Research Article

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Effects of nanoparticles on the activity and resistance genes of anaerobic digestion enzymes in livestock and poultry manure containing the antibiotic tetracycline

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Abstract: Taking chicken manure containing antibiotic oxytetracycline (OTC) as the research object, nano-Fe_2O_3 with a concentration of 300 mg/kg TS and nano-C_{60} with a concentration of 100 mg/kg TS as carriers were used for a 55-day sequential batch mesophilic anaerobic digestion experiment. The gas production performance, chemical parameters, antibiotic content, enzyme concentration, and resistance gene changes during anaerobic digestion were analyzed to clarify the effect of nanoparticles on anaerobic systems containing antibiotic chicken manure and provide a reliable basis for pollution reduction capacity of livestock and poultry manure. The results showed that (1) adding nano-Fe_2O_3 and nano-C_{60} promoted anaerobic gas production in chicken manure with different concentrations of OTC. The cumulative gas production from days 1 to 10 was 2,234(T5) > 2,163(T4) > 1,445(T2) > 1,289(T3) > 1,220(T1) > 1,216(CK) mL. The cumulative gas production of T4 and T5 increased by 77.29 and 83.11%, respectively. The final cumulative gas production for each group was 3,712(CK), 3,993(T1), 4,344(T2), 4,447(T3), 4,671(T4), and 4,849(T5) mL. The final OTC residue concentrations were 15.25, 20.40, 56.56, 17.67, and 16.89 μg/L, with degradation rates of 98.31, 98.80, 98.29, 99.07, and 99.11% respectively; (2) antibiotic OTC increased the activities of dehydrogenase, amylase, protease, and urease, while adding nanoparticles increased the activities of dehydrogenase and amylase, with no significant impact on cellulase, urease, and protease activities; (3) antibiotic resistance gene (ARGs) of various types were found in groups T2, T4, and T5, with the multidrug resistance gene (ARGs)-Multidrug accounting for a high proportion of 33.54, 35.63, and 37.43%, respectively, while the proportions of the other four types MLS, tetracycline, glycopeptide, and peptide ranged from 8.18 to 12.98%.

Keywords: nanoparticles, oxytetracycline, anaerobic digestion, antibiotics, resistance genes

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1 Introduction

The expansion of the population has increased the demand for animal-derived food, and industrialization and intensification have become important features of modern livestock farming. The concentrated discharge of large amounts of animal waste is causing increasingly severe environmental pollution problems [1]. According to the second national pollution source survey, the chemical oxygen demand emissions reached 21.44 million tons in 2017, with livestock excrement being the main source of pollution, contributing 46.67% [2]. The use of antibiotics in the livestock industry is a common phenomenon, with China's total antibiotic use in 2013 estimated at around 162,000 tons, of which 52% were for veterinary use. Commonly used antibiotics include tetracyclines, sulfonamides, quinolones, macrolides, and β-lactams. Livestock farms discharge bacteria containing resistant genes into the environment through treated wastewater and solid waste. Antibiotics have biological toxicity, strong inhibitory effects on microbial activity, and may induce resistant bacteria and antibiotic resistance genes (ARGs). The transfer of resistance genes can occur between antibiotic-resistant bacteria and local bacteria through conjugation, transformation, and transduction. Antibiotic-resistant bacteria may enter the human body through various means, including consuming contaminated food or water, as well as through occupational exposure such as inhalation [3], posing a threat to human life and health [4–7].

Anaerobic digestion technology is becoming increasingly mature, which can effectively reduce, harmlessly treat, and resource livestock and poultry manure. The clean energy biogas produced can alleviate the pressure of today's energy shortage, and utilizing livestock and poultry manure to produce biogas can meet 4–5% of China's annual energy demand [8]. By starting a biogas project using livestock and poultry manure as raw materials, the antibiotic content and concentration have a certain impact on the gas production performance of anaerobic systems. Ke et al. [9] found that tetracycline at concentrations of 20, 50, and 80 mg/L caused reductions in biogas production from mesophilic anaerobic digestion of cattle manure by 43.83, 65.10, and 77.79%, respectively. Ma et al. [10] reported that lincomycin and chloramphenicol at 25 mg/L led to decreases in total methane production of 29.39 and 19.88%, respectively, further decreasing to 51.27 and 49.46% at a concentration of 50 mg/L. Mitchell et al. [11] demonstrated that tylosin at concentrations below 100 mg/L had no inhibitory effect on overall biogas production, but as the dosage was gradually increased to 130, 260, 520, and 913 mg/L, biogas yields decreased by 10, 20, 30, and 38%, respectively. Zhao et al. [12] observed that norfloxacin at 500 mg/kg inhibited methane production rate without affecting the total methane yield. According to Cetecioglu et al. [13], sulfamethoxazole at concentrations between 100 and 250 mg/L significantly suppressed gas generation. Nanoparticles refer to materials with at least one dimension within the 100 nm range or composed of nanostructural units in a three-dimensional space spanning the nanoscale (1–100 nm). Nanoparticles exhibit surface effects, characterized by a dramatic increase in surface area, surface energy, and surface tension as particle size decreases. This renders nanoparticles highly chemically reactive, making them prone to interaction with other chemical substances. In this context, this study focuses on chicken manure containing tetracycline antibiotics, using nano-Fe_2O_3 and nano-C_{60} as carriers for a 55-day batch anaerobic digestion experiment. By analyzing gas production performance, chemical parameters, antibiotic content, enzyme concentration, and resistance gene changes during the anaerobic digestion process, the impact of nanoparticles on antibiotic-containing chicken manure anaerobic systems is clarified. This research provides important insights for maintaining ecological integrity, improving energy structure, and promoting sustainable development of ecological organic agriculture.

