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Deeper insights into effect of activated carbon and nano-zero-valent iron addition on acidogenesis and whole anaerobic digestion

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Abstract: Conductive materials presented promising advantages for enhancing anaerobic digestion (AD) performance. This study evaluated the effects of activated carbon (AC) and nano-zero-valent iron (nZVI) on the acidogenesis and whole AD to explore their potential mechanisms. AC increased the content of lactic and propionic acids in acidogenesis. nZVI increased the production of formic acid, acetic acid and H_2 in acidogenesis, thus significantly promoted the methane yield in the whole AD. Mechanism exploration proved that AC enriched *Trichococcus*, and *norank_f__Bacteroidetes_vadinHA17*, and then improved the activity of enzymes involved in the production of lactic and propionic acids. nZVI buffered the pH to increase the activity of pyruvate formate-lyase (PFL) in formic acid production. Furthermore, nZVI enriched the *Methanobacterium* which use H_2 and formic acid as substrate. The research paves pathway for the efficient enhancement of conductive materials added novel AD process.

Key words: Anaerobic digestion, Activated carbon, Nano-zero-valent iron, Acidogenesis, Microbial community.

1. Introduction

Anaerobic digestion (AD) presented promising advantage in industrial and agricultural waste treatment via converting organic waste into biogas in presence of microorganism (Li et al., 2019). However, research showed that the process was still limited by low acidification efficiency and the accumulation of volatile fatty acids (VFAs) caused by slow syntrophic metabolism (Appels et al., 2011). Many studies have shown that adding conductive materials to anaerobic digesters can accelerate and

stabilize the conversion of organic matter to methane (Dang et al., 2016; Zhang et al., 2018). Previous research indicated that carbon-based materials including biochar, activated carbon (AC) and carbon cloth or iron-based material including nano-zero-valent iron (nZVI), magnetite and iron powder could promote the performance of AD (Lim et al., 2020; Zhou et al., 2019; Li et al., 2019; Meng et al., 2013). Nevertheless, the potential mechanism involving different conductive materials to improve the performance of different stages of AD remains unclear.

Carbon materials are always used directly as an electron carrier to improve AD efficiency by promoting electron transfer between syntrophic and methanogenic partners (Liu et al., 2012a). Zhao et al. (2017) reported that after supplementing granular activated carbon (GAC) in methanogenic phase, the methane production rate improved by about 34%. Moreover, with a honeycomb pore structure, large specific surface area and good adsorption performance, AC provides attachment sites for microorganisms and helps reduce the impact of organic shock loads on the methane production process (Aziz et al., 2011; Liu et al., 2012a). Also, it was reported that AC could be used directly as an electron carrier to improve AD efficiency by promoting electron transfer between syntrophic and methanogenic partners (Zhao et al., 2017; Dang et al., 2016). nZVI, as a reducer, has high specific surface area, which is also often used to enhance AD. Su et al. (2013) observed that adding 0.1% nZVI in anaerobic system increased CH_4 production by 40.4%. As a reducing substance, it could lower the ORP to provide a better anaerobic environment for methanogens (Liu et al., 2011). On the other hand, nZVI can produce hydrogen through chemical

corrosion (Eq. (1)), which improves the efficiency of hydrogenotrophic methanogenesis (Hao et al., 2017). The produced Fe^{2+} from nZVI is an important element to many oxidoreductases, which can improve the metabolic activity of microorganisms.

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + H_2 + 2OH^- \quad \Delta G^0 = -5.02 \text{ kJ/moL } Fe^0 \quad (1)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^0 = -131 \text{ kJ/moL} \quad (2)$$

$$8H^+ + Fe^0 + 4CO_2 \rightarrow CH_4 + 4Fe^{2+} + 2H_2O \quad \Delta G^0 = -150.5 \text{ kJ/moL } CH_4 \quad (3)$$

Previous studies have compared the mechanisms between different types of conductive materials that enhance AD. However, the potential mechanisms of the conductive materials in each stage of AD are also different. In general, AD mainly involves four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Firstly, the complex organic matter is hydrolyzed into simple organic matter by fermentation bacteria, and then these simple organics are converted into VFAs (lactic acid, acetic acid, formic acid, propionic acid, butyric acid and H_2/CO_2). VFAs can be converted into acetic and formic acids by acetogens, and finally they will be used by methanogens to produce methane in methanogenesis. However, there are few specifically produced substrates that can be used by methanogens, including H_2 (Eq. (4)), formic acid (Eq. (5)) and acetic acid (Eq. (6)). It can be seen that VFAs are important intermediates produced in the process of acidogenesis, acetogenesis and methanogenesis. Therefore, the concentration and composition of VFAs directly affects the efficiency of AD.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^0 = -135 \text{ kJ/moL} \quad (4)$$

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O \quad \Delta G^0 = -130\ kJ/moL \quad (5)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \Delta G^0 = -33\ kJ/moL \quad (6)$$

According to our knowledge, previous researches mainly focused on the influence of conductive materials on the performance of AD, especially for the methanogenesis. However, there has been less investigations into the potential connection between the acidogenesis and the methanogenesis. Xie et al. (2020) found that AC increased short-chain fatty acids production from algae during alkaline anaerobic fermentation. Furthermore, it was reported that nZVI promoted VFAs production, and acetic acid dominated in the batch system (Jin et al., 2019). AC improves the activity of key hydrolase enzymes and the number of coding genes. nZVI changes the community and metabolism of microorganisms by adjusting pH and reducing ORP. However, there was no consensus on how conductive materials affect the conversion of various products in the acidogenic phase. Additionally, the relationship between microorganisms and materials are still unclear.

According to the above, AC and nZVI were selected as conductive materials to deeply explore their effects on acidogenesis and whole AD in this study. One aim was to explore the effects of different conductive materials on the conversion of metabolic products during AD. Another objective was to dissect the microbial community structure and metabolic mechanism in response to the addition of conductive materials.

2. Material and methods

2.1 Substrates and inoculum

The granular sludge used as seed sludge was collected from the starch wastewater treatment plant in Xuzhou, China. The mixed liquor volatile suspended solid (MLVSS) of inoculum was 23.33 g/L, and mixed liquor suspended solid (MLSS) was 40.22 g/L.

In this experiment, artificial wastewater was used as the substrate. Glucose was the carbon source and the chemical oxygen demand (COD) concentration was 2000 mg/L. In addition, NH_4Cl and KH_2PO_4 were used as nitrogen source and phosphorus source, respectively. The ratio of COD: N: P in influent was 800: 5: 1. Trace metal solution and mineral elements were added to the reaction system to ensure the growth conditions of microorganisms. Their compositions were as follows: trace metal solution (mg/L): $FeCl_2 \cdot 4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2 \cdot 2H_2O$, 0.038; $MnCl_2 \cdot 4H_2O$, 0.05; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2 \cdot 6H_2O$, 0.05; $NiCl_2 \cdot 6H_2O$, 0.09; $Na_2WO_4 \cdot 2H_2O$, 0.05; mineral elements (mg/L): $CaCl_2$, 50; $MgCl_2 \cdot 6H_2O$, 100; NaCl, 100.

