial population was observed between mesophilic and thermophilic AD system. This implied that the live-LDNA bacteria which played crucial role as an active member of community dominating thermophilic AD system using carnivorous animal manure.

3.5 Contribution of HDNA and LDNA to dead bacteria

In this study, the abundance of HDNA was about three times of LDNA, demonstrating the major contribution of total HDNA bacteria in AD. We speculate that the high abundance of HDNA bacteria might come from the LDNA bacteria. In previous research, bacteria with low DNA content are considered inactive cells which were dormant form in oligotrophic conditions but started to fast replicate in nutrient-rich conditions and were detected as HDNA cells [27]. But increased temperature reduced the HDNA abundance of hippopotamus (omnivorous) and lion sample (carnivorous) without any significant change in elephant sample (herbivorous) and orangutan sample (omnivorous).

Further study was taken to explore the composition and type of the dead bacteria. The contribution of HDNA and LDNA to the dead bacterial population was presented in Fig. 5. HDNA predominated the dead bacteria (69.6-87.1%), independent of the two tested temperatures (35 °C, 55 °C, one-sided *t*-test, $p > 0.05$). The abundance of live bacteria shown in section 3.4 demonstrated that the live-LDNA bacteria content was higher than live-HDNA which could infer that HDNA bacteria were easy to die at thermophilic AD condition. This could be due to more sensitiveness of HDNA bacterial population to the changes of nutritional environment especially the levels of phosphates, nitrates, conductivity and manganese [45]. The proportion of HDNA dead bacteria rose significantly in lion and hippopotamus manure at high temperature and was marginally different from dead cells in elephant and orangutan manure. It was

observed that the contribution of HDNA to dead cells was unchanged with the increased temperature in AD of elephant manure. The highest contribution of HDNA to the dead bacterial population was observed for AD of orangutan manure. However, the orangutan and hippopotamus samples were both omnivorous animals, and the reasons for the difference in abundance of DNA after AD anaerobic digestion at elevated temperatures remain to be undisclosed and more studies are required to decode this.

3.6 Functional enzymes analysis

As a biocatalyst, the activities of enzymes are close reflection of rate of substrate degradation and methane production in the biological systems. This study analyzed five key enzymes in mesophilic and thermophilic AD of animal manure, including microbial coenzyme F420 (CoF420), microbial phosphorus dehydrogenase (DHO), acetate kinase, phosphotransacetylase (PTA) and microbial protease (POR).

Table 2 showed that the activity of the five examined enzymes in manure sample of lion was promoted at high temperature of 55°C. However, high temperature significantly inhibited CoF420 activities in samples H and OU of omnivorous, falling from 74.25 IU/L and 82.44 IU/L to 61.62 IU/L and 64.69 IU/L, respectively.

Moreover, the AK activity in E55 (45.18 mU/L) of herbivorous sample was lower than E35 (53.62 mU/L). The CoF420 is a specific coenzyme of methanogens in hydrogenotrophic pathway whose concentration can reflect the methanogenic activity of anaerobic granular sludge. In the process of carbon dioxide reduction, CoF420 play

an important role as an electron transfer ($\text{CoF420} + \text{Hydrogen} \leftrightarrow \text{Reduced CoF420}$) [46, 47]. As the reaction ($\text{acetyl phosphate} + \text{ADP} \leftrightarrow \text{acetate} + \text{ATP}$) shows, AK is the key to the conversion of acetyl coenzyme A (acetyl-CoA) into acetic acid. The phosphorus dehydrogenase was found to utilize the $\text{NAD}^+/\text{NADP}^+$ as electron acceptors for dehydrogenation ($6\text{-Phospho-D-gluconate} + \text{NAD(P)}^+ \leftrightarrow 6\text{-phospho-2-dehydro-D-gluconate} + \text{NAD(P)H} + \text{H}^+$) [46, 47].

DHO activities which could provide indications of cell metabolic activity [48] showed an increasing trend when temperature was raised. Especially in the L sample, the DHO activity increased by 140% from 150.59 IU/L to 211.5 IU/L compared with the other three samples.

