EFFECTS OF RESIDUAL SULFAMETHOXAZOLE ON BIOGAS PRODUCTION CHARACTERISTICS AND HYDROLYTIC ACIDIFICATION BACTERIA IN ANAEROBIC DIGESTION OF CHICKEN MANURE

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Abstract. To investigate the effects of sulfonamide antibiotics on the anaerobic digestion performance and hydrolytic acid-producing bacterial population at residual concentrations, this study was carried out in a sequential batch anaerobic digestion experiment with chicken manure as substrate at different concentrations (CK: 0, S1: 10 mg/kg·TS, S2: 20 mg/kg·TS, S3: 40 mg/kg·TS) of sulfamethoxazole (SMX) at a medium temperature of 37 °C. The biogas yield, chemical parameters, hydrolytic enzyme activity and changes in hydrolytic acidification bacteria were analyzed. The results showed that S1 and S2 significantly promoted biogas production, with 14.16% and 6.62% increase, respectively, compared with CK, and the delay period was shortened, while S3 had no significant effect on gas production; SMX could stimulate enzyme activity, and S2 had the highest level of enzyme activity; S1 and S2 bacterial community diversity increased, while S3 was inhibited in the early stage of the experiment, and the bacterial community diversity recovered with the degradation of sulfamethoxazole; the bacterial community structure was more stable. SMX was easily degraded in the digestive system, and the 5d removal rate was 99.64%- 99.89%, proving that medium temperature anaerobic digestion is a feasible solution for treating livestock manure containing sulfa drugs. This study can provide a research basis for efficient utilization and harmless treatment of livestock manure.
Keywords: *chicken manure; antibiotics; anaerobic digestion; microbial community; enzyme activity*

Introduction

Veterinary antibiotics, as therapeutic drugs and growth promoters for livestock and poultry, are heavily invested in the process of large-scale farming, but livestock and poultry cannot absorb them completely. Therefore, antibiotics and metabolites are frequently detected within the excreta. It has been noted (Zhao et al., 2017) that the introduction of antibiotics into the external environment may increase microbial resistance and pose a threat to human life and health. Therefore, antibiotic-contaminated livestock manure needs to be handled with care. Sulfonamide antibiotics are commonly used veterinary drugs in agricultural farming and are able to competitively inhibit amino benzoic acid in bacteria, thus preventing folic acid synthesis (Bilkova et al., 2019) and show strong antibacterial activity against Gram-positive and some Gram-negative bacteria (Al-Ahmad et al., 1999). The survey showed that the highest concentration of sulfamethoxazole in livestock manure in Northeast China reached 18 mg/kg (An et al., 2015).

The use of anaerobic digestion is economical and efficient for the treatment of organic solid waste and also provides clean energy biogas, but the residues of veterinary drugs in manure can impact on the digestive system, and the type and variation of antibiotics are

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the main factors affecting anaerobic biogas production (Ding and He, 2010). Cetecioglu found that sulfamethoxazole (SMX) at concentrations of 100-250 mg/L significantly inhibited gas production (Cetecioglu et al., 2012). However, it has also been shown that antibiotics have no significant negative effect on the anaerobic reaction and even biogas production was somewhat enhanced (Pan et al., 2019). Wen et al. (2020) found that 50 mg/L sulfamethoxazole increased methane production by 22% in high-temperature anaerobic digestion. Among them, gas production was doubled at 120 mg/kg concentration. Based on the different gas production results, it was concluded that there may be inhibition thresholds for antibiotics in anaerobic digestion systems.

Studies have shown that sulfonamide antibiotics can promote hydrolytic acidification reactions (Guo et al., 2012). To investigate the influence of SMX on the anaerobic digestion of chicken manure, we conducted a sequential batch anaerobic digestion experiment based on the residual concentration of SMX in chicken manure in Northeast China, and analyzed the changes of biogas production and chemical parameters during anaerobic digestion, focusing on the changes of hydrolytic enzyme activity and hydrolytic acid-producing bacterial community structure, to investigate the influence of SMX on the anaerobic digestion of chicken manure and hydrolytic acid-producing bacterial community, which can help to improve the anaerobic digestion gas production potential.

Materials and methods

Materials

The chicken manure used in this experiment was obtained from a broiler breeding base in Beipiao City, Liaoning Province, and the hard-to-degrade materials such as eggshells, feathers and stones were picked out from the chicken manure and stored in a refrigerator at 4 ℃ for use. The level of sulfamethoxazole in chicken manure was tested to be below the detection limit, and the experiment was conducted by exogenous dosing of antibiotics. Sulfamethoxazole (98%, CAS: 723-46-6) was purchased from Shanghai Aladdin Company, with chemical formula $C_{10}H_{11}N_3O_3S$ and relative molecular mass of 253.28. The sludge was stored in a refrigerator at 4 ℃ before use. The inoculated sludge was taken from the sludge thickening tank of the northern sewage treatment plant in Shenyang and used after being domesticated with chicken manure at 37 ℃ for 2 weeks. The characteristics of chicken manure and inoculated sludge are shown in *Table 1*.