2.2 Batch Experimental design

2.2.1 Effect of conductive materials on acidogenesis

To elucidate the effect of materials on the acidogenesis, 2-bromoethane sulfonate (BES) was added with concentration of 30 mM at the beginning to inhibit the activity of methanogens. The experiment was performed in 120 ± 4 mL serum bottle with 40 mL of substrate and 10 mL of inoculum. For AC group, 0.25 g AC (10-15 mm, Sigma) was supplemented into the bottle. For nZVI group, 0.25 g nZVI (99.9% metals basis, 100 nm, Sigma) was added into the bottle. The group without materials addition

was used as blank. For each group, the experiment was performed in triplicates. In order to maintain strict anaerobic conditions, pure nitrogen (99.99%) was blown into the reactor for 15 minutes, and then the bottles were sealed with rubber stoppers. Finally, the reaction bottles were placed in a water bath shaker at 37°C and stirred at 160 rpm for 54 h. For chemical analysis, pH, VFAs and biogas components were measured at 6, 9, 12, 15, 18, 30 and 54 h.

2.2.2 Effects of conductive materials on whole AD

For the whole AD process, the experimental operation was consistent with the acidogenesis but without adding 30 mM 2-BES to the bottles. All of these experiments were conducted in triplicate. The bottles were incubated in a shaker at 37℃ and stirred at 160 rpm for 31 days, and the biogas composition was monitored every day.

2.3 Analytical methods

Gas composition were analyzed by gas chromatography (FULI, GC9790II, China) equipped with a TDX-01 (2 m long and 3 mm in inner diameter) as a separation column and argon as a carrier gas. The GC parameters were set as follows: column temperature, 120℃; detector temperature, 160℃; injection temperature, 160℃.

For the measurement of VFAs, the mixed solution was first centrifuged at 8000 rpm for 5 min, then filtered through a 0.22 μm filter, and the supernatant was acidified with 2 M HCl to a pH below 2.0, and then stored at 4°C. Finally, ion chromatography (ICS-3000, Dionex, USA) equipped with anion exchange column (Dionex IonPacTM

AS11) including analytical column (Dionex IonPacTM AS11-HC, 4 mm×250 mm) and protect column (Dionex IonPacTM AG11-HC, 4 mm×50 mm) were used for VFAs measurement. The MLSS, MLVSS, COD, pH and ORP values were determined according to Standard Methods (APHA, 2012).

The cumulative volume of CH_4 was calculated by multiplying the headspace volume (74 mL) by the percentage of CH_4 (mL CH_4/mL) in the headspace as determined by GC analysis. The gas sample taken from the batch reactor should take into account the pressure in the batch reactor, which should equilibrate in the batch reactor. Additionally, the obtained cumulative CH_4 production value was normalized according to the ideal gas law (PV = nRT) in Standard Temperature and Pressure (STP) conditions (0°C and 1 atm). The methane yield was expressed in NmLCH_4/g COD (Pan et al., 2019).

The scanning electron microscope and X-ray energy dispersive spectroscopy (SEM–EDS, S-4800, Japan) were used to determine morphology and chemical composition of the sludge after acidogenesis. At the end of acidogenic phase, 5 mL sludge sample was taken and centrifuged at 3000 rpm for 3 min, and then discard the liquid supernatant and fixed in 2.5% glutaraldehyde for 4 h at 4℃. Then the samples were washed twice times with 0.1 M phosphate buffer (pH=7.4), 10 minutes for each step. After that, samples were dehydrated through a graded ethanol series, by 50%, 70%, 80% and 90%, 10 minutes for each step and keep samples at 4℃. Then, it was dehydrated twice with 100% ethanol for 15 minutes each time. Finally, samples were dried by critical point drying method for 24 h.

2.4 Microbial community analysis

Sludge samples were collected from the original inoculum, 9 h and 54 h of the acidogenesis. In addition, the sludge samples were taken for microbiological analysis at the end of whole AD. All samples were refrigerated at -20°C for subsequent microbial analysis. DNA was extracted using Fast DNA Spin Kit for Soli (MP, USA) method. The DNA concentration was determined by Nanodrop2000 (Thermo Scientific, USA), and the purity was determined by measuring the absorbance ratio at 260/280 nm. The extracted DNA was stored at -20°C before measurement. The microbial communities of the sludge were analyzed by high-throughput sequencing on an Illumina MiSeq platform, which was performed by Majorbio Bio-Pharm Technology Co. Ltd (Shanghai, China). The primers used for PCR amplification of bacteria and archaea were 515Fmodf (GTGYCAGCMGCCGCGGTAA) and 806RmodR (GGACTACNVGGGTWTCTAAT), respectively, for the V4 region of 16S rRNA gene in each sludge sample.

Prediction of the metabolic function of bacteria and archaea in AD through PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was based on the Galaxy platform against the KEGG (Kyoto Encyclopedia of Gene and Genome) Orthology database (Langille et al., 2013). Specific steps are as follows: 1) The gene function spectrum of their common ancestor was inferred based on the full-length 16S rRNA sequence of the tested bacterial genome; 2) The gene function spectrum of other untested species in the Greengenes database was inferred, and the gene function prediction spectrum of the entire lineage

of archaea and bacteria was constructed; 3) The sequenced 16S rRNA gene sequence data was compared with the Greengenes database to found the "reference sequence nearest neighbor" of each sequencing sequence, and was classified as a reference OTU; 4) The obtained OTU abundance matrix was corrected according to the rRNA gene copy number of the "reference sequence nearest neighbor"; 5) The KEGG Orthology (KO) information corresponding to the OTU was obtained through the greengene id corresponding to each OTU; 6) According to the information in the KEGG database, KO information can be obtained, and the abundance of each functional category can be calculated according to the OTU abundance.

2.5 Calculation

Acidification efficiency (AE, %) was evaluated by the following Eq. (1):

$$AE = \frac{COD_{VFAs}}{COD_{Influent}} \times 100\% \quad (1)$$

Where $COD_{Influent}$ is the concentration of influent COD (mg/L), COD_{VFAs} is the concentration of effluent VFAs (mgCOD/L) (Lu et al., 2012).

2.6 Statistical analysis

Statistical analysis was conducted using EXCEL 2016 and SPSS 26 software. In addition, Origin 8.0 was used for graphics drawing. Free online platform (http://www.i-sanger.com/) was Majorbio I-Sanger Cloud platform. The operational taxonomic unit (OTU) table was normalized, and the functional genes in AD were analyzed by the KEGG Orthology (KO) (http://www.kegg.jp/kegg/ko.html). A p value of <0.05 indicates statistical significance.

3. Results and Discussion

3.1 Effect of conductive materials on VFAs, pH and gas content in acidogenesis

The composition of VFAs and biogas content in each group were measured (Fig. 1). As shown in Fig. 1a-b, the main products were VFAs (including lactic acid, formic acid, acetic acid and propionic acid), and H_2/CO_2 after blocking the methanogenesis. In the initial 9 h, the pH of blank, AC and nZVI groups dropped sharply from 8.2, 8.1 and 8.1 to 6.2, 6.0 and 6.7 (Fig. 1c), respectively. It indicated that glucose was quickly converted into acidic products after experiments. The lactic and formic acids both accumulated in the three groups, while acetic and propionic acids showed small generation. Lactic acid increased by 22.05% in AC group, but there has been little change in nZVI added group. The formic acid in the three groups reached the maximum value at 9 h with 169.57 mg/L, 192.95 mg/L and 193.99 mg/L, respectively. Formic acid increased by 13.79% and 14.40%, in AC and nZVI added systems, separately. This result indicated that AC mainly promoted the production of lactic acid, while nZVI mainly promoted the formic acid generation in the early stage. After 9 h, acetic acid and propionic acid gradually accumulated in the reactors. The acetic acid content of the blank, AC and nZVI groups at 54 h were 513.60 mg/L, 531.55 mg/L and 613.50 mg/L, respectively. Acetic acid increased by 19.45% in nZVI added systems, which was consistent with the previous research (Liu et al., 2012b). Interestingly, the lactic acid and formic acid were completely consumed at the end of reaction.