Similarly, the activity of the PTA was increased in thermophilic AD system. It was especially in the H sample, rising from 20.68 to 27.61 U/mL, an increase of 134%. PTA is mainly used to determine the potency of coenzyme A (CoA) and the CoA plays a vital role in microbial metabolism. The acetyl-CoA was transferred to acetyl phosphate by PTA ($\text{acetyl-CoA} + \text{phosphate} \leftrightarrow \text{CoA} + \text{acetyl phosphate}$) and then the AK converted acetyl phosphate to acetate [46, 47].

POR participated in the conversion of CoA into acetyl-CoA ($\text{pyruvate} + \text{CoA} + 2 \text{ oxidized ferredoxins} \leftrightarrow \text{acetyl-CoA} + \text{CO}_2 + 2 \text{ reduced ferredoxins} + 2\text{H}^+$) [46, 47]. Moreover, POR is also important in the process of protein degradation [49] which facilitates protein catabolism by hydrolysis of the peptide bonds [50]. Temperature showed a promoting effect on protease concentration during AD, an improvement of 103-105% in the L, H and OU samples, and a large jump of 117% in the E samples,

[from 865.18 to 1016.03 ($\mu\text{g/ml}$)] was observed. The previous literature has reported higher degradation of proteins in thermophilic AD system to than mesophilic one [50, 51].

These results indicated the activities of DHO, PTA and POR of the four-animal manure in thermophilic AD system had been increased and thus enhanced the production of methane. Nevertheless, the AK activities of herbivorous animal manure and CoF420 activities of omnivorous animal manure were inhibited at high temperature, which may be well explained by different temperature optima of different enzymes.

3.7 Implications

AD has been used extensively in waste biomass treatment to produce biogas. The methane yield and stability of AD process are closely related to microbial conditions. The microbial community composition and abundance in mesophilic and thermophilic AD were well documented. However, the live microorganisms in the whole microbes community composition in AD process need to be further studied. Moreover, the functional enzymes in the mesophilic and thermophilic AD of animal manure and bacteria survival ratio should also be studied should be investigated.

This study investigated the proportion of live and dead bacteria in the mesophilic and thermophilic AD of animal manure by FCM. Fig. 6 illustrates the potential enzymes function in methanogenesis and viability of bacteria. Firstly, the two pathways of methane production in AD are hydrolytic methane production and acidification methane production under different kinds of enzymes. The five essential enzymes, microbial coenzyme CoF₄₂₀, microbial phosphorus dehydrogenase (DHO),

microbial protease (POR), acetate kinase (AK) and phosphotransacetylase (PTA) played an important part in methanogenesis, which was related to the synthesis of key intermediates. All four enzymes except CoF₄₂₀ are associated with the acidified methane-producing pathway. CoA forms acetyl-CoA under the action of POR, and then converts to acetyl phosphate by PTA. After AK realizes the final acidification to acetate, the acetate generated from this process and other formed by simple organic matter under the catalytic action of biological dehydrogenation of DHO together serve as the substrate of acetoclastic methanogens. Similarly, CoF₄₂₀ is considered to be a key electron carrier that plays a role in the process of hydrogenotrophic methane production, which determines the rate of utilization of hydrogen and carbon dioxide, and its concentration can be regarded as one of the indicators of methanogenic activity [26]. Secondly, live-LDNA bacteria are the major component of the microbial community, while live-HDNA accounts for less.

The activity of enzymes was improved in the thermophilic AD system which enhanced the methanogenesis, further increased biogas yield. On the other hand, the influence of thermophilic AD on live and dead cells and their metabolism was discussed, and it was supposed that there were two sources of dead bacteria, one from normal metabolism of bacteria, and the other from bacteria that could not adapt to the environment temperature changes and hard to grow. Due to the sensitivity of HDNA to high temperature, its survival rate was much lower than LDNA. Therefore, more in-depth studies should be carried out to explore the maintenance of HDNA survival rate while the enhancement of enzyme activity at high temperature. The interaction between bacteria viability and enzyme activity also required further study.

In AD, disturbances of the microbial metabolism could lead to process instability, and the dynamic changes of the microbial community may indicate AD

performance[52]. Thus, the evaluation of AD performance and microbial viability will improve the management and utilization of animal manure. It is important to enhance the efficiency of AD and converts animal waste to bioenergy, which is an essential component for achieving sustainable management of livestock.