Table 1. *Characteristics of chicken manure and inoculated sludge*

Material	Total solid (TS)/%	Volatile solid (VS)/%	C/%	H/%	O/%	N/%	S/%	C/N	SMX/mg·kg^{-1}
Chicken manure	27.32	24.49	47.338	6.256	43.786	1.981	0.639	23.896	ND
Inoculated sludge	2.21	0.87	19.970	3.270	74.888	1.180	0.692	16.930	ND

ND: not detected

Experimental method

The volume of the anaerobic digestion reactor was 1.2 L. The experimental setup is shown in *Figure 1*. In this study, four experimental groups CK, S1, S2 and S3 were set up, and 120 g of chicken manure and 300 g of inoculated sludge were added to each

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group, and different concentrations (0 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg) of sulfamethoxazole were added to the chicken manure according to its dry weight. After the end of feeding, the volume was fixed to 1 L with deionized water, and the tank was sealed by blowing off with nitrogen for 2 min to exclude air, and the actual antibiotic concentration was 400 μg/L (S1), 700 μg/L (S2), and 1300 μg/L (S3). The anaerobic digestion reactor was placed in a constant temperature water bath at 37 ± 0.5 °C and shaded. Two parallel experiments were set up for each group, one of which was used to supplement the material lost by the extraction test, and the results were taken as the mean value of the two groups. Manual stirring was performed twice a day for 1 min each time during the experiment.

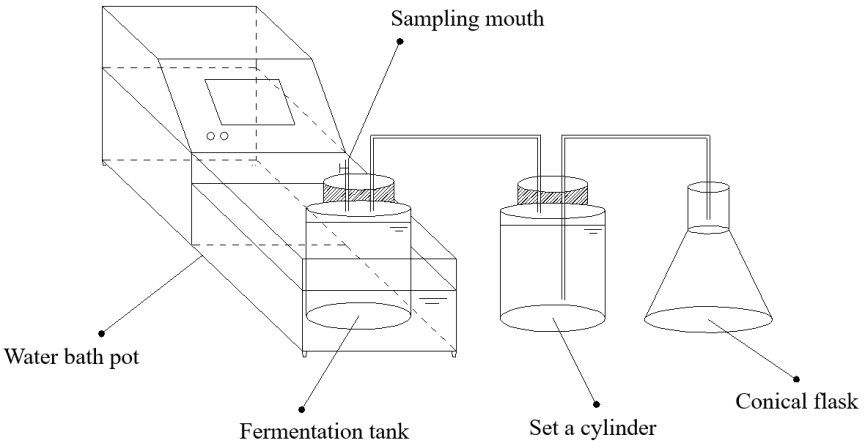

Figure 1. *Diagram of the device*

Measurement method

Determination of physical and chemical parameters

TS and VS were measured by the drying weight method and high-temperature cautery method, respectively. Daily gas production was measured by the drainage method, and fermentation broth pH was determined using a Remagnet PHS-2F pH meter (Shanghai Yidian Scientific Instruments Co., Ltd., China). 5 mL of digest was taken and centrifuged at 8000 r/min for 10 min, and the supernatant was passed through a 0.45 μm filter membrane and collected for chemical analysis. TAN (total ammonia nitrogen) and SCOD (soluble chemical oxygen demand) were determined by international standard methods (Cabrera et al., 2019). The concentration of TVFA was carried out according to the recommendations of the Standard Methods for the Examination of Water and Wastewater (Colmenarejo et al., 2004). The frequency of testing TAN concentration, SCOD and TVFAs concentration in the fermentation broth was 3 days, and the frequency of testing pH was 1 day in the first period and adjusted to 3 days on day 40.

Enzyme activity determination

The digests of day 1, 5, 15, 30 and 50 were extracted from four groups of reactors and crushed under ice bath conditions using an ultrasonic cell crusher (750 W, 40% power, 5 s sonication, 9 s interval) for 90 min. The crushed samples were centrifuged at a low speed of 10,000 r/min (4 °C) for 20 min, and the supernatant was stored at -20 °C

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for measurement. Amylase, cellulase, protease, urease and dehydrogenase activities were determined using the corresponding enzyme kits (Shanghai Enzyme Link Biotechnology Co., Ltd., China) according to the manufacturer's instructions.

The standard wells were first set up and 50 μL of enzyme standard reagents at different concentrations (400, 200, 100, 50, 25, 0 IU/L) were added to each of the standard wells. Set up blank control wells, sample wells to be tested, no sample and enzyme standard reagents are added to the blank control wells, and the rest of the operations are the same. The sample wells to be tested were first filled with 40 μL of sample dilution, and then 10 μL of sample to be tested, i.e. the final dilution of the sample was 5 times. Add 100 μL of enzyme reagent to each well, seal the plate with sealing film and incubate at 37 °C for 60 min. Remove the sealing film, discard the liquid and shake dry, fill each well with 20 times diluted washing solution, leave for 30 s and discard, repeat 5 times and pat dry. The color developer was added to each well first, shaken and mixed, and the color development was carried out at 37 °C for 15 min with the addition of 50 μL of the termination solution, and the blue color was immediately changed to yellow and measured within 15 min. The absorbance of each well was measured at 450 nm using a Rayto RT-6100 ELISA (Shenzhen Redu Life Science Co., Ltd., China), and the standard curve was plotted by zeroing in the blank control wells.

16S rRNA sequencing

The digests were extracted from four groups of reactors on days 1, 5, 15, 30 and 50, passed through 0.22 μm filter membranes, and the membranes were collected and stored at -20 °C for testing. Samples were extracted using Fast DNA® Spin Kit for Soil Extraction Kit (MP Biomedicals, USA) and DNA extraction was performed according to the instructions for use. Primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3'), 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3-V4 region of the bacterial 16S rRNA gene; primer 524F10extF (5'-TGYCAGCCGCCGCGGTAA-3') was used. The high-throughput sequencing platform was Miseq PE300 (Illumina, USA). Bacterial community Alpha diversity data (sobs, chao, shannon, simpson) were obtained by sequencing the bacteria of the samples to assess the sample group flora composition. The sobs, chao, and ace indices reflect community richness, and the shannon, simpson indices reflect community diversity.