Lactic acid was the initial product of anaerobic fermentation. In the metabolism of mixed bacteria, lactic acid was first produced through catalytic reduction of

pyruvate with lactate dehydrogenase. Afterwards, lactic acid was reduced to propionic acid by propionate dehydrogenase (Lee et al., 2008). At 54 h, the contents of propionic acid in the blank, AC and nZVI groups were 317.99 mg/L, 343.93 mg/L and 310.28 mg/L, respectively. Due to promotion in lactic acid generation by AC, it's the propionic acid reached the highest concentration at the end of the reaction. On the other hands, ORP also affects fermentation products. The ORP of the blank, AC and nZVI groups at the end of the reaction were -310 mV, -264 mV, and -335 mV, respectively. Wang et al. (2006) reported that propionic acid was the main product at ORP of −280 mV. As AC improved the ORP of the system, the production of propionic acid was also increased. It was noteworthy that the proportion of propionic acid dropped from 37.02% to 32.93% after adding nZVI. Since the fermentation products in the anaerobic system were affected by ORP, the addition of nZVI to reduce ORP may promote the conversion of propionic acid. Meng et al. (2013) reported that the reduced ORP after adding ZVI can effectively increase the activity of dehydrogenase, so the conversion rate of propionic acid increases from 43-77% to 67-89%.

Formic and acetic acids are substrates that can be utilized by methanogens, and formic acid is more easily used by methanogens than acetic acid in granular sludge (Pan et al., 2016). The results showed that formic acid mainly accumulated in the initial 9 h, while acetic acid was the dominant product at the end of the reaction. This may be due to formic acid is not stable and is easily cracked into H_2 and CO_2 by formate dehydrogenase (FDH) enzymes (Crable et al., 2011). Then H_2/CO_2 can be

converted into acetic acid as a substrate for homoacetogenic bacteria. As shown in Fig. 1b, the H_2 content in nZVI group was higher than that of AC and blank groups. After adding nZVI, a large amount of H_2 was generated in the system. At 54 h, CO_2 in the nZVI group was converted into acetic acid by reacting with H_2, and only little CO_2 was remained in nZVI group. Therefore, the content of acetic acid in the nZVI group was the highest at the end of the reaction. Obviously, it suggested that adding nZVI significantly promoted the generation of VFAs. This was consistent with the previous research results, which obtained the maximum VFAs content after adding 30 mM nZVI (Zhou et al., 2020). At the end of reaction, the acidification rates in the blank, AC and nZVI groups were 53.64%, 56.21% and 57.77%, respectively. Hence, adding nZVI in AD could promoted the conversion of more organic matter into VFAs and increase the substrates (H_2, formic acid and acetic acid) for methanogens.

Fig. 1

3.2 Effects of conductive materials on methane production in whole AD

Methane yield is an important indicator in reflecting the efficiency of AD. As shown in Fig. 2, the methane yield in AC added group was 241.18 mL CH_4/g COD, which was lower than that of blank group of 250.11 mL CH_4/g COD. This result was different from the previous research that adding GAC could promote methane yield in methanogenesis by stimulating direct interspecies electron transfer (DIET) (Zhao et al., 2017). This probably resulted from that AC mainly promoted the production of lactic and propionic acids in the acidogenic phase, while DIET was not formed to accelerate the syntrophic conversion of propionic acid to methane in this study.

277 Interestingly, the cumulative methane yield in nZVI added group was 309.89 mL
278 CH_4/g COD, which was significantly higher than that of the blank group. The
279 methane yield of nZVI added group increased by 23.9%, suggesting nZVI addition
280 could promote methane production. This could be mainly resulted from the following
281 three aspects. Firstly, nZVI created an environment conducive to the growth of
282 methanogens (Zhou et al., 2019). After the reaction, the pH value in nZVI group
283 (8.48) was higher than that of the AC group (6.87) and the blank group (6.88) (Table
284 1). The ORP value in nZVI group was -345 mV which was much lower than that of
285 AC (-197 mV) and blank group (-206 mV) (Table 1). Previous research also reported
286 that nZVI addition could lower the ORP value, providing a reductive condition (< -
287 200 mV) for methanogens (Liu et al., 2011). Secondly, Fe^{2+} released by the corrosion
288 of nZVI under anaerobic conditions (Eq. (1)) was an important element of various
289 redox enzymes. Hence, adding nZVI could improve the metabolic activity of
290 microorganisms (Wang et al., 2016). Thirdly, adding nZVI increased the available
291 substrates for methanogens (Feng et al., 2014). More formic acid was produced in
292 acidogenic phase, which could be used as a substrate for H_2-utilzing methanogens. In
293 addition, the H_2 produced by nZVI corrosion of in the early stage could further
294 convert CO_2 into CH_4 (Eqs. (1), (2)). However, due to the high Gibbs free energy of
295 the process of releasing H_2 through the corrosion of nZVI (Daniels et al., 1987), it was
296 necessary for H_2-consuming microorganisms to continuously consume hydrogen to
297 pull the corrosion of nZVI and keep the hydrogen partial pressure at a low level.
298 Additionally, iron can be used as an extracellular electron donor to participate in the

299 electron transfer of microbial metabolism (Newman & Kolter., 2000). nZVI also can
300 be used as a direct electron donor for methanogens (Eq. (3)) to accelerate the
301 corrosion of nZVI for methane enhancement (Karri et al., 2005).

302 **Fig. 2**

303 **Table 1**

304 ***3.3 Microbial community analysis***

305 ***3.3.1 Effects of conductive materials on microbial community in acidogenesis***

306 The microbial community in acidogenesis process was investigated and shown in
307 Fig 3a-b. In phylum level (Fig. 3a), *Firmicutes*, *Bacteroidetes* and *Chloroflexi*
308 dominated in conductive materials added conditions. These three phyla were also
309 reported to be common hydrolysis fermentation bacteria in solid waste, wastewater
310 and sludge treated by AD (Zhang et al., 2018). In addition, they can convert organic
311 matter into VFAs, following acetic acid and H_2/CO_2 generation. *Firmicutes* can
312 greatly promote the degradation of organic matter by proteases, cellulases and other
313 extracellular enzymes (Chen et al., 2017). *Bacteroidetes* is also a common hydrolysis
314 and fermentative bacteria in anaerobic systems, which can convert organic matter into
315 short-chain fatty acids (Chouari et al., 2005). Moreover, the bacteria in *Chloroflexi*
316 can degrade complex organic compounds (Roest et al., 2005). At 9 h, the total
317 abundance of the three bacterial phyla in the blank, AC and nZVI groups increased
318 from the original 60.26% to 77.94%, 83.72% and 81.52%, respectively, which were
319 consistent with the fermentation products. Correspondingly, AC added group obtained
320 the highest VFAs concentration, followed by nZVI and blank group. At 54 h, the

relative abundance of *Firmicutes* significantly increased in AC and nZVI groups with 44.35% and 43.70%, respectively. However, the *Bacteroidetes* decreased sharply in the nZVI group (13.63%), and lower than the AC group (26.45%) and the blank group (27.56%). The *Chloroflexi* decreased sharply in the AC group (10.85%) compared to the blank (21.21%), while it was barely influenced by nZVI addition (20.68%).

