IMPACT OF NANOPARTICLES ON THE ANAEROBIC DIGESTION CHARACTERISTICS OF ENROFLOXACIN-CONTAINING MANURE OF LIVESTOCK AND POULTRY AND ANALYSIS OF MICROBIAL COMMUNITY EVOLUTION

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Abstract. Enrofloxacin-containing chicken manure was taken as the research object for a 55-day batch mesophilic anaerobic digestion experiment using carriers of 300 mg/kg·TS nano-Fe_2O_3 and 100 mg/kg·TS nano-C_{60}. Based on the analysis of the biogas production characteristics, chemical parameters, microbial diversity, and changes in antibiotic content during the anaerobic digestion process, the impact of nanoparticles on the anaerobic system of antibiotic-containing chicken manure was clarified, and the degradation laws of antibiotic were extracted to shed light on the pollution reduction and energy production of livestock and poultry manure. The results showed that: (1) The adding of nanoparticles nano-Fe_2O_3 and nano-C_{60} had a promotion effect on the anaerobic biogas production with manure containing different concentrations of ENR (enrofloxacin). The cumulative biogas output from day 1 to 10 was in the following order: 2968 (R5) > 2490 (R4) > 1375 (R1) > 1216 (CK) > 1203 (R2) > 1121 (R3) mL, and the cumulative biogas output of R4 and R5 increased by 81.09 and 115.8%, respectively. The degradation rates reached 82.7%, 89.18%, 86.69%, 92.12%, and 94.33%, respectively; (2) On the first day of the reaction process, Firmicutes and Bacteroidota were the dominant bacterial phyla, with relative abundance of 46.56–66.18% and 29.32–49.52%, respectively. The bacterial communities in R4 and R5 containing nanoparticles did not significantly change; The quantity of Bacteroidota in the nanoparticle-containing groups was slightly larger than that in R2. On the 50th day, Firmicutes became dominant with a relative abundance of 83.81 to 93.44%, with small difference between the groups; (3) Euryarchaeota and Halobacterota were the dominant phyla of archaea in the groups during the experiment. On the first day, the relative abundance of Halobacterota in the CK group was 74.56%, 14.11–12.56% higher than that in the groups R2, R4, and R5. In the middle stage of the experiment, Euryarchaeota was dominant with a relative abundance of 71.36–99.01%. On the 50th day, Halobacterota became the dominant phylum in the CK group again, while Euryarchaeota was still dominant in the groups R2, R4, and R5.

Keywords: *nanoparticle, antibiotic enrofloxacin, fermentation, microbial diversity*

Introduction

The expansion of human population leads to a growing demand for animal-derived food, and modern livestock and poultry breeding has large-scale ntensifyin. The concentrative discharge of a large amount of livestock and poultry manure has become an urgent environmental issue (Balat and Balat, 2009). The annual discharge of livestock and poultry manure in China is 3.8 billion tons, approximately, and pig manure, cow manure, and poultry manure account for 47, 37, and 16%, respectively (Gurmessa et al.,

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2020). China is a major producer and consumer of veterinary antibiotics. In 2013, the total quantity of antibiotics consumed in China reached ca. 162000 tons, about 9 times the total quantity consumed in the United States. Among them, veterinary antibiotics account for 52% (Zhang et al., 2015). Tetracyclines, sulfonamides, quinolones, macrolides, and β-lactamides are the common antibiotics in breeding. Li et al. (2012) measured the contents of residual antibiotics in 54 animal fecal samples from 9 cities in Northeast China. Tetracycline, sulfonamide, and quinolone antibiotics were dominant residue. Among these antibiotics, the maximum detection concentrations of tetracycline, sulfamethoxazole, and enrofloxacin in chicken manure reached 13.39, 7.11, and 15.43 mg/kg. An et al. (2015) investigated the antibiotic contamination situation of livestock and poultry manure in Shenyang City, and the results showed that the maximum detection concentrations of tetracycline-type aureomycin and oxytetracycline reached 143.97 and 47.25 mg/kg, respectively. Sulfamethoxazole is a typical sulfonamide-type antibiotic contaminant, and its maximum detection concentration reached 18.00 mg/kg. Zhang et al. (2005) reported that oxytetracycline was the dominant antibiotic in chicken manure in Beijing, with a detection concentration of 3.96-23.43 mg/kg. Zhao et al. (2010) reported the content of enrofloxacin in chicken manure was up to 1420.76 mg/kg. Antibiotics have biological toxicity and strongly inhibit microbial activity. Meanwhile, the selective pressure from antibiotics may also induce antibiotic-resistant microorganisms, antibiotic-resistant genes (ARGs), and even superbacteria. Among them, ARGs can spread between different organisms through horizontal transfer. This special mechanism increases the possibility that antibiotic-resistant bacteria access the food chain, threatening human life and health (Sui et al., 2016; Sun et al., 2019; Zhi et al., 2019; Li et al., 2020).

Anaerobic digestion technology has been mature, and livestock and poultry manure can be effectively reduced, or can be transformed into harmless substances or resource. Clean energy source, biogas, can be produced to alleviate the pressure of energy source shortage. In 2017, the energy source potential of livestock and poultry manure was 5.74×1012–6.73×1012 MJ, approximately, equivalent to 213 million tons of standard coal, 149 million tons of crude oil, or 161 billion cubic meters of natural gas. The biogas produced from livestock and poultry manure can meet 4–5% of annual energy demand of China (Feng et al., 2015). If livestock and poultry manure is taken as the starting material to produce biogas, the contents and concentrations of antibiotics have an impact on the biogas production performance of anaerobic systems. Nanoparticles are the materials that have a size of less than 100 nm in at least one dimension in the three-dimensional space in the nanoscale range (1–100 nm) or are composed of nanostructured units. Nanoparticles have excellent properties that are different from other general materials. They are usually much smaller than the diameters of most cells, so they may access the cells. Nanoparticle-containing cells continuously undergo cyclic metabolism in the organism, causing damage to the functions of organism (Zhang et al., 2010). Meanwhile, nanoparticles have surface effects, and the surface area, surface energy, and surface tension of nanoparticles increase dramatically due to the decrease of particle size (Wang et al., 2015), endowing nanoparticles with highly chemical activity, so nanoparticles are prone to react with other chemicals. Based on this background, enrofloxacin-containing chicken manure was taken as the research object in the present study, and nano-Fe_2O_3 and nano-C_{60} particles served as the carriers to conduct a 55-day batch mesophilic anaerobic digestion experiment. After analyzing the biogas production performance, chemical parameters, microbial diversity, and changes in the content of antibiotic during the anaerobic digestion process, the impact of nanoparticles on the anaerobic system of antibiotic-containing chicken manure was clarified, and the laws of antibiotic degradation were extracted. This paper is beneficial for the stable operation of biogas engineering and safe transport of biogas slurry and sludge to the field. This paper is of great significance for maintaining the ecological environment, improving energy structure, and promoting the sustainable development of ecological organic agriculture.