2 Materials and methods

2.1 Experimental equipment

The experiment uses a self-designed anaerobic digestion potential test device, as shown in Figure 1. The test system consists of two wide-mouth bottles (1 L) and 1 capacity bottle (1 L), which serve as the raw material digestion tank, biogas collection bottle, and water collection bottle, respectively. They are connected by anti-aging treated rubber tubes to form a set of gas communication devices. When connecting the device, it is necessary to ensure air tightness.

2.2 Experimental materials

The chicken manure used in this experiment was taken from a certain meat chicken breeding base. The difficult-to-degrade substances such as eggshells, feathers, and

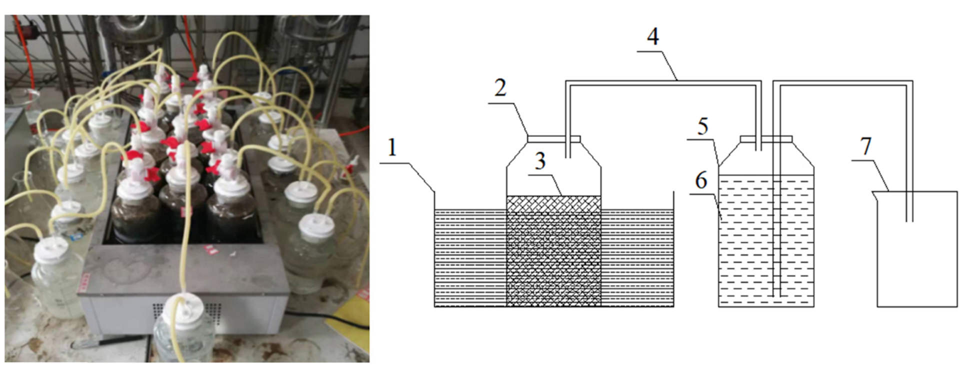

Figure 1: Physical and schematic illustration of the apparatus: (1) water bath; (2) digestion-reaction bottle; (3) mixture of chicken manure and acclimated sludge; (4) air duct; (5) gas cylinder; (6) distilled water; (7) beaker.

stones in the chicken manure were picked out and stored in a refrigerator at 4°C for later use. It was tested that the chicken manure did not contain any antibiotics.

The anaerobic digestion inoculum used in the experiment comes from the residual sludge of the surrounding sewage treatment plant. The residual sludge is transferred to a large sealed plastic container, with a temperature of about 20°C during transportation. After returning to the laboratory, the residual sludge is domesticated under the condition of 37°C. The domestication method is to place 5 L of recovered activated sludge in a 25-L sealed plastic container and incubate at a constant temperature of 37°C for 3 days. After the adaptation period, 2.5 kg of chicken manure taken out in advance to return to room temperature is added to the activated sludge for a 10-day incubation. Then, 5 kg of kitchen waste returned to room temperature is taken for domestication and incubation for 10 days, completing the domestication. The characteristics of chicken manure and inoculated sludge are shown in Table 1.

2.3 Experimental methods

2.3.1 Reagents

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

Preparation of nano-C_{60} suspension: Fullerene C_{60} was purchased from Shanghai Maclin Biochemical Technology Co., Ltd.: purity, 99.9%. Antibiotics were purchased from Shanghai Aladdin Co., Ltd.: Tetracycline (97%, CAS: 6153-64-6) – chemical formula, $C_{22}H_{24}N_2O_9{\cdot}2H_2O$; relative molecular weight, 496.46, purity ≥98%, and stored in a 4°C refrigerator before use.

2.3.2 Experiments

The study set up a total of 6 groups of experiments, each group adding 120 g of chicken manure and 300 g of inoculated sludge; the maximum residual concentration of oxytetracycline (OTC) was 47.25 mg/kg TS, approximately taken as 50 mg/kg TS, and added at 50, 100, and 200% of the maximum residual concentration, respectively. In experiments T4 and T5, under a concentration of 50 mg/kg TS of OTC, 300 mg/kg TS of nano-Fe_2O_3, and 100 mg/kg TS of nano-C_{60} were added. The materials for each group are as shown in Table 2. After the feeding, deionized water was added to reach a final volume of 1 L, followed by nitrogen stripping for 2 min to remove the air. The tank was then sealed. The anaerobic digestion reactor was placed in a thermostat water bath at 37 ± 0.5°C and shielded from light. Four replicates were set up for each group. One replicate was only used for supplementing the material lost for the sampling detection, and the final result of each group was the average of the remaining three replicates. Throughout the experiments, the slurry was stirred manually twice daily, for 1 min each time.

2.4 Monitoring method

2.4.1 Analysis of physicochemical properties

The conventional indicators tested in this study mainly focus on the supernatant of the digestive fluid, including volatile fatty acids (VFAs) and pH value. The slurry supernatant was obtained by centrifugation of an appropriate amount of the digestion mixture using a high-speed refrigerated centrifuge at 9,000 rpm for 10 min, followed by filtration using a 0.45 μm filter membrane.

The specific testing method is as follows:

(1) VFAs: In this study, the composition of VFAs was determined using a gas chromatograph. The supernatant samples of sludge were further filtered through a 0.22-μm filter membrane, 1 mL of sample was added to the injection bottle along with 100 μL of chromatographic grade formic acid, and the sample was acidified to pH < 2.

The biogas production is measured using the drainage metering method, with measurements taken once daily.