SMX concentration measurement

Accurately weigh 5 g of the sample, add 20 mL of acetonitrile, homogenize, vortex and sonicate for 10 min each. Add 5 g of anhydrous sodium sulfate, vortex (do not clump), and centrifuge at 10,000 r/min for 10 min. The supernatant was transferred to a chicken heart flask, 7 mL of isopropanol was added, sonicated for 1 min, spun at 45 °C to about 5 mL, and the above operation was repeated. The chicken heart flask was washed with 15 mL of 0.1 mol/L HCl in three portions and transferred to a 50 mL centrifuge tube, and then the chicken heart flask was washed with 5 mL of n-hexane. The hexane was also transferred to a centrifuge tube, vortexed for 2 min, centrifuged at 10000 r/min for 5 min, and the lower layer of the hexane layer was discarded for purification.

The solid phase extraction was performed on an MCX column (60 mg, 3 mL), and the column was activated with 3 mL of methanol and 3 mL of 0.1 mol/L HCl in turn, and 8 mL of the upper sample was accurately measured. Wash with 3 mL of 0.1 mol/L HCl, 3 mL of 50% methanol in water, draw dry, and then elute with 5 mL of 5%

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ammonia in methanol. After nitrogen blowing at 50 °C, the solution was re-dissolved with 0.2% formic acid water: methanol (1:1), passed through 0.22 μm organic system filter membrane and determined on the machine.

Experimental equipment

Electric thermostatic water bath M246857 (Beijing Haifuda Technology Co., Ltd., China), High Temperature Oven 0030 (Suzhou Juxing Oven Equipment Co., Ltd., China), Magnetics PHS-2F pH Meter (Shanghai Yidian Scientific Instruments Co., Ltd., China), High Speed Centrifuge TG-16W (Shandong Boke Scientific Instruments Co., Ltd., China), Enzyme Reagent Kit (Shanghai Enzyme Union Biotechnology Co., Ltd., China), Rayto RT-6100 ELISA (Shenzhen Redu Life Science Co., Ltd., China), FastDNA® Spin Kit for Soil Extraction Kit (MP Biomedicals, USA), high-throughput sequencing platform Miseq PE300 (Illumina, Inc., USA).

Results and discussion

Effect of different concentrations of SMX on biogas production

The results of SMX content determination are shown in *Table 2*, and the degradation rate of SMX can reach 99.64%~99.89% at 5 d and 99.78%~99.94% at 15 d. Due to the rapid rate of SMX degradation, there was a lack of sufficient amount of data to fit the kinetics to it. The SMX was almost completely removed in the pre-experimental period, indicating its low environmental persistence within the anaerobic system, which is consistent with the results of other scholars. Mohring et al. (2009) found that a 34 day medium-temperature anaerobic digestion experiment of pig manure resulted in almost complete removal of SMX. Wen et al. (2020) obtained a half-life of 3 days for SMX at a concentration of 50 mg/L in a high temperature batch anaerobic digestion experiment in swine manure.

Table 2. *Changes in the content of sulfamethoxazole*

	0d μg/L	5d μg/L	5d removal rate	15d μg/L	15d removal rate
S1	400	1.45	99.64%	0.89	99.78%
S2	700	1.61	99.77%	0.79	99.89%
S3	1300	1.39	99.89%	0.81	99.94%

The daily gas production rate of anaerobic digestion with different concentrations of SMX is shown in *Figure 2*. After the experiment was started, the hydrolytic acid-producing bacteria decomposed the soluble organic matter rapidly, and at this time, the methanogenic bacteria had sufficient nutrients and suitable pH, and the methanation efficiency was high, and the peak gas production was reached on the first day. The rate of gas production in S1 was significantly higher than the other three groups at the beginning of the experiment, indicating a higher activity of the bacterial flora in their systems. After the first peak of gas production, the gas production of each group gradually stagnated, and the pH was adjusted to above 6.5 by adding NaOH on day 21~23. After day 24, the gas production of each group gradually recovered and entered the second peak of gas production. At this stage, insoluble macromolecular organic

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matter such as cellulose is gradually broken down into VFAs by hydrolytic acid-producing bacteria, which provide nutrients for methanogenic bacteria. The gas production rates of S1 and S2 groups increased rapidly, with S1 reaching a peak gas production of 231 mL on day 34 and S2 reaching a peak of 185 mL on day 40. While S3 peaked at day 45 with 390 mL, CK peaked at day 48 with 345 mL. It can be seen that CK and S3 have longer delay periods, higher peak gas production and shorter peak periods than S1 and S2. It was shown (Hu et al., 2018) that sulfonamide antibiotics are able to act as solubilizers by disrupting EPS and cell membranes, while enhancing acid and acetic acid production processes. This could be the reason for the high gas production efficiency of S1 and S2, while S3 may be due to higher SMX concentration and the onset of inhibition expression.

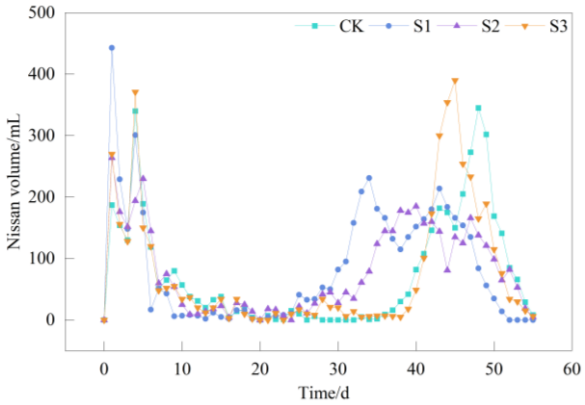

Figure 2. *Variation of daily gas production rate during anaerobic digestion with different SMX concentrations*