Materials and methods

Experimental setup

The sizes of the various constituents of the digestive apparatus should be presented in *Fig. 1*. The apparatus consisted of two jars (1 L) and a volumetric flask (1 L), which functioned as the feedstock digestion tank, biogas collecting vessel, and effluent collecting vessel. These vessels were connected by anti-aging rubber tubes to compose the apparatus with high airtightness.

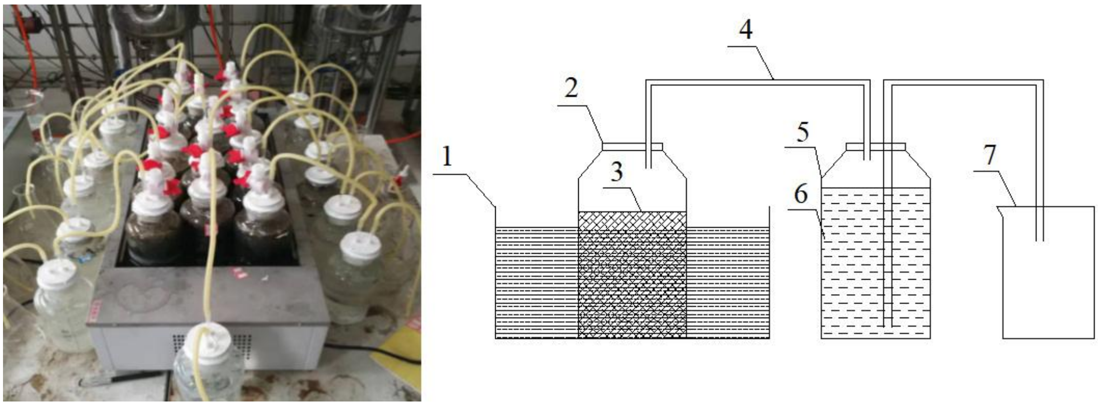

Figure 1. *Physical and schematic illustration of the apparatus. (1) Water bath; (2) digestion-reaction bottle; (3) mixture of chicken manure and acclimated sludge; (4) air duct; (5) biogas cylinder; (6) distilled water; (7) beaker*

Materials

The kitchen waste used in this study was taken from the school canteen. The kitchen waste was finely crushed by a blender and thoroughly mixed to achieve the preparation of homogenized samples. In order to ensure the consistency of the sample, the kitchen waste prepared with 5 kg of samples at a time should be frozen at -20 °C and placed at 4 °C for 12 h before use, so that it is completely thawed for reserve use.

The chicken manure used in this experiment was taken from a chicken breeding base. The persistent substances such as eggshells, feathers, and stones were extracted from the chicken manure, which was then stored in a refrigerator at 4 °C. According to testing result, this chicken manure did not contain antibiotics.

The anaerobic digestion inoculation microorganisms used in the experiment were sampled from the excess sludge of a surrounding sewage treatment plant. The excess sludge was stored in a large sealed plastic container, and then was transferred at 20 °C, approximately, to the laboratory, where the excess sludge was cultured and domesticated at 37 °C. The domestication was performed as follows: 5 L of the activated sludge was placed in a 25-L sealed plastic container and was incubated at 37 °C for 3 days. After the adaptation period, 2.5 kg of room-temperature chicken manure was added to the activated sludge. The domestication took 10 days. Then, 5 kg of room-temperature kitchen waste was added for successive domestication for 10 days. The characteristics of chicken manure and inoculated sludge are shown in *Table 1.*

Table 1. *Characteristics of Chicken Manure and Inoculated Sludge*

Sample	TS/%	VS/%	C/%	H/%	O/%	N/%	S/%	C/N
Chicken manure	27.32	24.49	47.338	6.256	43.786	1.981	0.639	23.896
Inoculated sludge	2.21	0.87	19.970	3.270	74.888	1.180	0.692	16.930

TS=total solid; VS= volatile solid

Experimental procedures

Reagents

Preparation of nano-Fe_2O_3 suspension: Nano-Fe_2O_3 (90 nm, purity = 99.8%) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd.

Preparation of nano-C_{60} suspension: Fullerene C_{60} (purity = 99.9%) was obtained from Shanghai Macklin Biochemical Technology Co., Ltd.

The antibiotic enrofloxacin (98%, CAS: 93106-60-6, $C_{19}H_{22}FN_3O_3$, molecular weight = 359.39; purity: ≥ 98%) was purchased from Shanghai Aladdin Company. It was stored in a refrigerator at 4 °C before use.

Procedures

A total of 6 experiments were set. 120 g of chicken manure and 300 g of inoculated sludge were added to each group; The maximum residual concentration of enrofloxacin was 15.43 mg/kg·TS, so the value 16 mg/kg·TS was taken. 50%, 100%, and 200% of the maximum residual concentration of enrofloxacin were added once. In the R4 and R5 groups, 300 mg/kg·TS nano-Fe_2O_3 and 100 mg/kg·TS nano-C_{60} were separately added in the presence of 16 mg/kg·TS ENR. The contents of materials in each group are shown in *Table 2.* After feeding, the volume was increased to 1 L with deionized water, and the air was removed with dinitrogen flow for 2 min. The jars were sealed. The anaerobic digestion reactors were placed in thermostatic water baths at 37 ± 0.5 °C, protected from light. 4 parallel experiments were conducted in each group, and one of the four was set to compensate the loss of materials due to the withdrawal and detection. The mean value of other 3 groups was taken as the real value. During the experiment, the systems were manually stirred for 1 min, twice in each day.

Analysis methods

Physical and chemical properties

The common indicators of the digestive supernatant, including volatile fatty acids (VFAs) and pH, were measured in this study. The supernatant of sludge was prepared via centrifugation at 9000 r/min for 10 min with a high-speed freezing centrifuge and filtration with a membrane (0.45 μm).

***Table 2.** Design of experiments*

Entry	Fresh chicken manure (g)	ENR (mg/kg·TS)	Nano-Fe_2O_3 (mg/kg·TS)	Nano-C_{60} (mg/kg·TS)	Original concentration (μg/L)	Mass of inoculated sludge (g)
CK	120	0	0	0	0	300
R1	120	8	0	0	300	300
R2	120	16	0	0	600	300
R3	120	32	0	0	1100	300
R4	120	16	300	0	600	300
R5	120	16	0	100	600	300

The detailed detection procedures are the following.