The components of biogas were analyzed by gas chromatography. The analysis conditions are as follows: chromatographic column: stainless steel packed column produced by the National Chromatography Research and Analysis Center of Dalian Institute of Chemical Physics, Chinese Academy of Sciences, with TDX-01 as the filler, 2 m × 3 mn; detector: TCD;

Table 1: Properties of the chicken manure and the acclimated sludge [14]

Substance	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
Acclimated sludge	2.21	0.87	19.970	3.270	74.888	1.180	0.692	16.930

TS, total solids; VS, volatile solid.

Table 2: Experimental design

Serial no.	Fresh chicken manure (g)	OTC (mg/kg TS)	Nano-Fe_2O_3 (mg/kg TS)	Nano-C_{60} (mg/kg TS)	Initial concentration (μg/L)	Amount of acclimated sludge (g)
CK	120	0	0	0	0	300
T1	120	25	0	0	700	300
T2	120	50	0	0	1,900	300
T3	120	100	0	0	3,300	300
T4	120	50	300	0	1,900	300
T5	120	50	0	100	1,900	300

carrier gas: He, flow rate of 20 mL min^{-1}; current, 100 mA; attenuation, 1; detection temperature, 200°C; column oven temperature, 180°C; injection temperature, 200°C.

(2) pH: The pH value is determined by the glass electrode method.

2.4.2 Determination of the content of antibiotic tetracycline

About 2 g of sample was added to a 50 mL centrifuge tube and then 10 mL of 0.1 mol/L EDTA-Mellvaine buffer; the mixture was homogenized for 1 min, sonicated for 10 min in an ice bath, and centrifuged at 10,000 rpm for 5 min at 4°C. The above steps were repeated, the supernatants from the two times were combined, and 15 mL of water-saturated *n*-hexane and vortex was added; centrifuge at 10,000 rpm for 5 min at 4°C; and set aside.

Solid phase extraction uses an HLB column (3 mL, 60 mg), sequentially activated with 5 mL of methanol and 5 mL of ultrapure water, accurately measures 10 mL of sample, washes with 5 mL of water, discards all liquid and dries, elutes with 6 mL of methanol, collects eluate in a 10 mL centrifuge tube, and dries. Dried with 45°C nitrogen, 0.2% formic acid water–methanol (50:50) made up to 1 mL, sonicated, passed through a 0.22-μm organic membrane filter, bottled, and tested on the machine.

3 Results and discussion

3.1 Effects of nanoparticles on the anaerobic digestion properties of OTC-containing chicken manure

3.1.1 Effects of nanoparticles on the anaerobic biogas production from OTC-containing chicken manure

Nanoparticles have a promoting effect on anaerobic gas production and gas accumulation in livestock and poultry manure containing OTC, as shown in Figure 2. In the 55-day anaerobic process, two gas production peaks appeared. The first gas production peak occurred from days 1 to 10 of the experiment, with T1–T3 reaching their peak values on day 1 without the addition of nanoparticles, at 271 ± 17, 390 ± 13, and 199 ± 15 mL, respectively. The blank group CK reached its first gas production peak on the day 3, at 166 ± 12 mL. In contrast, the T4 group with added nanoparticles reached its peak gas production on day 1, at 423 ± 21 mL, and the T5 group reached its peak on day 2, at 445 ± 31 mL. The cumulative gas production during this stage was: 2,234(T5) > 2,163(T4) > 1,445(T2) > 1,289(T3) > 1,220(T1) > 1,216(CK) mL. It can be seen that nano-Fe_2O_3 and nano-C_{60} have a promoting effect on anaerobic gas production in livestock and poultry manure containing different concentrations of OTC. In the first 10 days of the reaction, the cumulative gas production of T4 and T5 increased by 77.29 and 83.11%, respectively. After the first gas production peak (days 1–10), the system began to acidify, and gas production gradually stagnated. After adding alkaline conditions, gas production in each group resumed on day 25. The addition of OTC caused differences in the appearance of the second gas production peak in each group, with T2 and T3 reaching their peak gas production on days 32 and 33, at 360 ± 17 and 410 ± 22 mL respectively, while CK and T1 had delayed peak periods on days 36 and 38, with daily gas production of 230 ± 26 and 181 ± 37 mL, respectively. The results indicate that OTC stimulates gas production in the system, with a more pronounced effect at higher concentrations. Wang [15] found through batch experiments that 5 and 10 mg/L of OTC, respectively, increased methane production in anaerobic digestion of chicken manure by 3.38 and 7.11%, and calculated an inhibition threshold of 24.32 mg/L, which is similar to the results of this study. The stimulating effect of tetracycline antibiotics has also been observed in plants. Pomati et al. [16] found that 0.1 mg/L of tetracycline promoted the growth of duckweed, while 1 mg/L had a significant inhibitory effect on its cultivation and development.