The bacterial community is closely connected with the metabolites formation (VFAs), which can further affect the methanogenesis process. At genus level (Fig. 3b), the dominant bacteria in the three groups were *Trichococcus* (phylum *Firmicutes*), *norank_f__Bacteroidetes_vadinHA17* (phylum *Bacteroidetes*) and *norank_f__Anaerolineaceae* (phylum *Chloroflexi*)). *Trichococcus* is common acid-producing bacteria, which mainly use glucose to produce lactic acid, formic acid, acetic acid, and CO_2 (Wang et al., 2018b). *norank_f__Bacteroidetes_vadinHA17* is reported to degrade glucose into acetic acid, propionic acid and H_2/CO_2 (Tan et al., 2010; Ueki., 2006). Moreover, *norank_f__Anaerolineaceae* can convert propionic acid into acetic acid (Vrieze et al., 2015). At 9 h, *Trichococcus* increased significantly compared with initial condition, which maybe resulted in the increase of lactic and formic acids (Fig. 1a). Furthermore, it was obvious that AC and nZVI addition enriched *Trichococcus* compared to the blank group, leading to the higher concentrations of lactic and formic acids. As a material with strong adsorption, high porosity and high surface area, AC is conducive to the adhesion of microorganisms (Aziz et al., 2011). Whereas, the enrichment of *Trichococcus* in nZVI-added system may be due to the buffered pH. Wang et al. (2018b) reported that *Trichococcus* was

common among neutral pH bacteria. In three groups, compared to 9 h, the relative abundance of *Trichococcus* and *norank_f__Bacteroidetes_vadinHA17* decreased at 54 h due to the consumption of substrates. However, the relative abundance of *norank_f__Anaerolineacea* increased due to the production of propionic acid during the reaction. The variation of VFAs was closely related to the microbial community. At 54 h, it was showed that the content of lactic and formic acids decreased, while the content of propionic and acetic acids increased significantly. Remarkably, nZVI addition reduced the abundance of *norank_f__Bacteroidetes_vadinHA17*, while enriched *norank_f__Anaerolineacea*, which indicated that nZVI may promote the conversion of propionic acid. nZVI rapidly reduced the ORP, and the released Fe^{2+} could promote sludge flocculation to form particles. Moreover, most of the *norank_f__Anaerolineacea* are strictly anaerobic multicellular filamentous microorganism. The flocculation of sludge can avoid destroying the structure of bacterial filaments during the stirring process, which is more conducive to the growth of *norank_f__Anaerolineacea* (Zhou et al., 2020). Therefore, at the end of the acidogenesis, the acetic acid content in the nZVI group was the highest and the propionic acid content was the least. Furthermore, it was worth noting that *Clostridium* (phylum *Firmicutes*), increased from 0.34%, 0.43% and 0.13% at 9h to 1.45%, 2.05% and 0.42% at 54h in blank, AC and nZVI, respectively. As the most common homoacetogen (Drake et al., 2006), the growth of *Clostridium* indicated that H_2 and CO_2 were used as substrates by through homoacetogenesis to generate acetic acid in the late stage of the reaction.

Fig. 3

3.3.2 Effects of conductive materials on the microbial community in whole AD

The relative abundances of methanogens at phylum and genus levels in each group were shown in Fig. 3c-d. At phylum level (Fig. 3c), *Euryarchaeota* was the main phyla in the blank, AC and nZVI groups (counting for 97.96%, 98.58% and 99.19%, respectively). At the genus level (Fig. 3d), *Methanosaeta* and *Methanobacterium* dominated in three groups. *Methanosaeta*, the well-known acetate-utilizing methanogens, accounted for 56.02% in nZVI group which was lower than that in AC group (58.52%) and blank group (64.03%). As typical hydrogen-utilizing methanogens, the abundances of *Methanobacterium* were 19.83%, 30.44% and 39.44% in the blank, AC and nZVI groups, respectively. Compared with blank, the increase of relative abundance of *Methanobacterium* in AC group was resulted from the enrichment of acid-producing bacteria to produce more H_2 in the early stage after AC addition. Chen et al. (2014) reported that GAC could accelerate the syntrophic conversion of VFAs to methane via DIET in defined co-cultures of *G. metallireducens* and *Methanosarcina barkeri* as well as in some mixed cultures (Zhao et al., 2015). However, the abundances of *Methanosaeta* and *Methanosarcina* in the AC group decreased compared to the blank group, while the abundance of *Methanobacterium* increased significantly. Thus, DIET was not formed to promote methane production in this study. Therefore, AC did not show a promoting effect in the whole AD process. Particularly, the significant increase of *Methanobacterium* in the nZVI group may be due to the generation of hydrogen from nZVI addition (Fig.

2). Moreover, nZVI promoted the production of formic acid and the increase of H_2 content during acidogenic phase (Fig. 1), which supplied substrate for *Methanobacterium*. Similarly, more methane generation in the nZVI group maybe resulted from the higher abundance of *Methanobacterium.* In the nZVI group, the content of H_2 and formic acid significantly increased in the early stage, while the accumulation of acetic acid occurred in the later stage of the acidogenesis. However, H_2 and formic acid were thermodynamically easier to be used by methanogens than acetic acid (Eqs. (4)-(6)). Therefore, the increase in H_2 and formic acid content in the early stage provided a suitable substrate for hydrogenotrophic methanogens.

3.4 Characteristics of conductive materials and mechanism analysis on AD

3.4.1 Impacts of conductive materials on sludge characteristics

SEM observation showed that the interior and surface of the sludge in the three groups were dominated by bacillus and cocci. The microorganisms in the blank group were scattered on the surface of the sludge. Interestingly, a pore structure was formed after the addition of AC, so bacillus and cocci were adsorbed on the inside and surface of the sludge. Remarkably, the microorganisms in the nZVI group were tightly connected and wrapped. Zhang et al. (2011) reported that the cocci-shaped on the surface of sludge in ZVI packed reactor was related to the acidogens. Due to the electrostatic interaction and high specific surface area, nZVI was adsorbed on the cell surface. Therefore, the elemental iron on the cell surface increased significantly after nZVI addition. The released Fe^{2+} might penetrate into the microbial cells and participate in the synthesis of key enzymes.