4. Conclusion

This study investigated the effects of mesophilic and thermophilic conditions on microbial communities, the ratio of living and dead cells and enzyme activity during AD of four animal feces. Compared to mesophilic AD, thermophilic AD had a significantly higher methane yield. Also, the result demonstrated that carnivore manure from lion had higher methane production than that of herbivores (elephant), and omnivores (hippopotamus and orangutan) during the AD process. The dominant microbial species were distinctly different at mesophilic and thermophilic AD. Bacterial communities examined by FCM were dominated by live-LDNA bacteria. The increased temperature had a negative effect on the HDNA abundance of omnivorous hippopotamus and carnivorous lion sample. The activities of functional enzymes (DHO, PTA and POR) were significantly increased in thermophilic AD system of all animal manure.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

J. Z.: Supervision, Writing - original draft, Funding acquisition, Project administration, Resources, Methodology, Software, Writing - Review & Editing, Visualization, Validation. **J. C.:** Methodology, Writing - original draft, Data curation, Software, Writing - Review & Editing, Visualization, Validation. **R. M.:** Methodology, Data curation, Software, Writing - Review & Editing, Visualization, Validation. **V. K.:** Supervision, Writing - original draft, Formal analysis, Investigation, Writing - Review & Editing, Visualization, Validation. **Y. W. T.:** Resources, Conceptualization, Formal analysis, Investigation, Writing - Review & Editing, Visualization, Validation. **F. M.:** Supervision, Writing - original draft, Writing - Review & Editing, Project administration, Resources, Writing - Review & Editing, Visualization, Validation. **Y. H.:** Supervision, Investigation, Funding acquisition, Project administration, Resources, Writing - Review & Editing, Visualization, Validation.

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Table captions

Table 1. Characteristics of seed sludge and four types of animal manure

Table 2. Activity of functional enzymes in AD of the samples.

Figure captions

Fig. 1. (A) methane yield and (B) COD of four animals' manure under mesophilic and thermophilic conditions

Fig. 2. Species taxonomy at the (A) phylum and (B) genus level

Fig. 3. Non-metric multidimensional scaling (NMDS) diagram

Fig. 4. Abundance of dead, live-HDNA and live-LDNA bacterial population

(Hippopo.: hippopotamus)

Fig. 5. Contribution of HDNA and LDNA to the dead cells

Fig. 6. The viability of bacteria and potential function of enzymes in Methanogenesis

Table

Table 1. Characteristics of seed sludge and four types of animal manure

Substrate	Elemental Composition (%)				TS (%)	VS (%)
	C	H	N	S		
Elephant	39.52	5.34	1.49	<0.5	25.46	18.78
Hippopotamus	42.40	5.43	0.99	<0.5	15.48	14.55
Lion	28.61	4.45	3.85	<0.5	48.04	24.39
Orangutan	40.49	5.98	4.50	<0.5	24.76	17.59
Sludge	37.66	4.98	2.33	<0.5	1.51	1.21

Table 2. Activity of functional enzymes in AD of the samples.

Samples	CoF420 (IU/L)	DHO (IU/L)	AK (mU/L)	PTA (U/L)	POR (µg/ml)
L35	75.44	150.59	38.92	21.92	874.87
L55	77.15	211.50	40.58	23.21	902.60
H35	74.25	210.16	54.90	20.68	1082.76
H55	61.62	226.73	56.69	27.61	1141.44
OU35	82.44	187.76	51.83	23.82	879.71
OU55	64.69	208.82	62.19	24.28	924.15
E35	59.92	158.65	53.62	23.27	865.18
E55	64.86	185.52	45.18	25.46	1016.03