The addition of nanoparticles T4 and T5 on day 37 reached a second peak of gas production, with volumes of 187 ± 12 and 198 ± 4 mL, respectively. The final

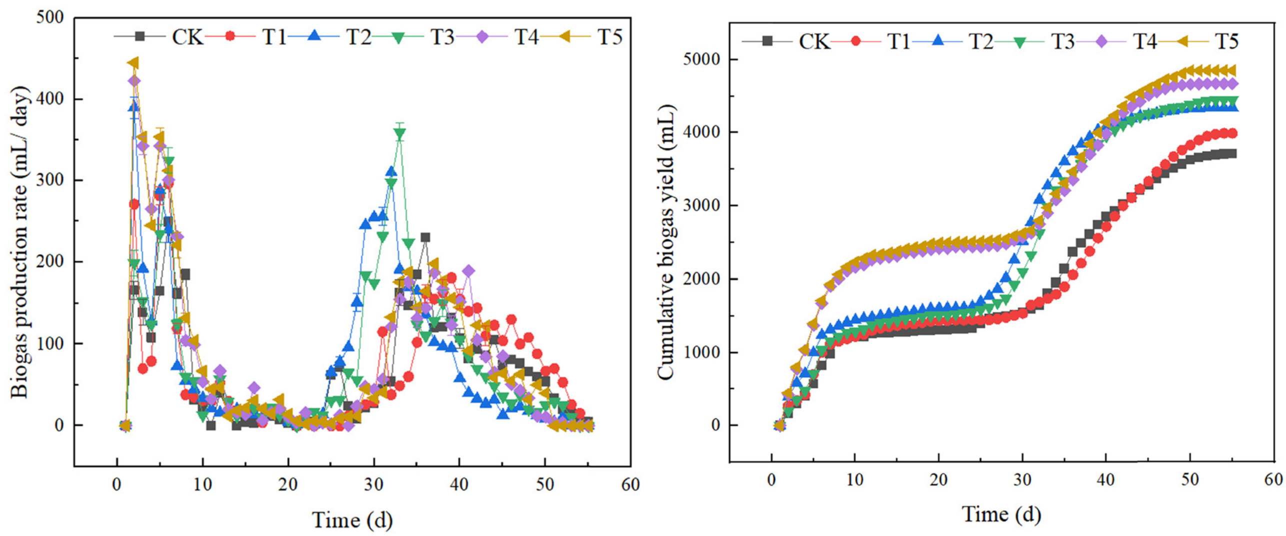

Figure 2: Effects of nanoparticles on changes in daily biogas production rate and cumulative biogas production from anaerobic digestion of OTC-containing chicken manure.

cumulative gas production for each group was 3,712(CK), 3,993(T1), 4,344(T2), 4,447(T3), 4,671(T4), and 4,849(T5) mL. The research results show that the addition of nano-Fe_2O_3 and nano-C_{60} has a promoting effect on anaerobic gas production in livestock manure with different concentrations of OTC. Adding carbon-based nanomaterials can enhance direct interspecies electron transfer between electron-supplying bacteria and methane-producing archaea, promote the conversion of carbon dioxide to methane, shorten the lag phase required for starting biogas production, and increase methane production. Nanoparticles have excellent properties different from other common materials. The ultra-small structure of nanoparticles results in a surface effect, where the surface area, surface energy, and surface tension of nanoparticles increase sharply with decreasing particle size. This makes nanoparticles highly chemically active and easily react with other chemicals, acting as a medium to promote the gas production effect of microbial hydrolysis of substrates. At the same time, the release of metal ions from nano-Fe_2O_3 and nano-C_{60} is considered one of the most common and important ways to affect the anaerobic digestion of sludge. Fe^{3+} is an essential trace element for anaerobic microbial growth and metabolism and is beneficial to microorganisms within a certain range. Mu et al. [17] and others have also reached similar conclusions, suggesting that when the concentration of nano-Fe_2O_3 is low, the released Fe^{3+} can chelate with negatively charged residues in extracellular polymeric substances (EPS), reducing ion toxicity.

3.1.2 Effects of nanoparticles on the changes in pH and TVFA levels during anaerobic digestion of OTC-containing chicken manure

Nanoparticles affect the pH and TVFAs concentration changes during the anaerobic digestion process of livestock and poultry manure containing different concentrations of OTC as shown in Figures 3 and 4. VFAs are produced by hydrolysis-producing acid bacteria, among which formic acid and acetic acid are

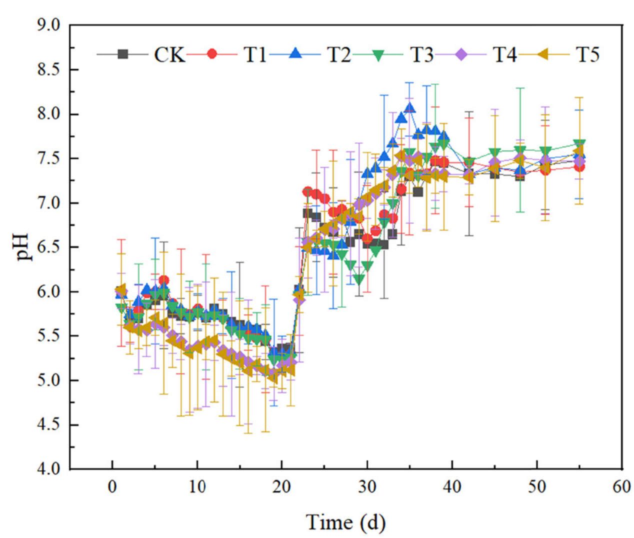

Figure 3: Effects of nanoparticles on changes in pH value during anaerobic digestion of OTC-containing chicken manure.