(1) Volatile fatty acids (VFAs): In this study, a gas chromatograph was used to determine the composition and content of volatile fatty acids. The sludge supernatant sample was further filtrated with a membrane (0.22 μm), and 100 μL of HPLC-grade formic acid was added to 1 mL of this sample for acidification until the pH was < 2.

The biogas output was measured once a day by using a drainage-metering method.

The composition of biogas was analyzed by gas chromatography(Shimadzu :SPD-10A) under the following conditions: a stainless-steel packed column (2m * 3m) with TDX-01 as the filler, produced by National Chromatographic Research and Analysis Center of Dalian Institute of Chemical Physics, Chinese Academy of Sciences; detector: TCD; carrier gas: He; flow rate: 20 mL·min-1; current: 100 mA; attenuation: 1; detection temperature: 200 °C; oven temperature: 180 °C; injection temperature: 200 °C.

(2) pH: pH was determined by a glass-electrode method.

Determination of content of antibiotic enrofloxacin

5 g of the sample was placed in a centrifuge tube, and 20 mL of 0.1 mol/L EDTA-Mellvaine buffer was added. After vortex for 1 min, homogenization for 1 min, and sonication for 10 min, the suspension was treated via centrifugation at 10000 r/min at 4 °C for 5 min. The supernatant was collected.

Solid-phase extraction was performed by using an HLB column (200 mg, 6 mL). The column was activated with 6 mL of methanol, followed by 6 mL of ultrapure water. The entire supernatant flowed through the column at a rate of 1 mL/min, approximately. The column was washed with 6 mL of ultrapure water, and the effluent was discarded. The wet column was dried in flowing air for 10 min. 6 mL of methanol flowed through the column at a rate of 1.0–3.0 mL/min for elution. The eluent was dried with flowing dinitrogen at 50 °C, and the residue was dissolved in 1 mL of 0.1% formic acid (aq). This suspension was filtrated with the membrane (0.22 μm), and the filtrate was detected by LC-MS with a liquid chromatograph equipped with a triple-quadrupole mass spectrometer. A Plus-C18-type chromatographic column (2.1×50 mm 1.8 μm) was employed. The oven temperature was 40 °C, and injection volume was 1.0 μL. The mobile phases A and B were 0.1% formic acid (aq) and acetonitrile, and a gradient-elution mode was adopted. An ES+ ionization mode and MRM acquisition mode were chosen, and the capillary voltage was 4 kV.

16S rRNA sequencing

The anaerobic digestion solution was passed through a 0.22 μm filter membrane, and the filter membrane was collected and stored at −20 °C. The 16S rRNA high-throughput sequencing was completed by Shanghai Meiji Biomedical Technology Co., LTD., using Illumina's Miseq PE300 sequencing platform. FastDNA® Spin Kit for Soil extraction kit was used for DNA extraction according to the instructions. Primer 338F(5'-ACTCCTACGGGAGGCAGCAG-3') was used. 806R(5'-GGACTACHVGGGTWTCTAAT-3') amplified the V3-V4 region of bacterial 16S rRNA gene using primer 524F10extF(5'-TGYCAGCCGCCGCGGTAA-3'). Arch958RmodR(5'-YCCGGCGTTGAVTCCAATT-3') amplified the V4-V5 region of archaea 16S rRNA gene.

Data analysis

In the experiment, the physical and chemical indicators were measured in triplicate. The data was analyzed for significance and correlation using single-factor analysis of variance and multiple comparisons in SPSS v.18.0 software, evaluating the significant differences between each experimental treatment. The least significant difference method (LSD, a=0.05) was used for multiple comparisons of the mean values. All data graphs were plotted using Origin-8.0.

Results and Discussion

Effects of nanoparticles on the anaerobic digestion characteristics of ENR-containing livestock and poultry manure

Anaerobic biogas production

The daily and cumulative biogas output values during the anaerobic digestion process of ENR-containing livestock and poultry manure in the presence of nanoparticles are shown in *Fig. 2.* In those six groups, two biogas production peaks took place during the 55-day anaerobic process. The first peak occurred in the days 1–10. The R1-R3 groups without nanoparticles reached their maximum values of 333 ± 17, 244 ± 13, and 200 ± 15 mL on the 1st day. The first peak of the control group took place over longer time, and the biogas output reached its maximum value of 249 ± 22 mL on the 5th day. In contrast, the R4 group containing nanoparticles reached its maximum value of 345 ± 11 mL on the 1st day. The R5 group reached its maximum value of 423 ± 11 mL on the 2nd day. The cumulative biogas output at this stage was ranked as follows: 2968 (R5) > 2490 (R4) > 1375 (R1) > 1216 (CK) > 1203 (R2) > 1121 (R3) mL, indicating that the adding of nano-Fe_2O_3 and nano-C_{60} had a promotion effect on the anaerobic biogas production of livestock and poultry manure containing different concentrations of ENR. In the first 10 days of the reactions, the cumulative biogas output of R4 and R5 was increased by 81.09% and 115.8%, respectively. From the 11th day, the biogas output in each group was gradually reduced, and the pH declined to 5.3. The activity of methane-producing bacteria was inhibited. A week later, the systems were not self-recovered. During the 21st to 23rd days, NaOH was continuously added to regulate the pH to above 6.5. From the 25th day, biogas production was gradually retrieved in each group. The second biogas output peaks (272 ± 15 and 310 ± 8 mL) took place on the 30th and 33rd days in the groups R1 and R2. The second biogas output peak (230±11 mL) took place on the 35th day in the CK group. The second biogas output peaks (148±12 and 132±4 mL) of the groups R4 and R5 containing nanoparticles took place on the 33rd and 37th days, respectively. Their second biogas output peaks were lower than those of the other four groups. The final cumulative biogas output of each group was 3712 (CK), 4281 (R1), 3968 (R2), 4061 (R3), 4498 (R4), and 4639 (R5) mL. The relevant research results showed that the adding of carbon-based nanomaterials enhanced the direct interspecific electron transfer between electron-donating bacteria and methanogenic archaea, promoting the conversion of carbon dioxide to methane, shortening the lag time required to trigger biogas production, and increasing the methane output.