As shown in *Figure 3*, the cumulative gas production of each group at the end of the experiment was 4774 (S1) > 4459 (S2) > 4245 (S3) > 4182 (CK) mL, and it can be seen that SMX at three concentrations promoted anaerobic digestion gas production to different degrees, among which S1 and S2 promoted significantly, and the biogas production increased by 14.16% and 6.62%, respectively, compared with the control. Ma et al. (2021) continuously injected sulfadimethoxine during the experimental anaerobic digestion of cattle manure and found that the gas production efficiency was significantly improved after 6 days of experimental operation and the final biogas production was increased by 44.8%, which was similar to the results of this experiment, i.e., the overall gas production was not significantly changed by the antibiotic injection and the gas production efficiency was improved after the system was adapted. Wen et al. (2020) found that 50 mg/L sulfamethoxazole increased methane production by 22% in high-temperature anaerobic digestion, and it is possible that SMX played a role as a reaction substrate. There are currently two explanations for the result that antibiotics can promote anaerobic digestion gas production, one is as a substrate to supplement the carbon source for the flora (Dantas et al., 2008), and the other is the stimulatory effect of antibiotics on the flora (Zhi et al., 2019). Although SMX was almost completely degraded in the pre-experimental period, the theoretical contribution of antibiotics as substrate was much lower than the actual biogas increment, suggesting that SMX has some positive effect on the flora, while the pathway of SMX to promote gas production needs to be further explored.

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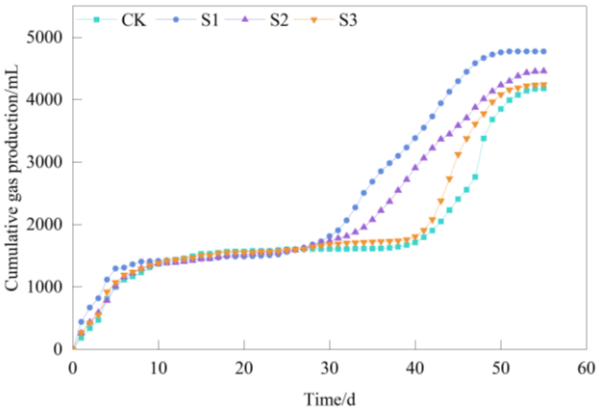

Figure 3. *Variation of cumulative biogas production during anaerobic digestion with different concentrations of SMX*

The gas production data showed that the residual concentration of SMX could anaerobically promote digestion and gas production, but as the promotion effect became less obvious with the increase of SMX concentration, the inhibition of antibiotics was gradually expressed, and the inhibition threshold of SMX was presumed to be higher than 40 mg/kg. It was determined that the residual amount of SMX in chicken manure was not sufficient to negatively affect the anaerobic digestion system.

Effect of different SMX concentrations on chemical parameters

The pH changes of anaerobic digestion with different concentrations of SMX are shown in *Figure 4*. After the first peak of gas production (day 1~5), the systems in each group began to acidify, and the pH showed a decreasing trend from day 6 to 20. The overall level of pH in the CK system was the lowest among the four groups, while the overall level of pH in the S1 and S2 systems was close to and higher than that in S3. After pH adjustment, all groups showed a decreasing trend, and then S1 and S2 were the first to increase on days 30 and 32, respectively, until pH recovered above 7 on days 34 and 38. CK and S3 were significantly lower than the other two groups from day 34 to 39, and pH started to recover significantly after day 39, which was consistent with the gas production results.

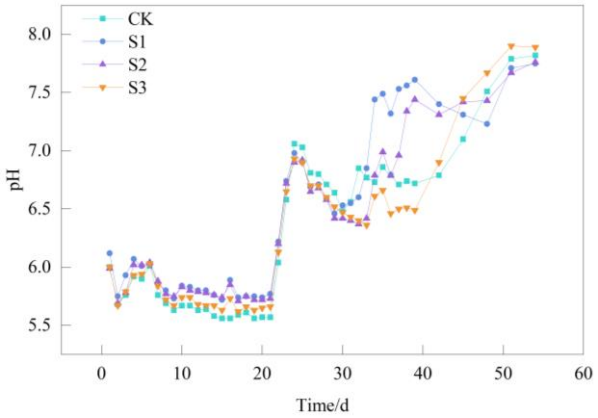

Figure 4. *pH change during anaerobic digestion with different concentrations of SMX*

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VFAs are both products of hydrolysis acid-producing bacteria and nutrients for methanogenic bacteria, which are in dynamic changes in the anaerobic digestion system, and the changes of TVFAs in anaerobic digestion with different concentrations of SMX are shown in *Figure 5*. TVFAs in each group showed a trend of increasing and then decreasing in the early stage of the experiment, and S1 had a lower level of TVFAs concentration in the early stage of the experiment, indicating that S1 colony had a higher substrate utilization rate. After adjustment with alkali, the concentration of TVFAs started to increase in each group, which corroborated with the previous trend of decreasing pH. S1 and S2 peaked again on day 30 at 8104.00 and 7689.55 mg/L, corresponding to pH values of 6.53 and 6.42. In contrast, CK and S3 continued to rise until day 34 when they reached their peak values of 10140.23 and 9187.95 mg/L, with corresponding pH of 6.73 and 6.61, while gas production was still stagnant at this time, presumably the activity of methanogenic bacteria in both groups was not fully recovered by systemic acid inhibition. Some studies have shown that some of the antibiotic derivatives are still biotoxic and SMX degradation products need to be further investigated (Langbehn et al., 2021).