Figures

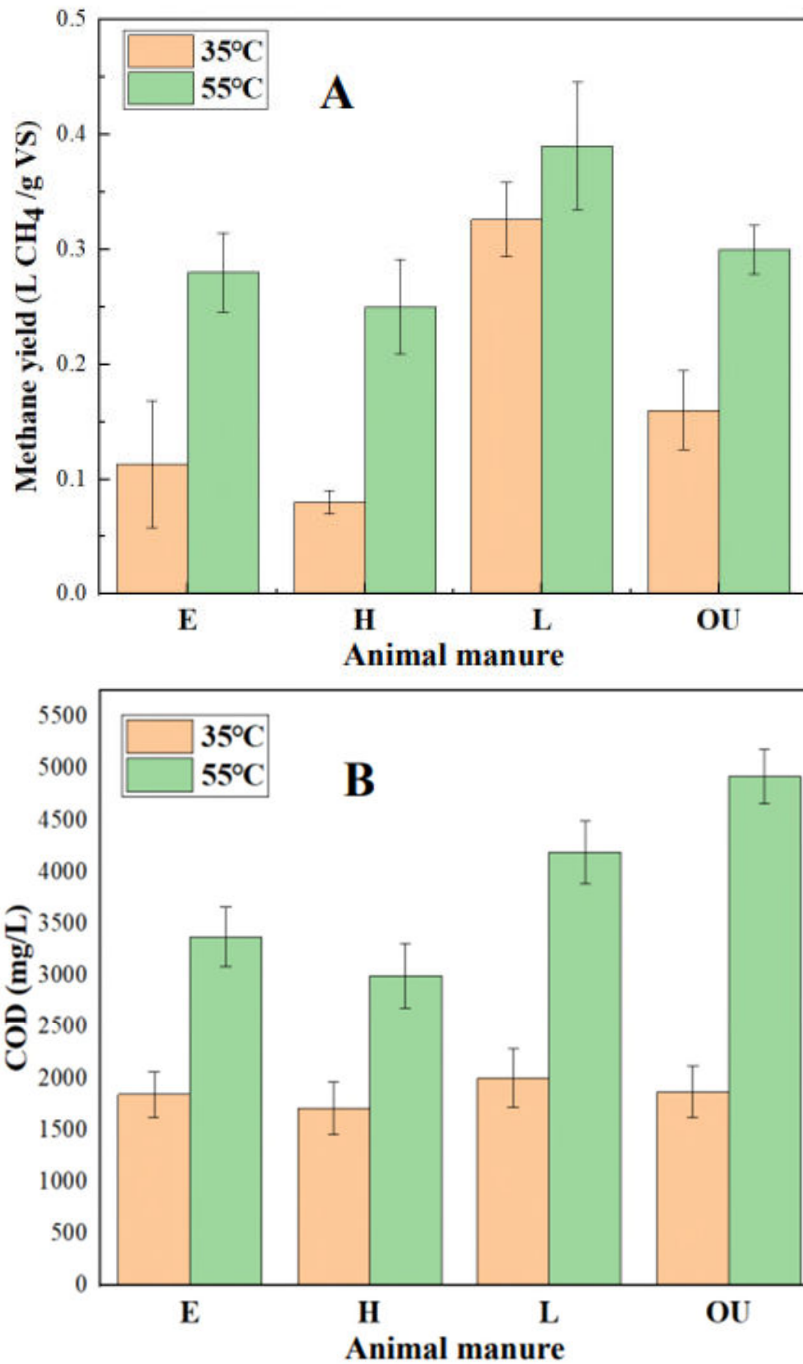