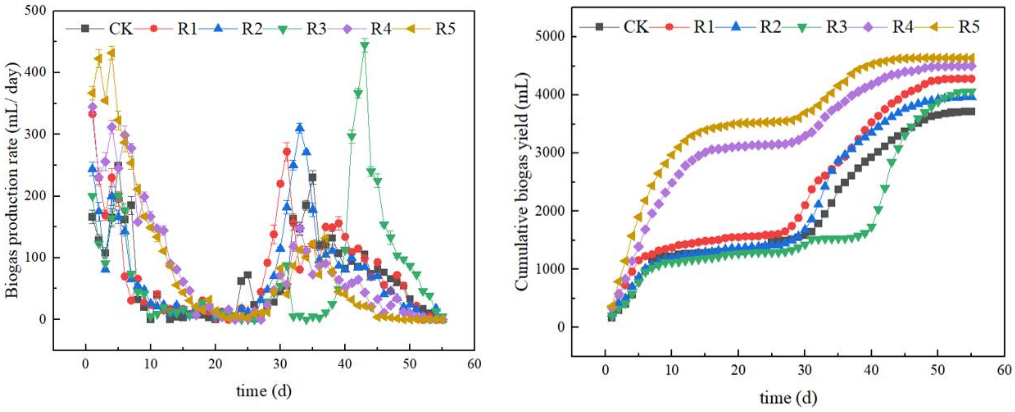

Figure 2. *Daily and cumulative biogas output of anaerobic digestion of ENR-containing livestock and poultry manure in the presence and absence of nanoparticles*

Zhao et al. (Zhao et al., 2018) reported that 500 mg/kg norfloxacin inhibited the methane production rate, while 10 mg/kg norfloxacin improved the biogas output. The positive impact of antibiotics on anaerobic digestion was also documented in other studies. Qiang et al. (2019) found that chloramphenicol (≤ 20 mg/L) inhibited early biogas production in a short period, but the microbial activity was reinforced in the later stage. The methane output was increased by 0.54–9.58% in the presence of different concentrations of chloramphenicol; Lu et al. (2014) investigated the effect of 0-2000 mg/L cephalosporin on the anaerobic digestion and biogas production with waste activated sludge through a 157-day experiment. The results showed that the antibiotic only had an inhibition effect in the first 25 days, and then the biogas production of the groups containing 600 mg/L and 1000 mg/L cephalosporin was recovered and even stimulated. The total biogas output was increased by 30.3% and 63.8% compared to the control group. Both studies revealed that the EPS (extracellular polymeric substances) in activated sludge could impede the diffusion of antibiotics in the cell through an adsorption effect, and the antibiotics stimulated the secretion of EPS, which protected the growth and reproduction of microorganisms.

The adding of nanoparticles nano-Fe_2O_3 and nano-C_{60} had a promotion effect on the anaerobic biogas production with livestock and poultry manure containing different concentrations of ENR. The biogas production efficiency in the first 10 days was significantly improved, and the cumulative biogas output was also higher than that of other groups. nano-Fe_2O_3 and nano-C_{60} have special structures and excellent properties that are different from other general materials. The ultra-small structures of nanoparticles lead to their surface effects. The surface area, surface energy, and surface tension of

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nanoparticles are largely increased with the decrease of particle size, endowing them with highly chemical activity to react with other chemicals. Therefore, nanoparticles can serve as a medium to promote the microbial hydrolysis of substrate for the production of biogas. The positive effects of nanomaterials on the metabolic rate of substrate and hydrogen output during anaerobic fermentation have been proven in certain relevant studies. The adding of an appropriate amount of nanomaterials to an anaerobic-fermentation system can enhance the activity of hydrogenase and optimize the composition of anaerobic microbial communities, further improving the output of biogas. In addition, the leaching of metal ions from the nanoparticles nano-Fe_2O_3 and nano-C_{60} has been considered as one of the most common and important pathways to affect the anaerobic digestion of sludge. Fe^{3+} is an essential trace element for the growth and metabolism of anaerobic microorganisms, and Fe^{3+} is beneficial to microorganisms under certain circumstances. Another similar conclusion has been drawn by Mu et al. (2012). If the concentration of nano-Fe_2O_3 was low, the released Fe^{3+} could chelate with negatively charged functional groups in EPS, thereby reducing the toxicity of ions.

pH and concentration of TVFAs

The pH and concentration of TVFAs during the anaerobic digestion process of livestock and poultry manure containing different concentrations of ENR are shown in *Figs. 3 and 4.* Volatile fatty acids are produced by hydrolytic acid-producing bacteria, and formic acid and acetic acid are the metabolic substrate of methanogens. However, an excess of acid accumulated inhibits the activity of methanogens and reduces the biogas production efficiency of anaerobic systems. The concentration of TVFAs in each group was rapidly increased from the 1st to 10th day, and the concentrations of volatile fatty acids in groups R4 and R5 were higher than those in the other 4 groups, indicating that the nano-Fe_2O_3 and nano-C_{60} nanoparticles had a promotion effect on the hydrolysis of ENR-containing livestock and poultry manure. Their ultrastructures served as a medium to facilitate the decomposition of substrate by hydrolysis-acidification microorganisms, resulting in the higher concentrations of TVFAs in the first 10 days compared to the other groups. Subsequently, the concentration of TVFAs in each group continued to increase and reached their maximum values on the 19th day: 10269.25 (CK), 10454.16 (R1), 9975.94 (R2), 10568.93 (R3), 10345 (R4), and 10987 (R5) mg/L; In this stage, the pH of each group continued to decrease, and the pH declined to 5.30–5.37 on the 19th day. The production of biogas was significantly suppressed, indicating that the inhibition effect of acids imbalanced the conversion of materials. Latif et al. (2017) reported that if the pH in an anaerobic digestion system was as low as 5.5, the severe accumulation of propionic acid and butyric acid led to a 50% reduction in methane output, and the proportion of incompletely degraded organic matter increased. When the pH was below 5.5, the quantity of methane production communities was significantly decreased, and these communities were transformed into propionic-acid-utilizing communities. The pH of groups containing nano-Fe_2O_3 and nano-C_{60} was lower, 5.08 ± 0.3 and 5.01 ± 0.1, respectively. This is consistent with the data of concentration of TVFAs. The 300 mg/kg·TS nano-Fe_2O_3 and 100 mg/kg·TS nano-C_{60} added promoted the hydrolysis of substrate to give organic acids to a certain extent, leading to the decrease in pH. After the adding of alkali, the pH of each group was increased to 6.64–6.72 on the 25th day, and biogas was produced in each group again. The concentrations of TVFAs rapidly decreased, and the biogas output of each group reached its maximum on the 30th day or later. The concentration of TVFAs in each group rapidly increased again during the days

34–37, indicating that the hydrolysis rates of persistent substances such as cellulose were higher than the utilization rate of methanogenic bacteria. After day 37, with the decrease of substrate, the density of acid-producing bacteria decreased, and the concentration of TVFAs was gradually reduced. The concentration of TVFAs in R3 was at a low level from the 25th to the 34th day, but the biogas output was not consistent with the concentration of TVFAs. The low content of volatile fatty acids in the system was not caused by the utilization activity of methanogens, and enrofloxacin might have a negative effect on the hydrolysis acid-producing bacteria. It has been documented that the methane output was inhibited by antibiotics without the accumulation of acetic acid (Li et al., 2013). Its rationale is similar to the non-competitive inhibition mechanism of enzymes. The antibiotics bind to the substrate complex of enzyme to deactivate the sites on enzyme, resulting in the inhibited methane production process from acetic acid.