3.4.2 The relationship between bacteria and environmental variables

Redundancy analysis (RDA) was carried out to explore the correlation between 6 environmental factors and the 3 main bacterial communities (*Trichococcus, norank_f__Bacteroidetes_vadinHA17 and norank_f__Anaerolineaceae*) in the acidogenesis process. As shown in Fig. 4, the distribution of the samples changed significantly, and the 7 samples were well separated in groups. The original sample was separated from the blank, AC and nZVI groups. The results showed that great difference occurred between different groups. Obviously, lactic acid and formic acid were clustered closely, and acetic acid and propionic acid were also positively correlated. This confirmed the accumulation of lactic acid and formic acid in the early stage of acidogenesis, while the accumulation of acetic acid and propionic acid in the later stage. In addition, pH and VFAs (lactic acid, formic acid) showed a strong negative correlation, indicating that the accumulation of lactic acid and formic acid in the early stage of acidogenesis rapidly reduced the pH. Lactic acid showed a strong positive correlation in the AC group (Fig. 4), indicating that AC had a significant promoting effect on lactic acid. Remarkably, the two groups with nZVI added were positively correlated with VFAs. These proved that conductive materials play a leading role in promoting the hydrolysis and acidogenesis of organics. In addition, RDA results can also reveal the distribution of microbial communities. *Trichococcus* was positively correlated with formic acid, acetic acid and H_2. At 9 h, compared with the original sludge, the abundance of *Trichococcus* in the three systems all increased significantly, so the content of fermentation products also increased accordingly. As

431 expected, *norank_f__Anaerolineaceae* showed a positive correlation with acetic acid.
432 Conversely, *norank_f__Bacteroidetes_vadinHA17* did not have a positive correlation
433 with propionic acid. It was speculated that the conversion of VFAs was not only
434 related to the abundance of microorganisms, but also related to the metabolic activity
435 of microorganisms. In addition, AC was located near the *Trichococcus* and
436 *norank_f__Bacteroidetes_vadinHA17*, indicating that AC had a positive effect on
437 thier growth. Furthermore, *Trichococcus* and *norank_f__Anaerolineaceae* were
438 distributed closely to nZVI, indicating that adding nZVI was beneficial to their
439 growth. The RDA analysis showed that the bacterial communities were affected by
440 AC and nZVI which explains the different performance in the AC and nZVI groups.
441 **Fig. 4**

442 ***3.4.3 Mechanism analysis of conductive materials on AD***

443 In the acidogenesis period, AC promoted the production of lactic and propionic
444 acids, while nZVI promoted the production of formic and acetic acids. In order to
445 reveal the promotion mechanism of AC and nZVI, based on the function prediction
446 analysis, the related functional enzyme-encoding gene in the blank, AC and nZVI
447 groups were analyzed. As shown in Fig. 5, the addition of materials enhanced the
448 activity of key enzymes compared to the blank group. The abundance of encoding
449 genes of lactate dehydrogenase, propionate CoA-transferase, butyryl-CoA
450 dehydrogenase and propionyl-CoA synthetase related to lactic and propionic acids
451 increased in the AC group, indicating that AC promoted the production of lactic and
452 propionic acids. Due to the adsorption and porous structure of AC, acid-producing

453 bacteria were enriched in the anaerobic system. Hence, the lactic acid content in the
454 AC group was the highest (Fig. 1a). Subsequently, as an intermediate product in the
455 process of reducing pyruvic acid to propionic acid, lactic acid was consumed by
456 producing propionic acid. The conversion from pyruvate to lactic and propionic acids
457 requires the consumption of NADH. It was reported that ORP can influent
458 intracellular NADH/NAD^+ ratio, which in turn affect enzyme activity and metabolites
459 (Liu et al., 2013). Tian et al. (2015) reported that when ORP was -250 mV, the highest
460 NADH appeared in the system. At the end of the reaction, the ORP value in the AC
461 group was -264 mV, so AC appropriately increased the ORP value of the system and
462 promoted the production of NADH. Therefore, the activity of the enzyme was
463 enhanced.

464 In addition, the products of concern were formic acid, acetic acid and H_2/CO_2,
465 which were substrates available for methanogens. In the absence of oxygen, pyruvate
466 can be catalyzed by pyruvate–ferredoxin oxidoreductase (POR) to acetyl-CoA and
467 CO_2, or by pyruvate formate-lyase (PFL) to formate and acetyl-CoA (Knappe et al.,
468 1974). In the initial 9 h, formic acid in the three groups accumulated to the maximum
469 value. At this time, the prediction of functional enzyme-encoding genes showed that
470 the abundance of PFL-encoding genes in the three groups increased significantly
471 compared to original sludge. The blank, AC and nZVI groups increased by 0.49-fold,
472 1.74-fold, and 1.94-fold, respectively. Due to nZVI decreased ORP, it provided a
473 favorable environment for the growth of acid-producing bacteria (Luo et al.,2014). On
474 the other hand, mainly enzymes contain iron-sulfur reducing clusters, the Fe^{2+}

475 released after the addition of nZVI could greatly increase the activity of the enzyme
476 (Yang & Wang, 2018). Also, it was reported that the optimum pH for PFL was 7.0
477 (Pecher et al., 1982). Therefore, nZVI buffered the pH of the system and provided a
478 more suitable condition for the expression of PFL. Consistently, the formic acid
479 production in the nZVI group was the highest (Fig. 1a). Whereas, when
480 methanogenesis was inhibited, formic acid can be cleaved into H_2/CO_2 by formate
481 dehydrogenase, and H_2/CO_2 can be converted into acetic acid via the Wood-
482 Ljungdahl pathway. At 54 h, the abundance of genes encoding formate dehydrogenase
483 and metheyltransferase increased slightly compared to 9 h, while other enzyme-
484 encoding genes have declined. Therefore, the results showed that in batch systems,
485 formic acid was mainly produced in the early stage, and acetic acid was generated in
486 the later stage of acidogenesis. The addition of nZVI could release more H_2, and the
487 increase of H_2 accelerated the consumption of CO_2 and promoted the homoacetogenic
488 process. Therefore, at the end of the reaction, CO_2 was completely consumed in the
489 nZVI group, and the acetic acid was higher than that of the other two groups.
490 From the perspective of the methanogenesis process, nZVI had a significant
491 promoting effect on the production of methane. The substrates produced in the
492 acidogenic phase that can be utilized by methanogens were mainly acetic acid, formic
493 acid and H_2/CO_2, which are mainly involved two methanogenic pathways, including
494 acetoclastic and hydrogenotrophic pathways. Obviously, 5-methyl-THMPT is a
495 common intermediate for two methanogenesis pathways, catalyzed by acetyl-CoA
496 decarbonylase during acetate-dependent methanogenesis and 5,10-

497 methylenetetrahydromethanopterin reductase during CO_2-dependent methanogenesis
498 (Wang et al., 2018a). In the process of acetoclastic methanogenesis, acetic acid is
499 finally converted into CH_4 and CO_2 by the action of various enzymes such as acetyl-
500 CoA decarbonylase, tetrahydromethanopterin S-methyltransferase and methyl-
501 coenzyme M reductase. In the hydrogenotrophic methanogenesis, H_2/CO_2 or formic
502 acid requires multiple steps to be converted into methane. CO_2 is gradually converted
503 into formyl, methylene and methyl groups under the action of various enzymes, and
504 then into methane (Guo et al., 2015). Moreover, the formic acid produced in the
505 acidogenic stage can also be used as a substrate for methanogens, but free formic acid
506 cannot be directly used by methanogens. Formic acid is first oxidized to CO_2 by FDH,
507 and accompanied by the production of $F_{420}H_2$, and then converted into methane
508 through the CO_2 reduction pathway (Crable et al., 2011). As shown in Fig. 5, after the
509 addition of nZVI, encoding gene of the FDH that catalyzed the conversion of formic
510 acid oxidation and CO_2 reduction had a higher abundance. Also, the abundances of
511 the encoding genes of functional enzymes for the hydrogenotrophic pathway was
512 richer than acetoclastic pathway. It indicated that nZVI promoted the production of
513 formic acid in the acidogenic phase, which could provide a rich substrate for
514 hydrogen-utilizing methanogens. The addition of nZVI greatly enhanced the
515 hydrogenotrophic pathway, and the hydrogen in the system may be produced by the
516 chemical corrosion of the nZVI or produced in acidogenesis process, which was
517 consistent with the results of Feng et al. (2014). Besides, nZVI can be used as a direct
518 electron donor to reduce CO_2 to methane.