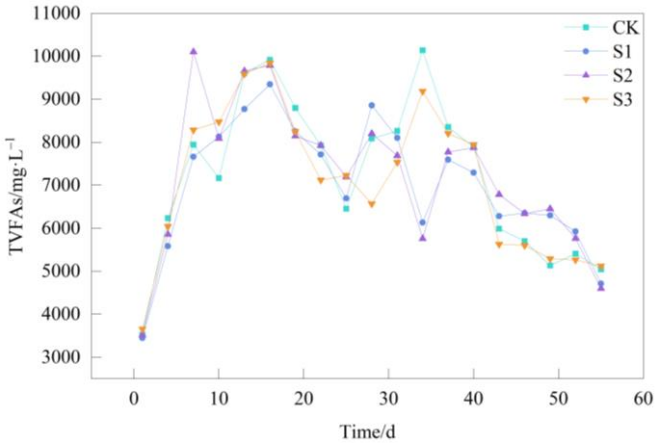

Figure 5. *Changes in TVFAs during anaerobic digestion with different concentrations of SMX*

Figure 6 represents the change of ammonia nitrogen concentration in the anaerobic system for each group, and the trend of TAN change was similar for each group throughout the experiment. The first peak of gas production (day 1~5), protein and urea were rapidly decomposed by the corresponding flora, and TAN concentration increased rapidly, reaching the first peak of 1112, 1169, 1126, 1103 mg/L on day 10, then the flora was affected by the acidification of the system, the TAN concentration changed less and showed a slow decreasing trend, on day 22 after alkali conditioning ammonia nitrogen concentration began to increase rapidly, reaching the maximum value of 1360, 1258, 1406, 1345 mg/L on day 31, then it has been decreasing trend, which indicates that the substrate was consumed. The difference in ammonia nitrogen concentration between the groups during the experimental run was not obvious, and the TAN concentration varied from 387 to 1406 mg/L, which was lower than 3000 mg/L, indicating that SMX has a small effect on ammonia nitrogen in the anaerobic digestion system (Niu et al., 2014). The changes of SCOD for anaerobic digestion at different concentrations of SMX are shown in *Figure 7*. The trend of SCOD concentration changes was similar in all groups, and the SCOD concentrations in S1 on days 1-10

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were lower than those in the other three groups, which corresponded to higher gas production rates, which indicated that the methanogenic bacteria in S1 utilized the substrate at a faster rate. From day 40 onwards S1 and S2 were the first to decline rapidly, indicating that the substrate in the system was completely depleted and the flora had entered the decay phase.

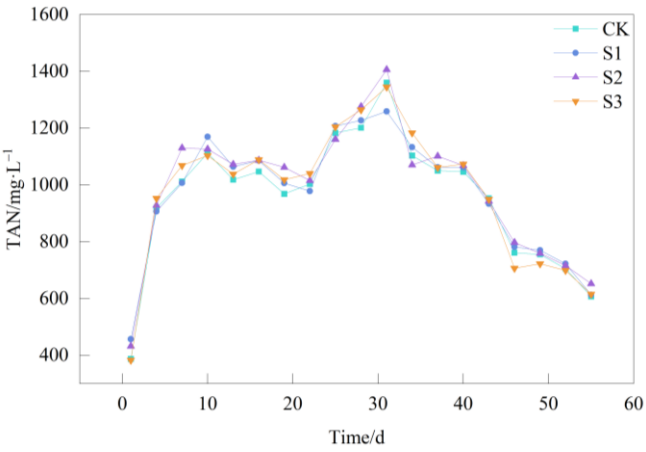

Figure 6. *Variation of TAN during anaerobic digestion with different concentrations of SMX*

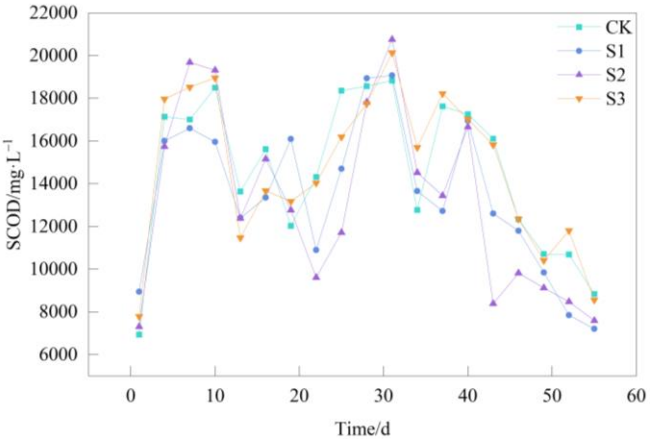

Figure 7. *Variation of SCOD during anaerobic digestion with different concentrations of SMX*

Effect of different concentrations of SMX on enzyme activity

The production of biogas by anaerobic digestion is the result of progressive enzymatic reactions under the action of different functional groups of bacteria, of which dehydrogenase activity is an important indicator to characterize the microbial status. The changes of dehydrogenase activity for different concentrations of SMX anaerobic digestion are shown in *Figure 8*. The dehydrogenase activity of the antibiotic group was higher than that of the control group throughout the experiment, and the mean values of dehydrogenase activity for each group were 119.38 (S2) > 114.88 (S3) > 111.57 (S1) > 100.78 (CK) IU/L, with S2 having the highest dehydrogenase activity.