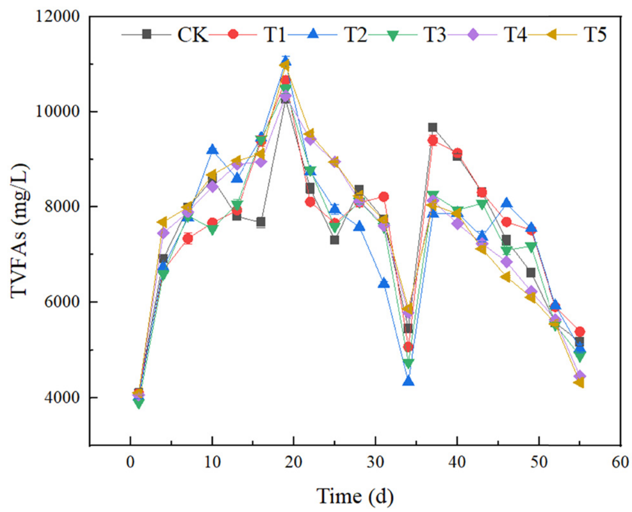

Figure 4: Effects of nanoparticles on changes in TVFAs level during anaerobic digestion of OTC-containing chicken manure.

metabolic substrates for methane-producing bacteria, but excessive acid accumulation will inhibit the activity of methane-producing bacteria, reducing the gas production efficiency of the anaerobic system. As shown in Figure 4, the TVFAs concentration of each group was in a rapid increase period from day 1 to day 10, and the VFA concentration of T4 and T5 was higher than the other four groups. It can be seen that the presence of nano-Fe_2O_3 and nano-C_{60} has a promoting effect on the hydrolysis of livestock and poultry manure containing antibiotics OTC, and their ultrafine structure as a medium is conducive to the decomposition of substrates by hydrolysis-acidifying microorganisms. In the early stage of the experiment, it showed a continuous rapid increase trend, reaching the maximum peak value of 10269.2(CK), 10673.61(T1), 11056.18(T2), 10488.7(T3), 11034.5(T4) and 10,987(T5) mg/L on day 19. At this time, a large amount of free H+ was accumulated in the system due to the accumulation of TVFAs, which severely inhibited the activity of methane-producing bacteria, leading to the cessation of methane production reaction. At this time, the pH of each group continued to decrease, and on day 19, the pH of the group without added nanoparticles decreased to 5.30–5.37, while the pH of the groups with added nano-Fe_2O_3 and nano-C_{60} was lower, at 5.05 ± 0.3 and 5.03 ± 0.1 respectively. Just like the TVFAs research, the addition of nanoparticles to a certain extent promotes the hydrolysis of substrates to produce organic acids, leading to a decrease in system pH. Latif et al. [18] found that when the pH in the anaerobic digestion system was 5.5, the severe accumulation of propionic acid and butyric acid led to a 50% reduction in methane production, and the proportion of incompletely degraded organic matter would increase. When the pH value was lower than 5.5, the methane-producing community significantly decreased and shifted to a propionic acid-utilizing community. Adjusting the pH of each group to above 6.5, the activity of methane-producing bacteria recovered, and volatile acids were rapidly utilized and converted into methane. On day 34, the TVFAs concentration of CK, T1, T2, T3, T4, and T5 decreased to 5457.87, 5063.61, 4329.29, 4737.36, 5785.4, and 5876.4 mg/L, respectively, and on day 37, they rapidly increased again to 9678.20, 9411.72, 7855.33, 8263.40, 8133.3, and 8043.2 mg/L, indicating that at this time, the hydrolysis rate of difficult-to-decompose substances such as cellulose was higher than the utilization rate of methane-producing bacteria. After day 37, as the substrate decreased, the density of hydrolysis-producing acid bacteria decreased, and the TVFA concentration gradually decreased. At the end of the experiment, the pH changes of each group were small, ranging from 7.30 to 7.67.

3.1.3 Effects of nanoparticles on the degradation rate of OTC

The addition of nanoparticles in the anaerobic digestion process of livestock manure antibiotic OTC degradation rate is shown in Table 3. The trends of T1–T5 are the same, with OTC being rapidly degraded on days 1–5. The addition of nano-Fe_2O_3 and nano-C_{60} groups has higher degradation rates of antibiotic OTC compared to the other three groups. The degradation rate of OTC in the group without nanoparticles on day 5 is 91.34–96.29%, while the degradation rates after adding nano-Fe_2O_3 and nano-C_{60} are 96.97 and 97.08%, respectively. The concentrations then tend to stabilize, with residual amounts on day 15 being 15.25, 20.40, 56.56, 17.67, and 16.89 μg/L, and degradation rates of 98.31, 98.80, 98.29, 99.07, and 99.11%, respectively. The results show that anaerobic digestion has a significant effect on the removal of OTC. Studies have shown that under conditions without nanoparticles, adsorption is the main removal pathway for anaerobic reaction systems to remove OTC drugs [19]. Yin et al. [20] found that

Table 3: Concentration and degradation rate of antibiotic OTC during anaerobic digestion of livestock and poultry manure

	0 day (μg/L)	5 day (μg/L)	Degradation rate on 5 day (%)	15 day (μg/L)	Degradation rate on 15 day (%)
T1	700	65.75	92.69	15.25	98.31
T2	1,900	63.13	96.29	20.40	98.80
T3	3,300	285.83	91.34	56.56	98.29
T4	1,900	57.66	96.97	17.67	99.07
T5	1,900	55.43	97.08	16.89	99.11

when the OTC addition concentration is less than 40 mg/kg TS, the anaerobic system can completely remove it. When the OTC concentration range is 40~100 mg/kg TS, the antibiotic removal rate follows the exponential equation $y = 6.6676x - 0.516$. Adding 300 mg/kg TS of nano-Fe_2O_3 and 100 mg/kg TS of nano-C_{60} can further degrade the antibiotic OTC on the basis of traditional mesophilic anaerobic digestion, indicating that adding nanoparticles can promote gas production in anaerobic digestion while also having a synergistic metabolic effect on antibiotic OTC. Other studies have found that tetracycline antibiotics cannot be completely degraded in the anaerobic digestion process, and their metabolites still have similar biological toxicity to the parent compounds and are easily adsorbed to solid phases, making them more environmentally persistent. Therefore, the degradation degree of antibiotics needs to be taken seriously [21–23].