519 **Fig. 5**

520 **4. Conclusions**

521 The present study systematically investigated the effect of AC and nZVI addition
522 on acidogenesis and whole anaerobic digestion process. AC enriched the
523 *Trichococcus* and *norank_f__Bacteroidetes_vadinHA17* by its pore structure,
524 indicating that AC promoted the production of lactic and propionic acids in
525 acidogenic phase. nZVI significantly improved the activity of functional enzymes by
526 buffering pH and releasing Fe^{2+}, thereby increased the production of formic acid in
527 acidogenic phase. In addition, nZVI enriched the hydrogenotrophic methanogens in
528 whole AD for higher CH_4 yield. Thus, adding nZVI is a promising strategy to increase
529 both acidogenesis and whole AD process for higher efficiency.

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688 **Credit Author Statement**

689 **Ruming Wang:** Conceptualization, Methodology, Formal analysis, Investigation, Data
690 curation, Writing-original Draft. **Chunxing Li:** Resources, Formal analysis, Validation,
691 Writing-review & editing. **Nan Lv:** Supervision, Validation. **Xiaofang Pan:** Resources,
692 Funding acquisition, Supervision. **Guanjing Cai:** Reviewing and Editing. **Jing Ning:**
693 Supervision, Formal analysis. **Gefu Zhu:** Project administration, Funding acquisition,
694 Supervision.

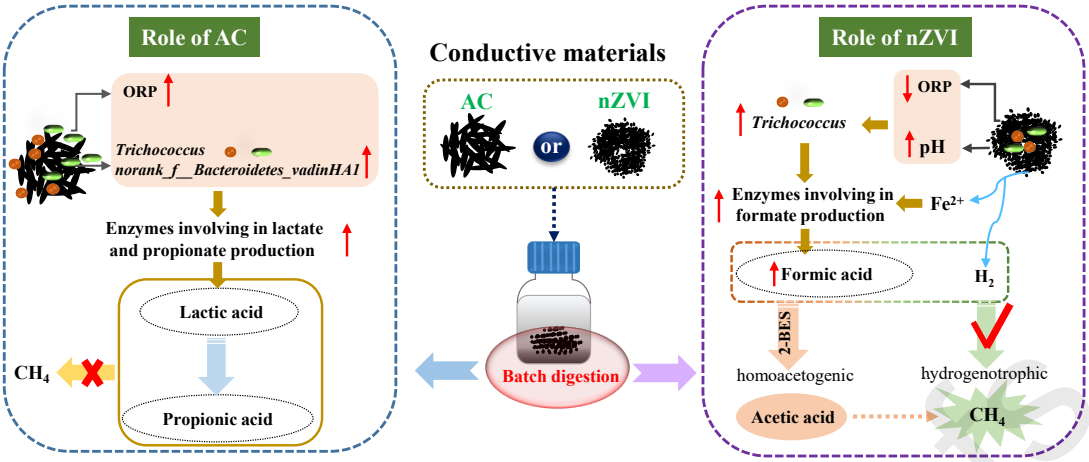
50.

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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

51.



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711 52.

712 **Figure Captions**

713 **Fig. 1** Variation in VFAs (a), gas content (b) and pH (c) during the acidogenesis.

714 **Fig. 2** Methane yield in blank, AC and nZVI added groups.

715 **Fig. 3** The distribution of bacterial communities at phylum level (a) and genus level

716 (b) in acidogenesis, and the comparison of archaea community structures of three

717 groups at phylum level (c) and genus level (d) in whole AD.

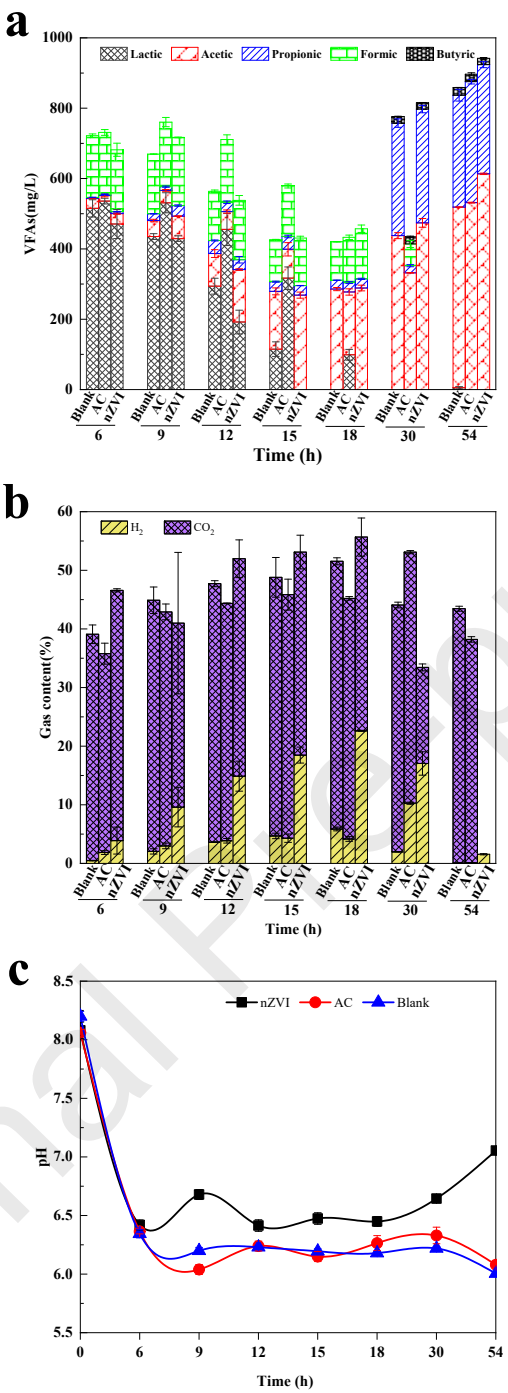
718 **Fig.4** The connection between different samples, bacterial communities and

719 environmental factors.