The hydrolysis rate governs the overall rate of anaerobic reaction and directly affects the gas production performance, while the hydrolysis status can be expressed visually

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by hydrolase activity. The activity states of four extracellular hydrolases, amylase, cellulase, protease and urease, were monitored at different stages according to the main components of the substrate. The changes of hydrolytic enzyme activity during anaerobic digestion with different concentrations of SMX are shown in *Figures 9-12*. The mean values of amylase activity during the experiment were 400.44 (S2) > 368.41 (S3) > 329.68 (S1) > 281.65 (CK) IU/L, all of which were higher in the antibiotic group than the control group. In the early stage of the experiment, S2 was significantly higher than the other three groups on day 1, while on day 5 it rapidly decreased and This may be due to the stimulating effect of antibiotics, which accelerated the decomposition of starch by the corresponding hydrolytic flora, and with the substrate being consumed rapidly, the density of flora decreased leading to the decrease of amylase activity. The enzyme activity of S2 was kept high in the middle and late stages of the experiment. Chicken manure is rich in crude fiber and other hard-to-decompose substances, and the cellulase activity status can reflect the cellulose decomposition in the anaerobic system. The mean value of cellulase activity during the experiment was 232.44 (S3) > 216.84 (S2) > 203.50 (S1) > 172.62 (CK) IU/mL, and the antibiotic group was significantly higher than the control group at all stages of the experiment. The cellulase activity of each group showed an overall trend of increasing and then decreasing during the experiment. In the early stage of the experiment, the cellulase activity increased correspondingly with the increase of SMX concentration, indicating that SMX would stimulate the secretion of cellulase by the colony. The cellulase activity reached maximum values of 199.64, 240.92, 242.39, and 280.85 IU/mL on day 15, respectively. The crystalline region of cellulose is difficult to degrade due to its dense structure, and the breakdown to glucose is slower. Cellulose was the main contributing substrate to the second peak of gas production, so the cellulase activity was significantly higher in the middle and late stages of the experiment than in the early stages. On the 50th day, the cellulase activity decreased in each group and the gap between groups narrowed, indicating that cellulose had been gradually and completely hydrolyzed.

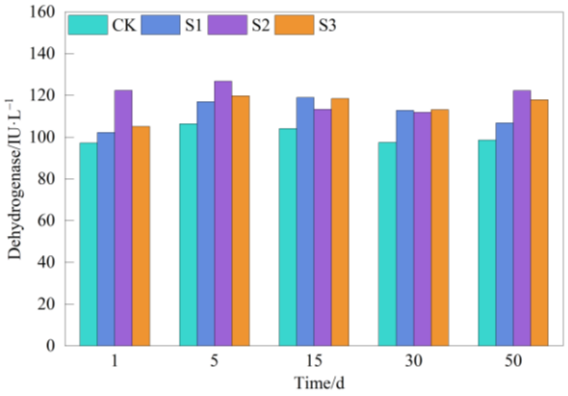

Figure 8. *Changes in dehydrogenase activity during anaerobic digestion with different concentrations of SMX*

Crude protein occupies a large proportion of the total organic matter in chicken manure, so protease activity is a key object to be monitored. The mean value of protease activity during the experiment was 216.99 (S2) > 201.75 (S3) > 190.81 (S1) > 160.62 (CK) IU/L, and SMX obviously had a promoting effect on protease activity. The S1

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protease activity was higher than S2 and S3 in the early stage of the experiment, which might be due to the inhibition of protein hydrolytic flora at 20 and 40 mg/kg·TS concentrations, while S2 and S3 maintained higher activity in the middle and late stage of the experiment as the inhibition was lifted by the continuous degradation of SMX.

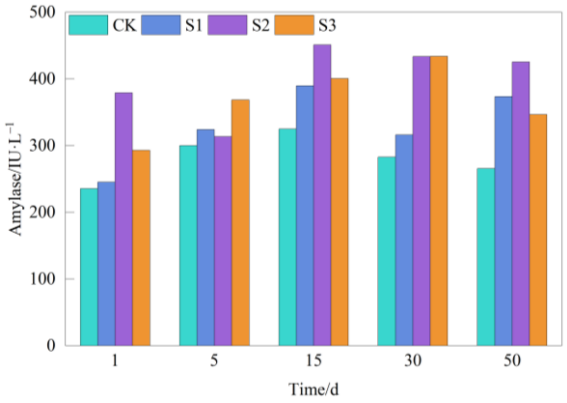

Figure 9. *Changes in amylase activity during anaerobic digestion with different concentrations of SMX*

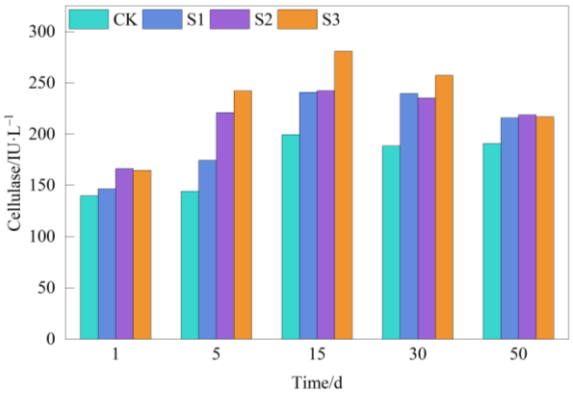

Figure 10. *Changes in cellulase activity during anaerobic digestion with different concentrations of SAM*

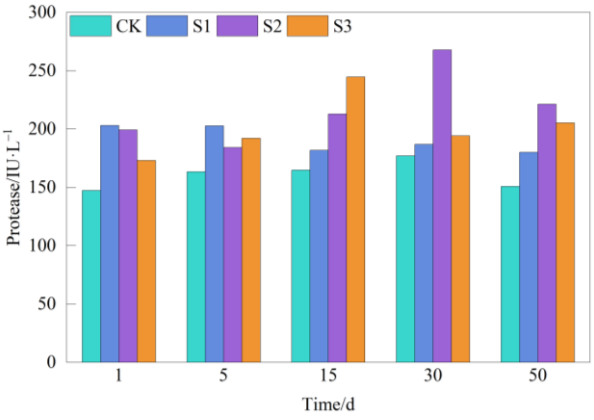

Figure 11. *Changes in protease activity during anaerobic digestion with different concentrations of SMX*