3.1.4 Effects of OTC on the activities of different enzymes

Anaerobic digestion produces biogas, which is the result of enzymatic reactions gradually carried out under the action of different functional microbial communities. The activity of dehydrogenase is an important indicator of microbial status. The changes in dehydrogenase activity during anaerobic digestion with different concentrations of OTC are shown in Figure 5. The concentration of dehydrogenase showed a trend of first increasing and then decreasing throughout the entire experimental period. The dehydrogenase activity in the antibiotic group was higher than that in the control group. The addition of 300 mg/kg TS of nano-Fe_2O_3 and 100 mg/kg TS of nano-C_{60} had the highest

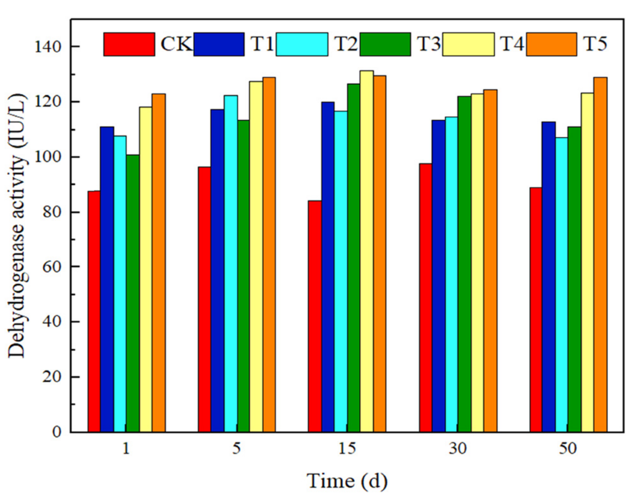

Figure 5: Changes in dehydrogenase activity at different OTC concentrations.

dehydrogenase activity. The average dehydrogenase activity of each group was 127.02(T5) > 124.74(T4) > 114.38(T2) > 114.98(T3) > 113.87(T1) > 90.88(CK) IU/L, indicating that the addition of nanoparticles stimulates microbial activity.

The changes in anaerobic digestion enzyme activity of different concentrations of OTC are shown in Figure 6. The addition of antibiotics and nano-particle groups showed a trend of first increase and then decrease in amylase activity, while the control group showed no significant changes. On the first day, there was no significant difference in amylase activity among the groups, all ranging 291.37–321.21 IU/L. On the fifth day, the amylase activity in the control group decreased to 242.47 IU/L, while T1–T3 were stimulated by OTC, with significantly increased amylase activity of 321.61, 313.84, and 366.44 IU/L, respectively, and the stimulating effect continued until the end of the experiment, with T4 and T5 groups at 324.3 and 332.5 IU/L, respectively. T3 reached its maximum value of 429.86 IU/L on day 15, while T1 and T2 reached their maximum values of 406.56 and 461.16 IU/L on day 30. The mean enzyme activity of each group was 384.08(T5) > 381.48(T2) > 378.12(T4) > 365.35(T3) > 364.79(T1) > 268.26(CK) IU/L. OTC has a stimulating effect on amylase activity, with the best promoting effect at a concentration of 50 mg/kg TS, and the addition of 100 mg/kg TS nano-C_{60} has a better stimulating effect on amylase activity.

Cellulose can be hydrolyzed into cellobiose by cellulase, and then further broken down into glucose. The change in cellulase activity can reflect the degradation of carbon substances during the fermentation process [24]. Chicken manure has a high cellulose content, with a longer hydrolysis time compared to starch and protein. The cellulase activity level in the system is significantly higher than other hydrolytic enzymes. As shown in Figure 5, on the first day, the cellulase activity in the antibiotic group was significantly enhanced. On the fifth day, it was the peak period of the first gas production, with enzyme activity levels of 131.77(CK), 251.21(T1), 292.50(T2), 198.96(T3), 273.6(T4), and 265.9(T5) IU/mL. The addition of 300 mg/kg TS of nano-Fe_2O_3 and 100 mg/kg TS of nano-C_{60} resulted in the highest cellulase activity. On day 30, each group was in the peak period of the second gas production, with enzyme activity data of 158.46(CK), 263.32(T1), 259.13(T1), 248.84(T3), 274.6(T4), and 289.9(T5) IU/mL. The difference in enzyme activity between the groups was small, but all were significantly higher than the control. On day 15, group T2 reached the maximum enzyme activity of 358.79 IU/mL. As cellulose was utilized, the density of cellulose-hydrolyzing bacteria decreased, leading to a significant decrease in enzyme activity. The average enzyme activity values were 275.43(T2) > 259.34(T5) > 253.41(T4) > 243.84(T3) > 242.26(T1) > 143.98(CK), indicating that tilmicosin stimulates cellulase