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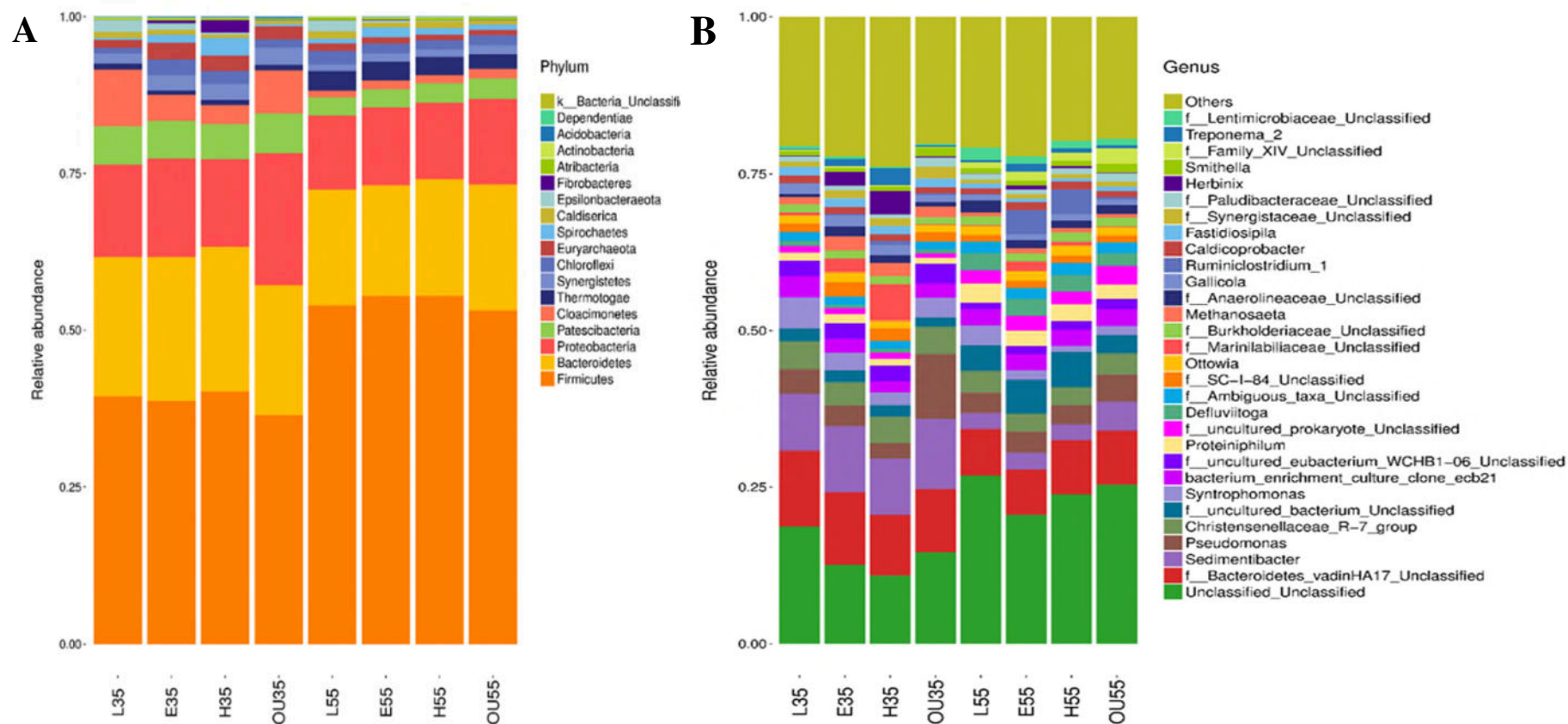