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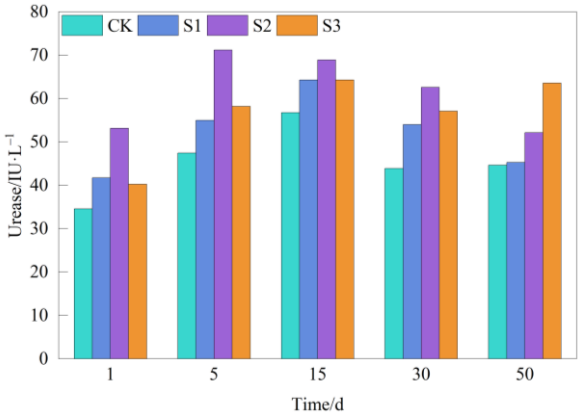

Figure 12. *Changes in urease activity during anaerobic digestion with different concentrations of SMX*

Urease is able to break down the amide C-N bond into ammonia to maintain the nitrogen cycle. The mean value of urease activity during the experiment was 61.59 (S2) > 56.69 (S3) > 52.06 (S1) > 45.46 (CK) IU/mL, and the urease activity of the control group remained lower than that of the antibiotic group throughout the experiment, which was similar to the results of other extracellular enzymes, and sulfamethoxazole would have a stimulating effect on urease activity. The trend of urease activity was similar in all groups, with a rapid increase in the early stage of the experiment. CK, S1 and S3 reached their peak values of 56.76, 64.28 and 64.3 IU/mL, respectively, on day 15 of the experiment, and S2 reached its maximum activity value of 71.18 IU/mL on day 5. The urease activity data showed that the highest level of T2 urease activity was observed during the experimental cycle. Han et al. (2019) found that digestate containing sulfadiazine was able to significantly increase soil urease activity.

Taken together, SMX stimulated dehydrogenase as well as all four hydrolytic enzymes, with the best stimulation effect at 20 mg/kg·TS concentration. As the hydrolysis process occurs more easily, hydrolytic acid-producing bacteria are less susceptible to antibiotic inhibition (Mustapha et al., 2016) and are well adapted to residual concentrations of SMX.

Microbial diversity and structural analysis

Alpha diversity analysis

The bacteria in the digest samples were sequenced and the sequences were clustered by OTUs (Operational Taxonomic Units) based on 97% similarity to obtain the bacterial community Alpha diversity data, as shown in *Table 3*. The sobs (the observed richness), chao, and ace indices reflect community richness, and shannon, and simpson indices reflect community diversity. Sobs indicates the actual observed value of richness, shannon index can reflect the relative abundance of microorganisms, simpson index reflects the proportion of dominant species biomass to the total biomass of the community, the larger the index indicates the smaller the proportion of dominant flora biomass to the total biomass. Chao value and ace can reflect the community richness, and the higher the index, the higher the microbial community richness.

On day 1, S2 had the highest level of Alpha diversity among the four experimental groups, followed by S1, CK, and S3. Alpha diversity within S1 and S2 systems was

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elevated at the beginning of the experiment, while S3 was significantly affected by SMX toxicity and decreased in diversity. On day 5, the highest level of Alpha diversity was observed in S1, and all indices increased significantly in S3, among which the sobs index was already higher than CK, which may be influenced by the degradation of SMX, the system was weakened by the toxic effect of SMX, and the bacterial diversity and abundance gradually recovered. On day 15, although all groups had started to be affected by systemic acidification, all indices showed an increasing trend, with S1 still leading, followed by S2 and S3, and the difference between S3 and CK was not obvious. On day 50, the level of Alpha diversity in S1 decreased significantly, indicating that the organic matter in the system was depleted, while S3 and CK maintained high bacterial diversity, which was consistent with the delayed phenomenon occurring in the anaerobic reaction when analyzed together with the gas production results. The dosing concentrations of 10 and 20 mg/kg·TS were able to increase the bacterial diversity during the experiment, while the negative effects produced at 40 mg/kg·TS were only present at the beginning of the experiment. Cetecioglu (2016) found that a staged increase in SMX concentration (0-45 mg/L) during semi-continuous anaerobic digestion experiments resulted in a significant decrease in bacterial species, and it was hypothesized that different dosing concentrations and dosing methods were the main factors contributing to the differences in results.

Table 3. *Alpha diversity of bacterial communities during anaerobic digestion with different concentrations of SMX*

	sobs	**shannon**	**simpson**	**ace**	**chao**	**coverage**
CK-1	662	2.935	0.147	1062.532	1044.462	0.993625
S1-1	793	3.225	0.161	1094.515	1097.595	0.994221
S2-1	824	3.248	0.124	1487.648	1268.175	0.993200
S3-1	605	2.278	0.301	1217.892	965.619	0.994271
CK-5	615	3.284	0.104	1133.976	901.251	0.995832
S1-5	768	3.967	0.047	1303.657	1116.165	0.994721
S2-5	654	3.230	0.120	1134.113	944.214	0.995269
S3-5	676	3.064	0.191	1197.289	1001.286	0.995440
CK-15	725	3.505	0.122	1192.509	1105.462	0.992243
S1-15	1031	4.436	0.038	1437.740	1399.725	0.993044
S2-15	816	3.998	0.062	1443.709	1188.508	0.991901
S3-15	731	3.473	0.096	1073.533	1034.836	0.992875
CK-30	966	4.751	0.021	1325.087	1366.481	0.992329
S1-30	1068	4.782	0.027	1410.866	1413.839	0.992071
S2-30	1075	4.572	0.035	1401.739	1413.903	0.992750
S3-30	1124	4.730	0.031	1684.079	1532.904	0.992831
CK-50	1095	4.553	0.050	1335.150	1373.244	0.992468
S1-50	883	3.943	0.110	1247.698	1238.952	0.991632
S2-50	1002	4.665	0.028	1270.430	1267.714	0.992195
S3-50	1117	4.668	0.026	1453.408	1438.006	0.993435