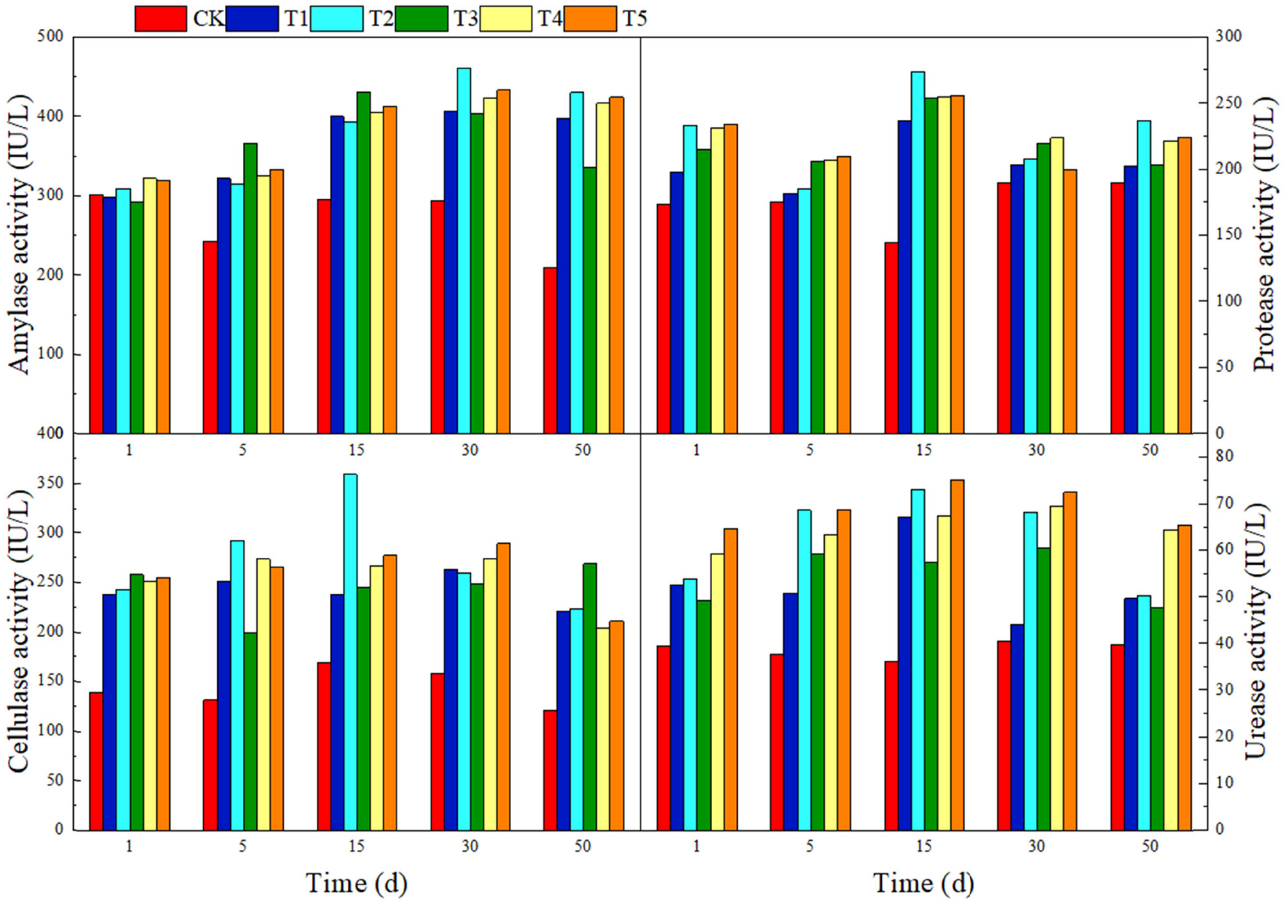

Figure 6: Changes in activities of hydrolytic enzymes at different OTC concentrations.

activity, with the best promoting effect at a concentration of 50 mg/kg TS.

Determining the activities of protease and urease is of great significance for understanding the decomposition and metabolism of nitrogenous organic matter in anaerobic digestion slurry and the changes in ammonia nitrogen concentration. Protein degradation is divided into two stages: extracellular and intracellular. Proteins are broken down into proteose and peptides under the catalysis of protease and eventually converted into amino acids that can be utilized by methane-producing bacteria. The average activities of protease are 227.56(T4) > 227.21(T2) > 224.21(T5) > 219.48(T3) > 204.17(T1) > 174.52(CK) IU/L. The protease activity in the antibiotic group is higher than in the control group, indicating that tetracycline stimulates protease. Among them, the protease activity level of T2 is the highest, and the addition of nanoparticles has no significant effect on protease activity. Urease activity data shows that the antibiotic group generally shows a trend of first increase and then decrease, while the control group shows little change over time. Throughout the experiment, the urease activity of CK has been maintained at 40 IU/mL. On the first day, the antibiotic group was significantly higher than the control group, and then, the difference in urease activity between T1–T3 and CK continued to increase until day 15. T1, T2, and T5 reached their maximum values at 15 days, which were 67.04, 73.06, and 75.05 IU/mL, respectively. Groups T3 and T4 reached their maximum values at 30 days, which were 68.20 and 69.53 IU/mL. The average activities of the enzymes in each group are 69.21(T5) > 64.79(T4) > 62.83(T2) > 54.84(T3) > 52.83(T1) > 38.78(CK) IU/mL. The addition of nanoparticles T4 and T5 has a promoting effect on urease activity. Pan et al. [25] found that 60 and 100 mg/kg tetracycline activates urease activity in the early stage of anaerobic fermentation and has a certain promoting effect on methane production. Gas production was significantly inhibited in the later stages of the experiment. They speculated that in the early stages of the experiment, urease may use the amide bond of tetracycline as a substrate and induce an increase in urease activity through substrate induction.