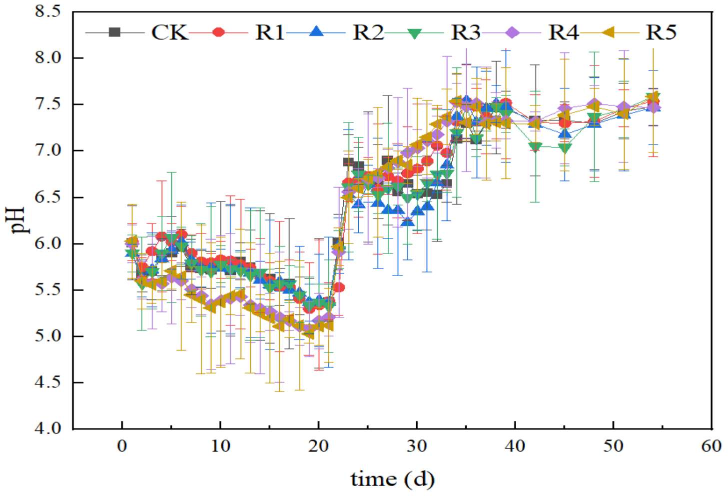

Figure 3. *pH values during the anaerobic process of ENR-containing livestock and poultry manure in the presence and absence of nanoparticles*

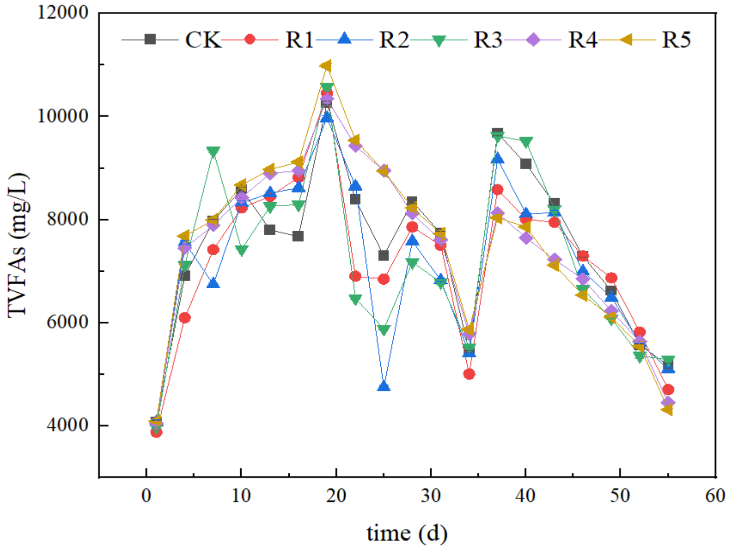

Figure 4. *Concentration of TVFAs during the anaerobic process of ENR-containing livestock and poultry manure in the presence and absence of nanoparticles*

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Degradation of antibiotic ENR

The degradation rates of antibiotic ENR during the anaerobic digestion of livestock and poultry manure with and without nanoparticles are shown in *Table 3.* The groups R1-R5 exhibited the same trend, and ENR was rapidly degraded from day 1 to 15. The degradation rates (84.56 to 87.41% on day 15) in the presence of nano-Fe_2O_3 and nano-C_{60} were higher than those in the other three groups (73.67 to 80.31% on day 15). Subsequently, the concentrations tended to level off. The residual concentrations were 51.91, 64.94, 146.45, 36.2, and 26.1 μg/L on the day 50, respectively, with the corresponding degradation rates of 82.7%, 89.18%, 86.69%, 92.12%, and 94.33%, respectively. It has been reported that adsorption is the principal pathway for the removal of fluoroquinolone drugs in an anaerobic reaction system in the absence of nanoparticles (Wang et al., 2017). Enrofloxacin has an n-octanol-water partition coefficient of 4.45 at 25 °C, indicating lipophilicity (Wu et al., 2005). In addition, it has been reported that fluoroquinolone drugs have zwitterionic properties, and they are susceptible to electrostatic interactions, resulting in the high adsorption capacity for them (Li and Zhang, 2010). The samples had been treated by solid-phase extraction, so the concentration of enrofloxacin measured was its total concentration (sum of quantity in the solid and liquid phases). The reactor was protected from light during the experiment, and it was not affected by photodegradation. Enrofloxacin could be effectively removed via mesophilic anaerobic digestion, and the removal was reinforced in the presence of 300 mg/kg·TS nano-Fe_2O_3 and 100 mg/kg·TS nano-C_{60}, indicating that these nanoparticles could not only promote the anaerobic digestion to produce biogas, but also have a synergistically metabolic effect on the antibiotic ENR.

Table 3. *Degradation rate of antibiotic ENR during the mesophilic anaerobic digestion process (%)*

	R1	R2	R3	R4	R5
5	17.52	17.43	17.09	22.37	24.51
15	73.67	80.31	80.08	84.56	87.41
30	81.21	83.37	83.56	89.43	91.22
50	82.7	89.18	86.69	92.12	94.33

Effects of nanoparticles on the anaerobic digestive microflora of ENR-containing livestock and poultry manure

Bacterial communities

The relative abundance of bacteria in the anaerobic digestion process of ENR-containing livestock and poultry manure at the phylum level is shown in *Fig. 5.* The bacterial community structures of groups CK, R2, R4, and R5 were similar at different stages, and their communities principally consisted of seven phyla: Firmicutes, Bacteroidota, Actinobacteriota, Spirochaetota, Proteobacteria, Chloroflexi, and Synergistota. Firmicutes, Bacteroidota, and Proteobacteria are common bacterial communities in anaerobic digestion, and they can metabolize various substrates for the hydrolysis of macromolecules and production of organic acids. Among them, a large quantity of extracellular enzymes such as proteases, cellulases, and lipases can be produced by Firmicutes (Chen et al., 2017; Zhao et al., 2017). Bacteroidota plays an