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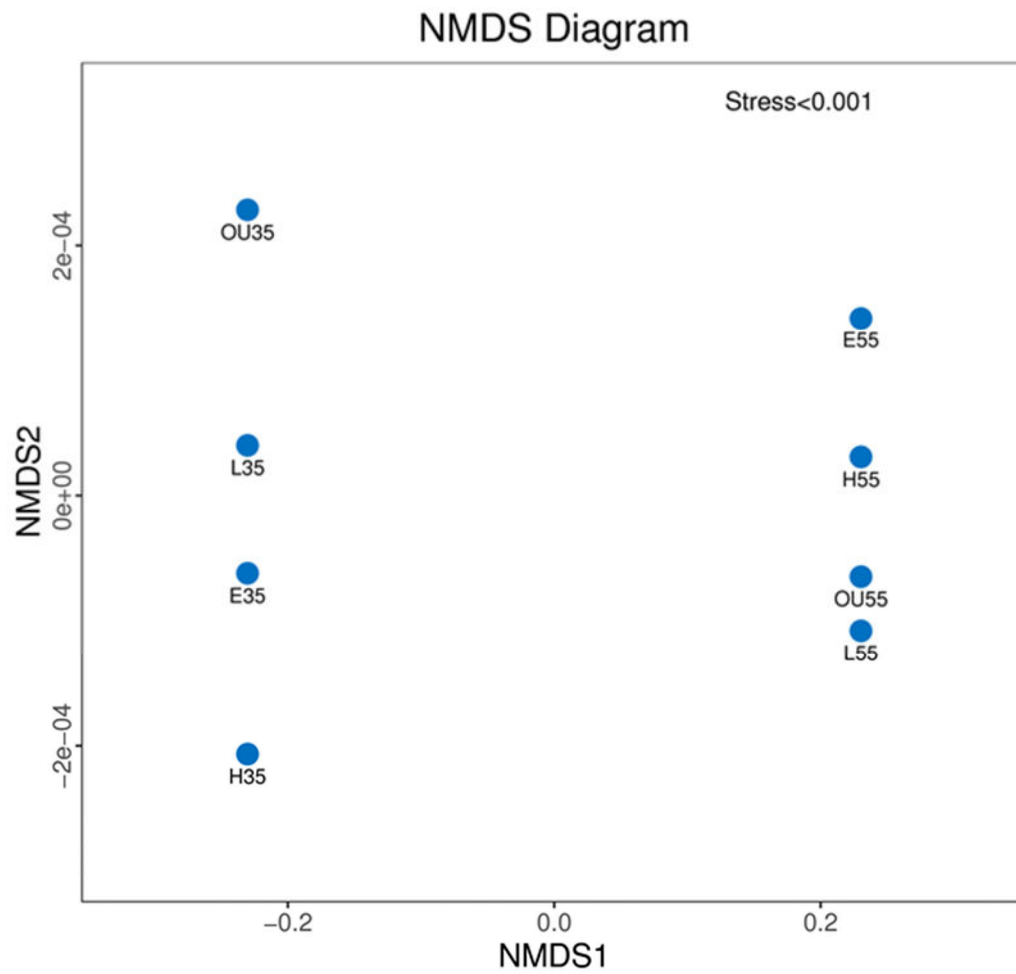


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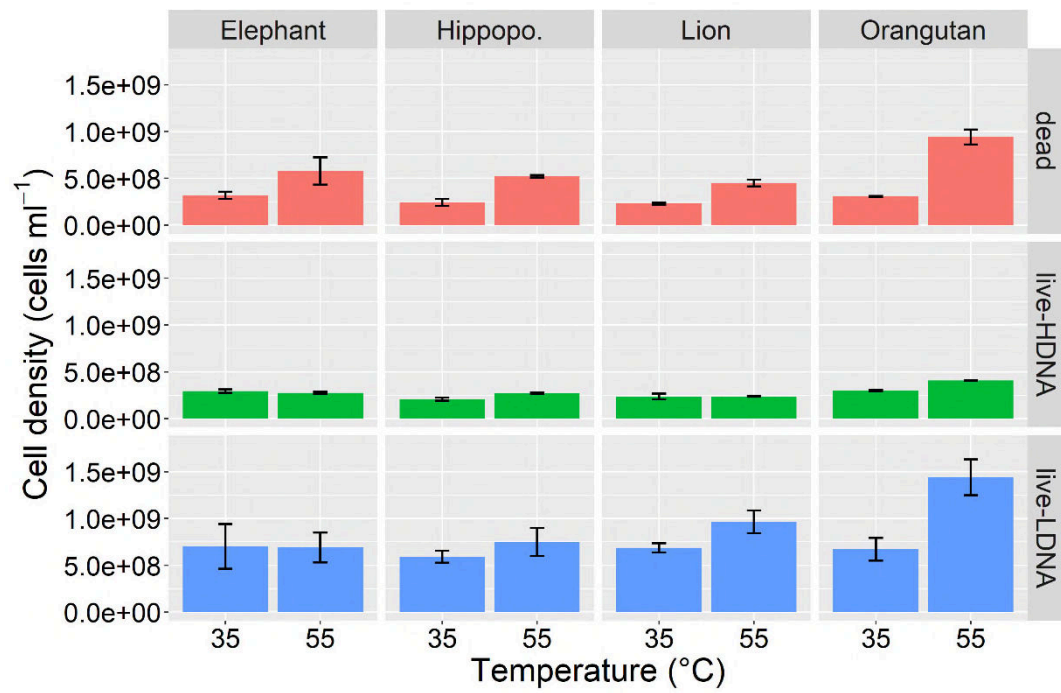