720 **Fig. 5** The abundance variations of relevant functional enzyme-encoding genes

721 involved in the pyruvate metabolism between acidogens and methanogens at three

722 groups.


a
Lactic
Acetic
Propionic
Formic
Butyric
VFAs(mg/L)
Blank
AC
nZVI
6
9
12
15
18
30
54
Time (h)
b
H2
CO2
Gas content(%)
Time (h)
c
nZVI
AC
Blank
pH
Time (h)

724

Fig. 1

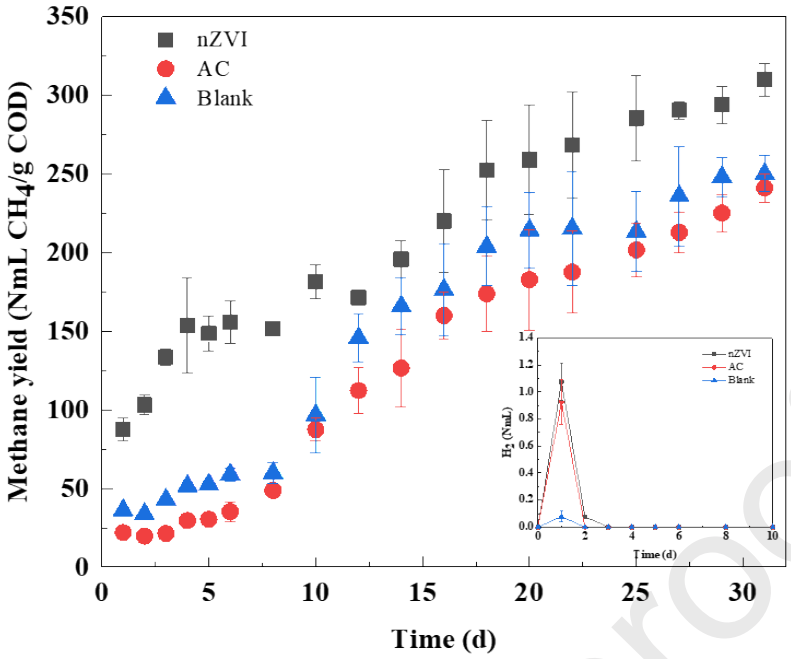


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Fig. 2

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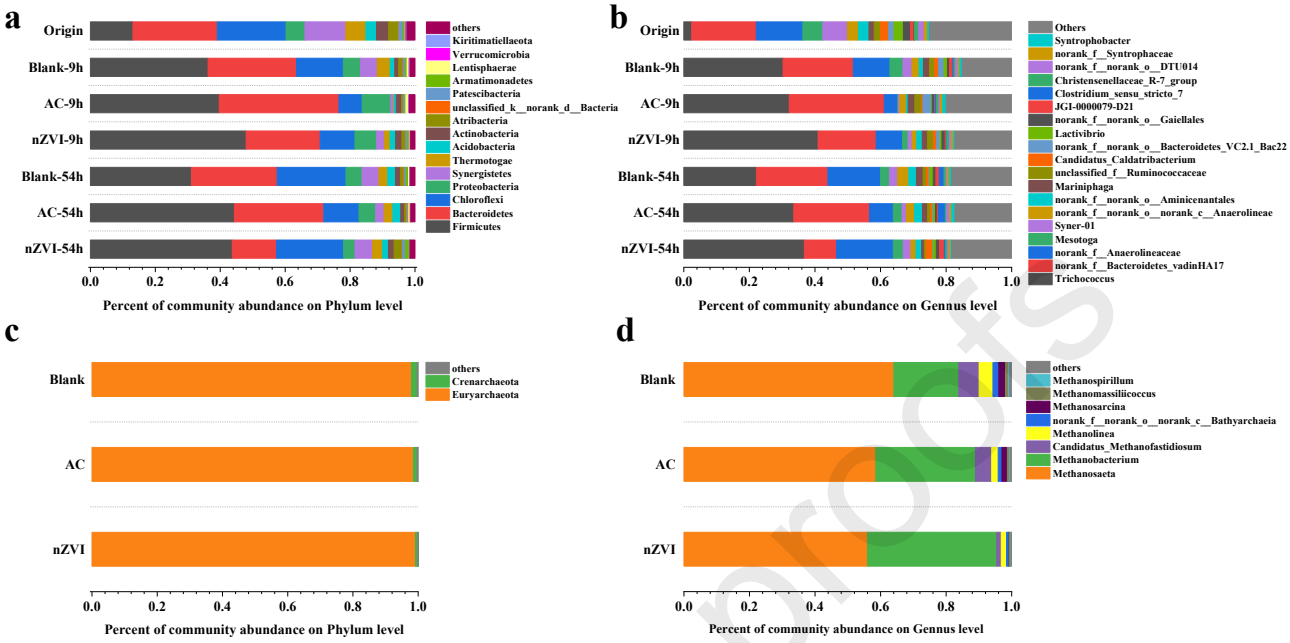