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important role in the degradation of carbohydrates and proteins into acetic acids and NH_3 (Tang et al., 2016). Some genera in Proteobacteria have the ability to reduce sulfates into H_2S. The organic acids produced by hydrolysis-acidification bacteria can be decomposed into acetic acid and H_2 by Spirochaeota, Synergistota, and Chloroflexi (Guo et al., 2012). On the first day, Firmicutes and Bacteroidota were the dominant bacterial phyla in each group, with relative abundance of 46.56–66.18% and 29.32–49.52%, respectively. The bacterial communities in the groups R4 and R5 containing nanoparticles did not exhibit special characteristics; On the 5th day, the relative abundance of Actinobacteriota and Bacteroidota was significantly increased to 6.11–21.75% and 35.20–54.49%, respectively. The abundance of Bacteroidota in the groups containing nanoparticles was slightly higher than that in R2. The nanoparticles functioned as a medium to promote the quantity of Bacteroidota. From the 15th to 50th day, Firmicutes showed higher competitiveness along with the proceeding of reactions. The relative abundance of Bacteroidota gradually decreased, which is possibly caused by lack of nutrients. However, the quantity of Bacteroidota in the groups R4 and R5 containing nanoparticles was larger than that in the other groups. On the 50th day, Firmicutes dominated the communities with relative abundance of 83.81–93.44%, and the difference between groups was insignificant.

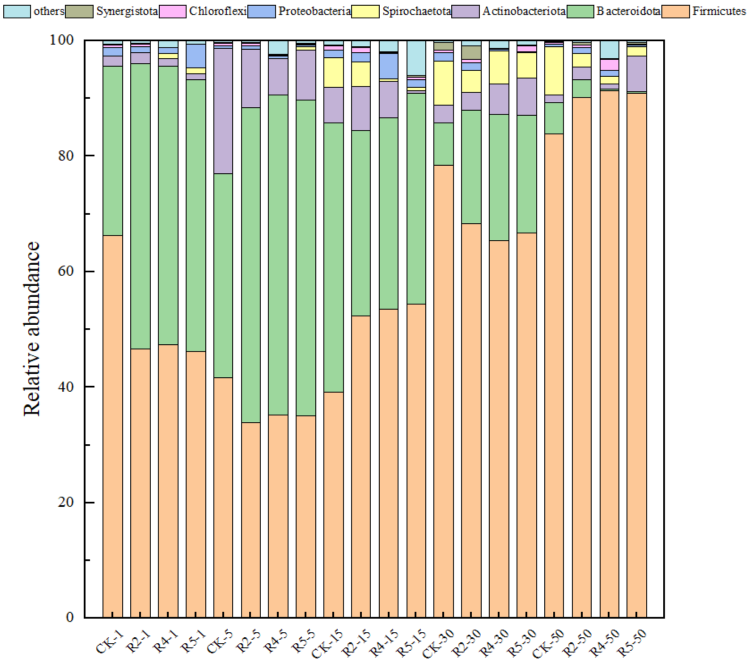

Figure 5. *Bacterial community structures at a phylum level during the anaerobic digestion process of ENR-containing livestock and poultry manure in the presence and absence of nanoparticles*

As shown in *Fig. 6.*, the bacterial structures at a genus level in different stages were obviously affected by substrate. With the consumption of nutrients, the original competitiveness of some bacterial communities disappeared. Prevotella belongs to Bacteroidota. On the 5th day, the relative abundance of Prevotella increased, indicating its strong tolerance to ENR. Especially, the relative abundance of Prevotella in the groups R4 and R5 containing nanoparticles was 2–3% higher than that in the R2 group. On the

15th day, compared to the control group CK, the relative abundance of Prevotella in the R2 group decreased, and those in the groups R4 and R5 declined to a larger extent. A possible reason is that the easily degradable nutrients in the substrate were rapidly degraded within 1-10 days. In contrast, the groups R4 and R5 underwent thorough hydrolysis and acidification reactions in the early stage, resulting in inadequate nutrients in the substrate and decrease in the relative abundance of Prevotella. The relative abundance of Treponema in R2, R4, and R5 was as high as 4.22–6.12%. Treponema belongs to Spirochaeota and can transform H_2 and CO_2 into acetic acid (Wang et al., 2013). At this time, the pH of R2, R4, and R5 was relatively low, which is consistent with afore-mentioned research results. Capriciproducens and norank_f_ Ethanolinenaceae belong to Firmicutes, and the abundance of both in R4 and R5 was higher than that in CK. This phenomenon was maintained until the 30th day. Capriciproducens converts sugars into H_2, which is the energy source of hydrogenotrophic methanogens (Qin et al., 2019; Ta et al., 2020). On the 50th day, the relative abundance of HN-HF0106 in CK was higher than that in R2, R4, and R5, indicating that the activity of this bacterial genus was inhibited by residual ENR. In contrast, Ruminiclostridium had stronger adaptability in the presence of residual ENR. Ruminiclostridium belongs to Bacteroidota and can secrete cellulose degrading enzymes to degrade lignocellulose (Dumitrache et al., 2016). At this time, easily decomposable organic matter was completely consumed, and thereby the competitiveness of the microbial community relying on persistent organic matter such as cellulose became stronger.

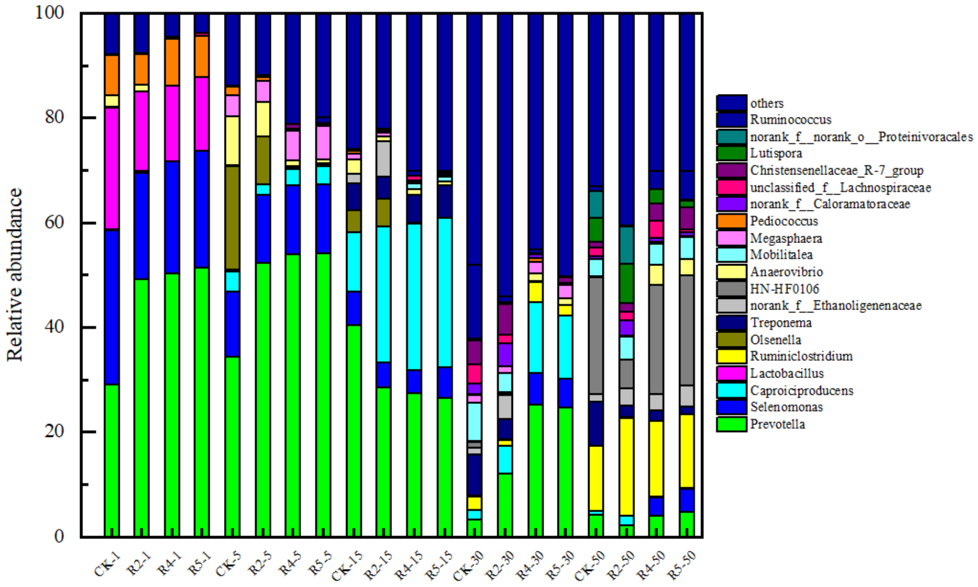

Figure 6. *Bacterial community structures at a genus level during the anaerobic digestion process of ENR-containing livestock and poultry manure in the presence and absence of nanoparticles*