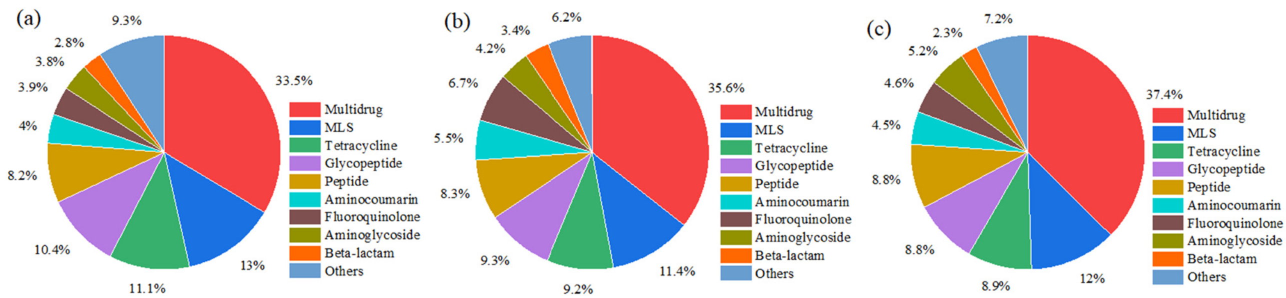

Figure 7: (a), (b) and (c) represent pie charts of ARGs for chicken manure and sludge in T2, T4 and T5 groups, respectively.

3.2 Nanoparticles on OTC-resistant genes in livestock and poultry manure

The anaerobic digestion system itself has a certain tolerance, and the self-regulation ability during operation is also a guarantee to cope with toxic environments. Yu et al. [26] found through a 180-h OTC waste sludge batch mesophilic anaerobic digestion experiment that 5 mg/L tetracycline did not have a negative impact on the anaerobic digestion system, while 50 mg/L tetracycline would acutely inhibit methane production activity in the system, with inhibition relieved at 141 hours and methane production reduced by 23.75%. Wei et al. [27] found through OTC pig manure mesophilic batch anaerobic digestion experiments that 10–1,000 mg/kg tetracycline could significantly increase biogas production, with 50 mg/kg showing the best promoting effect. When the OTC addition concentration was 2,000 mg/kg, it would severely inhibit microbial activity. The inoculum used by Wei was domesticated with antibiotics before use, showing a certain degree of resistance. Although the antibiotic content in the chicken manure and activated sludge used in this study was below the detection limit, the metagenomic sequencing results in Figure 7 showed that there were various types of ARGs in groups T2, T4, and T5, with the multidrug resistance gene (ARGs)-Multidrug accounting for a high proportion of 33.54, 35.63, and 37.43%, respectively, indicating a certain resistance of the anaerobic digestion system to tetracycline. The proportions of the other four types of MLS, tetracycline, glycopeptide, and peptide were between 8.18% and 12.98%. It can be seen that the addition of 300 mg/kg TS of nano-Fe_2O_3 and 100 mg/kg TS of nano-C_{60} can increase the proportion of multidrug resistance genes, while not reducing the proportions of MLS, tetracycline, glycopeptide, and peptide resistance genes, to a certain extent enhancing the inhibitory effect of the anaerobic digestion system on tetracycline. The use of antibiotics in agricultural breeding processes can lead to the presence of resistant bacteria and resistance genes in the intestines of livestock and poultry, which are then excreted with feces. Wastewater treatment systems serve as both a "sink" and a "source" of antibiotics, with activated sludge becoming the main carrier of ARGs. The higher biological density in anaerobic reactors may promote gene transfer, with resistant bacteria gaining an advantage under the selection pressure of antibiotics [28]. In addition, research [29] has shown that when microorganisms are exposed to toxic substances, EPS can release substrates to bind with toxic substances and form a protective barrier, which may be an important reason why anaerobic systems can resist toxic substances.

4 Conclusions

The addition of nano-Fe_2O_3 and nano-C_{60} has a promoting effect on the anaerobic gas production of livestock and poultry manure containing different concentrations of OTC. The nanoparticle group T4 reached peak gas production on the first day, with 423 ± 21 mL, while group T5 reached peak gas production on the second day, with 445 ± 31 mL. The cumulative gas production from day 1 to day 10 was 2,234(T5) > 2,163(T4) > 1,445(T2) > 1,289(T3) > 1,220(T1) > 1,216(CK) mL. The cumulative gas production of T4 and T5 increased by 77.29 and 83.11%, respectively. The cumulative gas production of each group was 3,712(CK), 3,993(T1), 4,344(T2), 4,447(T3), 4,671(T4), and 4,849(T5) mL.

The trend of changes in the degradation rate of antibiotic OTC during anaerobic digestion of livestock and poultry manure in T1–T5 is the same. The degradation rate of OTC without the addition of nanoparticles on the fifth day is 91.34–96.29%. The degradation rates after adding nano-Fe_2O_3 and nano-C_{60} are 96.97 and 97.08%, respectively. The concentrations then tend to stabilize, with residual amounts on day 15 being 15.25, 20.40, 56.56, 17.67, and 16.89 μg/L, and degradation rates being 98.31, 98.80, 98.29, 99.07, and 99.11%, respectively.

Over-the-counter antibiotics can increase the activity of dehydrogenase, amylase, protease, and urease. Adding nanoparticles can increase the activity of dehydrogenase and amylase, with no significant impact on cellulase, urease, and protease activity.

There are multiple types of ARGs in groups T2, T4, and T5, with the proportion of multidrug resistance genes (ARGs)-Multidrug being relatively high at 33.54, 35.63, and 37.43%, respectively. The proportions of the other four types of ARGs, MLS, tetracycline, glycopeptide, and peptide range from 8.18–12.98%.

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Data availability statement: The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval: The conducted research is not related to either human or animal use.

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