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Fig. 3

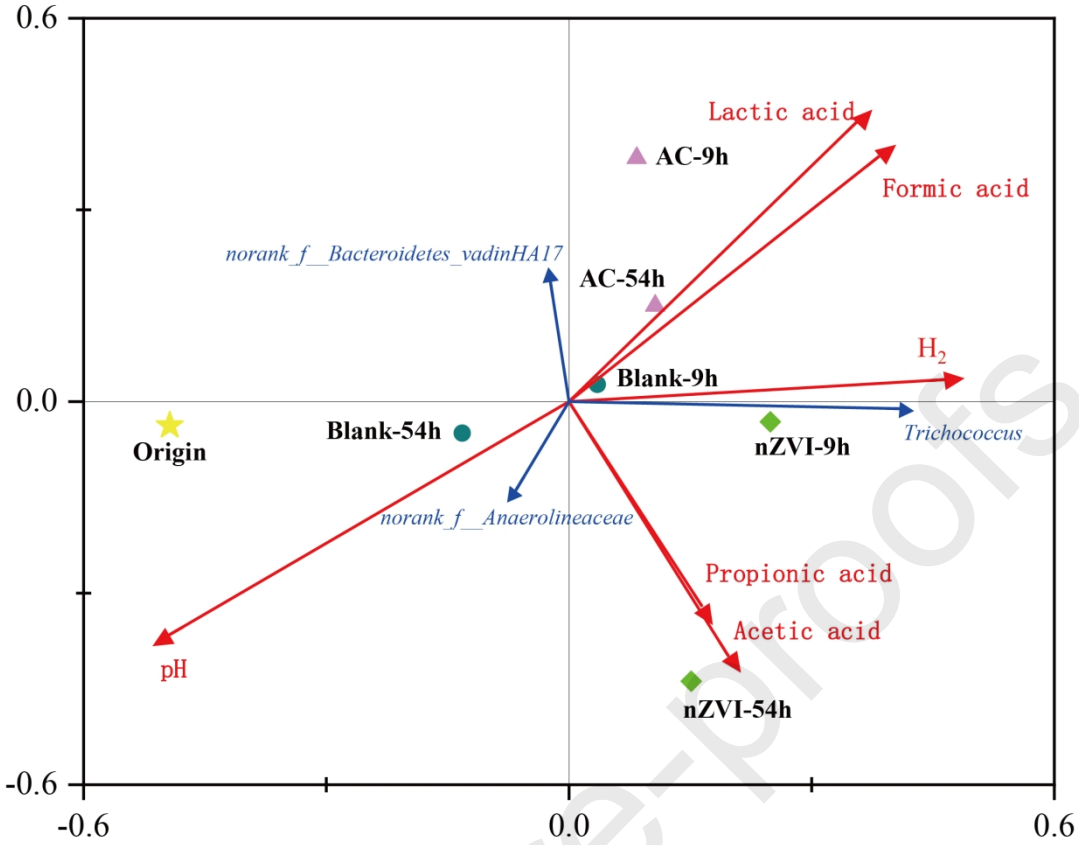

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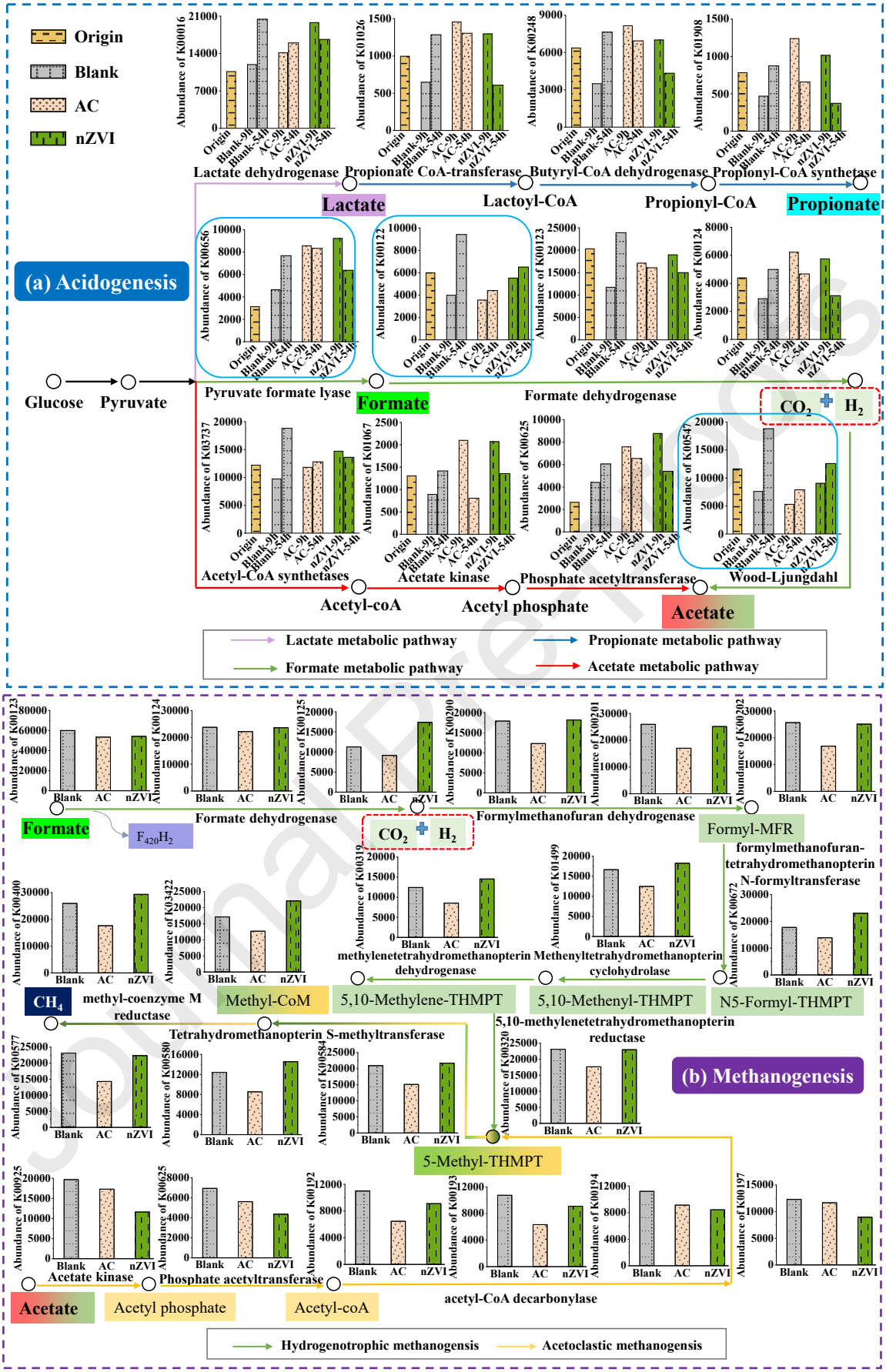
Fig. 4


Origin
Blank
AC
nZVI
Abundance of K00016
Abundance of K01026
Abundance of K00248
Abundance of K01908
Lactate dehydrogenase
Propionate CoA-transferase
Butyryl-CoA dehydrogenase
Propionyl-CoA synthetase
Lactate
Lactoyl-CoA
Propionyl-CoA
Propionate
(a) Acidogenesis
Abundance of K00656
Abundance of K00122
Abundance of K00123
Abundance of K00124
Glucose
Pyruvate
Pyruvate formate lyase
Formate
Formate dehydrogenase
CO2
H2
Abundance of K03737
Abundance of K01067
Abundance of K00625
Abundance of K00547
Acetyl-CoA synthetases
Acetate kinase
Phosphate acetyltransferase
Wood-Ljungdahl
Acetyl-coA
Acetyl phosphate
Acetate
Lactate metabolic pathway
Propionate metabolic pathway
Formate metabolic pathway
Acetate metabolic pathway
Abundance of K00123
Abundance of K00124
Abundance of K00125
Abundance of K00200
Abundance of K00201
Abundance of K00202
Formate
F420H2
Formate dehydrogenase
CO2
H2
Formylmethanofuran dehydrogenase
Formyl-MFR
formylmethanofuran-tetrahydromethanopterin N-formyltransferase
Abundance of K00400
Abundance of K03422
Abundance of K00319
Abundance of K01499
Abundance of K00672
methylenetetrahydromethanopterin dehydrogenase
Methenyltetrahydromethanopterin cyclohydrolase
CH4
methyl-coenzyme M reductase
Methyl-CoM
5,10-Methylene-THMPT
5,10-Methenyl-THMPT
N5-Formyl-THMPT
Tetrahydromethanopterin S-methyltransferase
5,10-methylenetetrahydromethanopterin reductase
Abundance of K00577
Abundance of K00580
Abundance of K00584
Abundance of K00320
(b) Methanogenesis
5-Methyl-THMPT
Abundance of K00925
Abundance of K00625
Abundance of K00192
Abundance of K00193
Abundance of K00194
Abundance of K00197
Acetate kinase
Phosphate acetyltransferase
acetyl-CoA decarbonylase
Acetate
Acetyl phosphate
Acetyl-coA
Hydrogenotrophic methanogenesis
Acetoclastic methanogenesis

735

Fig. 5

736 53.

737 **Table 1** The performance of batch reactors under different conditions after the whole AD

Batch	pH	ORP (mV)	COD removal efficiencies (%)	Cumulative CH_4 production (NmL)	CH_4 yield (NmL CH_4/g COD)
Blank	6.88	-206.00	95.48%	23.88	250.11
AC	6.87	-197.00	96.89%	23.37	241.18
nZVI	8.48	-345.00	95.89%	29.72	309.89

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740 54.

741 ## Highlights

742 1. Both AC and nZVI increased the production of VFAs in acidogenesis.

743 2. nZVI strengthened the formation of formic and acetic acids in acidogenesis.

744 3. nZVI is superior to AC on the enhancement of methane in whole AD.

745 4. *Methanobacterium* was selectively enriched with nZVI addition.

746 5. Mechanism of AC and nZVI on acidogenesis and whole AD was analyzed and

747 compared.

748

749 55.