Archaeal communities

The relative abundance of archaea at a phylum level is shown in *Fig. 7.* During the experiment, the dominant bacterial phyla were Euryarchaeota and Halobacterota. The bacterial community structure of each group was similar in the early and middle stages of the experiment. On the first day, Halobacterota was dominant, and the relative abundance of Halobacterota in the CK group was 74.56%, higher than that in the groups R2, R4, and R5 by 14.11–12.56%, indicating that the presence of antibiotic ENR had an inhibition effect on Halobacterota. In the middle stage of the experiment, Euryarchaeota was dominant with relative abundance of 71.36–99.01%, indicating that Euryarchaeota had ENR tolerance. In particular, after the adding of nanoparticles, the abundance of Euryarchaeota in R4 and R5 was higher than that in R2, indicating that the nanoparticles added provided a medium for the decomposition of substrate by Euryarchaeota. The ultra-small structures of nanoparticles led to surface effects, reducing the activation energy required by biological reactions, and thereby the nanoparticles had highly chemical activity. On the 50th day, Halobacterota became the dominant phylum in the CK group again, while Euryarchaeota was still dominant in R2, R4, and R5.

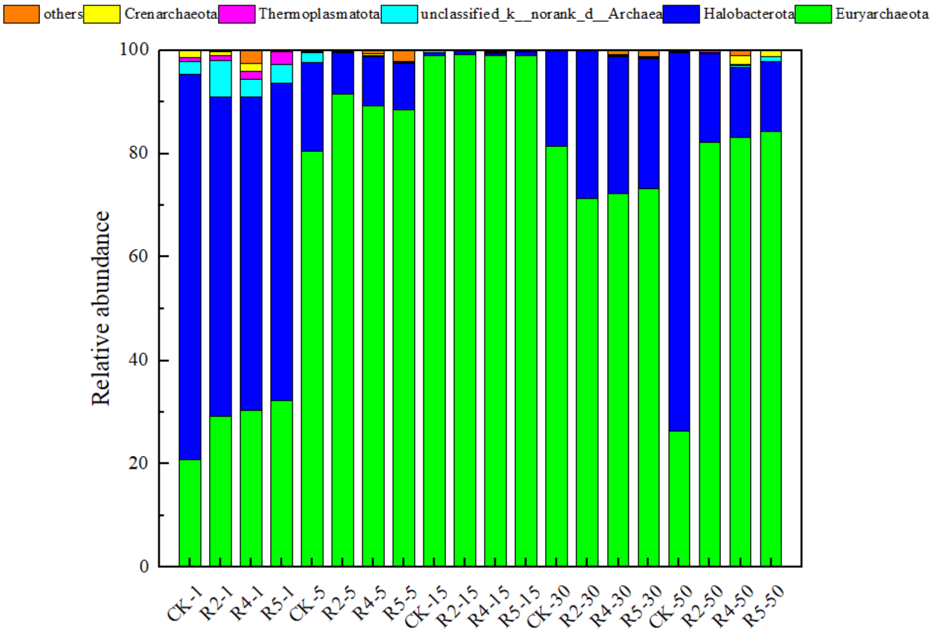

Figure 7. *Archaeal community structures at a phylum level during the anaerobic digestion process of ENR-containing livestock and poultry manure in the presence and absence of nanoparticles*

The relative abundance of archaea at a genus level is shown in *Fig. 8.* The dominant bacterial species was varied at different stages, and the dominant species included Methanosphaera, Methanobrevibacter, Methanosaeta, Methanobacterium, and Methanosarcina. Among them, Methanosphaera, Methanobrevibacter, and Methanobacterium belong to the Halobacterota phylum. In the initial stage of the experiment, the archaeal structure of each group genus was similar at a genus level, and the dominant bacterial communities were Metanosaeta (45.98–56.65%), Metanobacterium (13.34–21.02%), and Metanosarcina (8.57–12.51%). On the 5th day, the relative abundance of Metanosphaera increased drastically, and this community became dominant. Its relative abundance reached 68.49, 84.06, 86.23, and 87.32% in the groups CK, R2, R4, and R5, respectively. Metanosphaera is a methylotrophic methanogenic bacterium, with significantly higher abundance in the groups R2, R4, and R5 compared to CK. Its competitiveness was evidently reinforced, indicating that

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Metanosphaera had strong adaptability and tolerance to ENR. The relative abundance of Metanosaeta was 14.39% (CK), 7.17% (R2), 1.79% (R4), and 1.39% (R5), and its relative abundance was much lower in the presence of antibiotic. This phenomenon is consistent with the research results of Zhi et al. (2019). Meanwhile, the relative abundance of Metanosaeta in the groups R4 and R5 was lower than that in R2, which may be ascribed to the inhibition effect of nanoparticles on Metanosaeta. This effect disturbed the metabolism of Methanosaeta, leading to the decrease in its abundance. Methanosaeta is a methane-producing archaea that utilizes acetic acid as its sole substrate. On the 5th day, the relative abundance of another methane-producing acetic-acid-utilizing archaea, Methanosarcina, was 1.36% (CK), 0.52% (R2), 0.51% (R4), and 0.08% (R5), lower than the value of the control group, indicating that the antibiotic ENR had an inhibition effect on acetic-acid-consuming methane-producing bacteria.