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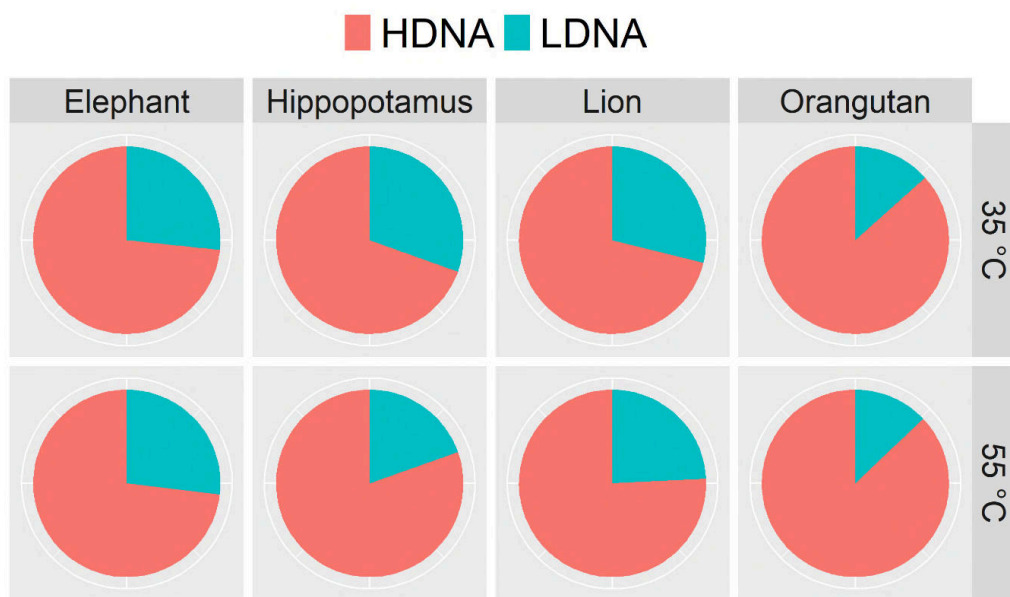


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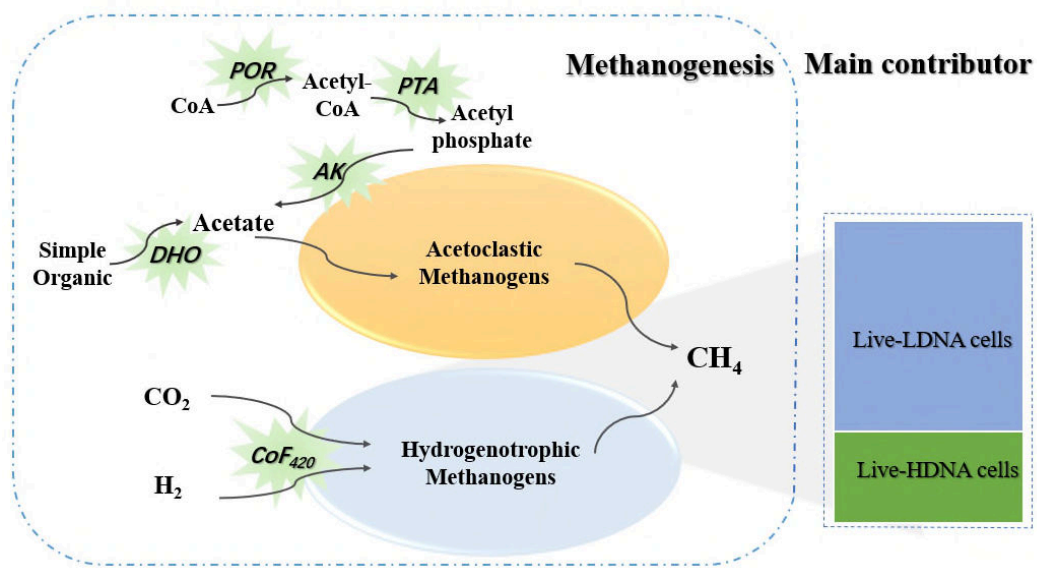


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