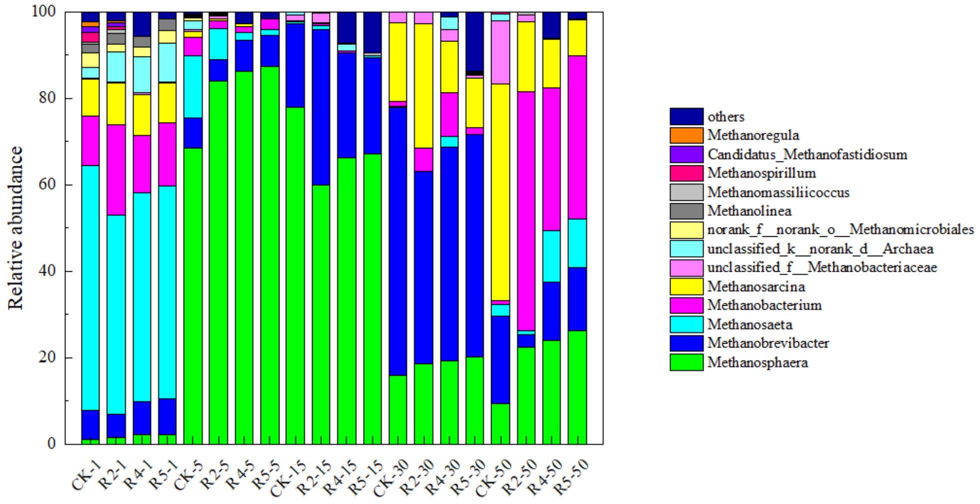

Figure 8. *Archaeal community structures at a genus level during the anaerobic digestion process of ENR-containing livestock and poultry manure in the presence and absence of nanoparticles*

Zhu et al. (2021) found that norfloxacin obviously suppressed acetic-acid-type methanogens, while hydrogenotrophic methanogens showed stronger tolerance. On the 15th day, Metanosphaera remained dominant, and its relative abundance in CK was increased to 77.94%. Its relative abundance in the groups R2, R4, and R5 was decreased to 59.89%, 66.34%, and 67.23% compared to the 5th day. Methanobrevibacter showed stronger competitiveness from the 15th day, and it was dominant on the 30th day. Meanwhile, the abundance of Methanosphaera decreased due to the consumption of methyl compounds. Methanobrevibacter belongs to hydrogenotrophic methanogens. On the 15th day, the abundance of Methanobrevibacter in CK was lower than that in the groups treated by antibiotic. On the 30th day, the difference in the abundance of Methanobrevibacter in all these groups was small, indicating high adaptability of hydrogenotrophic methanogens to ENR. On the 50th day, the relative abundance of Metanobacterium in the groups R2, R4, and R5 was 55.28%, 33.11%, and 37.75%, while that in the CK group was as low as 7.36%, when Metanosarcina was dominant with abundance of 72.7% in the CK group. Although over 80% of ENR was removed via anaerobic reactions, the residual ENR might still inhibit acetic-acid-consuming methane-producing bacteria. It has been reported that in the presence of concentrated antibiotics, hydrogenotrophic methanogens exhibited a higher substrate utilization rate, growth rate, and cell output (Aydin et al., 2015; Gan, 2019). Zeng et al. (2021) found that under anaerobic conditions, the activity of two types of hydrogen-producing bacterial communities, Anaerolineaceae and Microtrichales, was inhibited by 10 mg/L clarithromycin, thereby weakening the hydrogenotrophic methane-producing process.

Conclusion

(1) The adding of nanoparticles nano-Fe_2O_3 and nano-C_{60} had a promotion effect on the anaerobic biogas production processes with livestock and poultry manure containing different concentrations of ENR. The biogas output of R4 group containing nanoparticles reached its maximum of 345 ± 11 mL on the first day, and the R5 group reached its maximum of 423 ± 11 mL on the second day. The cumulative biogas output during days 1 to 10 was in this order: 2968 (R5) > 2490 (R4) > 1375 (R1) > 1216 (CK) > 1203 (R2) > 1121 (R3) mL, and the cumulative biogas output of R4 and R5 was increased by 81.09% and 115.8%, respectively. Finally, the cumulative biogas output of each group was 3712 (CK), 4281 (R1), 3968 (R2), 4061 (R3), 4498 (R4), and 4639 (R5) mL, respectively.

(2) The ENR degradation rates in the groups R1-R5 exhibited a similar trend during their anaerobic digestion processes of livestock and poultry manure. ENR was rapidly degraded from day 1 to 15, and the ENR degradation rates in the groups without nanoparticles on day 15 were in the range of 73.67% to 80.31%. The degradation rates in the groups containing nanoparticles nano-Fe_2O_3 and nano-C_{60} on day 15 were 84.56% and 87.41%, respectively. Subsequently, the ENR concentrations leveled off, and its residual contents at day 50 were 51.91, 64.94, 146.45, 36.2, and 26.1 μg/L, respectively, with corresponding degradation rates of 82.7%, 89.18%, 86.69%, 92.12%, and 94.33%, respectively.

(3) For the bacterial communities, Firmicutes and Bacteroidota were the dominant bacterial phyla at a phylum level on the first day of the reactions, with relative abundance of 46.56–66.18% and 29.32–49.52%, respectively. The bacterial communities in the groups R4 and R5 containing nanoparticles did not show obvious changes on the first day; On the 5th day, the relative abundance of Actinobacteriota and Bacteroidota was significantly increased to 6.11–21.75% and 35.20–54.49%, respectively. The quantity of Bacteroidota in the groups containing nanoparticles was slightly higher than that in R2. On the 50th day, Firmicutes was dominant with relative abundance of 83.81–93.44%, and the abundance difference between these groups was small.

(4) For the archaea communities, Euryarchaeota and Halobacterota were the dominant phyla at a phylum level in all the groups during the experiment. On the first day of the reactions, the relative abundance of Halobacterota reached 74.56% in the CK group, 14.11–12.56% higher than that in the groups R2, R4, and R5. In the middle stage of the experiment, Euryarchaeota was dominant with relative abundance of 71.36–99.01%. On the 50th day, Halobacterota became the dominant bacterial phylum again in the CK group, while Euryarchaeota was still dominant in the groups R2, R4, and R5; At a genus level, the dominant archaea communities were Metanosaeta (45.98–56.65%), Metanobacterium (13.34–21.02%), and Metanosarcina (8.57–12.51%) in the early stage of the experiment. On the 5th day, the relative abundance of Metanosphaera increased dramatically, and this community was dominant with relative abundance of 68.49%, 84.06%, 86.23%, and 87.32% in the groups CK, R2, R4, and R5, respectively. On the 50th day, the relative

abundance of Metanobacterium in the groups R2, R4, and R5 was 55.28%, 33.11%, and 37.75%, while its abundance in the group CK was only 7.36%. At this time, Metanosarcina was dominant in the CK system with abundance of 72.7%.

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