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Analysis of bacterial community structure

The relative abundance of bacteria at the phylum level is shown in *Figure 13*. The structural composition of the bacterial community at the phylum level was similar for each group during the experimental cycle, and consisted mainly of the following seven phyla: *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, *Spirochaetota*, *Proteobacteria*, *Chloroflexi*, and *Synergistota*, which are functional bacteria in the process of hydrolytic acidification reactions (Peng et al., 2018). *Firmicutes* and *Bacteroidota* had an absolute advantage throughout the experiment, with *Firmicutes* being able to extensively utilize digestive substrates to break down a variety of substrates such as proteins, cellulose, and lipids, and *Bacteroidota* mainly degrading various proteins into VFAs (Yi et al., 2014). In the first and middle stages of the experiment *Firmicutes*, *Bacteroidota* achieved a clear competitive advantage. Day 1 Firmicutes abundance showed that the control group (68.53%) was significantly higher than the antibiotic group (40.74%~56.89%), while *Bacteroidota* abundance was higher in the antibiotic group (34.74%~55.37%) than in the control group (26.16%), and this phenomenon continued until day 5, indicating that *Bacteroidota* had a higher SMX strong tolerance to SMX. On day 15, the acidification in the system was gradually obvious, and the relative abundance of *Firmicutes* in the antibiotic group was higher than that in the control group, and the degradation of SMX may have allowed *Firmicutes* to regain the competitive advantage. *Firmicutes* dominated absolutely in the system at the later stage of the experiment, and the relative abundance of each group was 85.06%~94.54% at day 50. The effect of SMX on the other phylum levels during the experiment was not significant and evolved mainly with nutrient consumption.

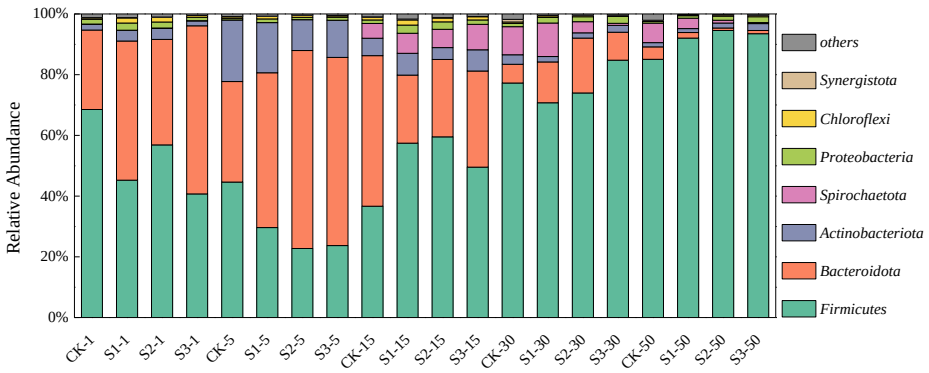

Figure 13. *Changes in bacterial community structure at the gate level during anaerobic digestion with different concentrations of SMX*

The structural composition of the bacteria at the genus level is shown in *Figure 14*, with *Prevotella*, *Selenomonas*, *Lactobacillus*, and *Olsenella* being the dominant genera at the beginning of the experiment. *Prevotella* belongs to *Bacteroidota*, which is the main acid-producing bacterium under mesophilic conditions and can adapt to low pH environments (Won et al., 2013), and its competitive advantage on days 1 and 5 was significantly higher in the antibiotic group (1d: 34.74% to 55.37%, 5d: 43.49% to 61.19%) than in the control group (1d: 26.01%, 5d: 36.50%), indicating that *Prevotella* was more tolerant to SMX. *Selenomonas* belonged to *Firmicutes* and the relative abundance tended to decrease by the substrate concentration, while the control group

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(10.92%) was higher than the antibiotic group (3.62%-6.60%), indicating that *Selenomonas* was more sensitive to SMX. The difference in relative abundance of *Lactobacillus*, *Olsenella* was small among the groups. *Lactobacillus* belongs to Firmicutes, a common lactic acid-producing bacterium, with obvious dominance on day 1 and relative abundance of 9.66~19.63%, and the competitive advantage gradually disappeared in the middle and later stages of the experiment. *Olsenella* belongs to *Actinobacteriota* and is capable of fermenting carbohydrates to lactic acid (Kraatz et al., 2011), reaching the highest relative abundance of 8.54% to 17.27% on day 5. *Prevotella*, *Caproiciproducens*, and *Treponema* were the dominant genera in the middle of the experiment. *Caproiciproducens* belonged to *Firmicutes*, and the system was in acid inhibition on day 15, with a maximum relative abundance of 15.22% (CK), 24.89% (S1), 22.51% (S2), and 24.60% (S3), respectively, which were elevated in the antibiotic group, indicating a strong tolerance in the face of toxic and harmful environments. *Treponema* belongs to *Spirochaetota* and is more common in medium temperature anaerobic digestion reactors (Li et al., 2015), with small differences between groups and relative abundances ranging from 4.64% to 8.40%. The dominant genera at the end of the experiment were *Ruminiclostridium*, *Mobilitalea*, *HN-HF01016*, and *Ruminococcus*, where *Ruminiclostridium*, *Ruminococcus*, and *Lutispora* all belonged to *Firmicutes*, and the groups changed similarly with the degradation of SMX. *Ruminiclostridium* was able to degrade xylan efficiently to generate VFAs and H2 (Ravachol et al., 2016), and gained a competitive advantage in the later stages of the experiment due to the single substrate, with relative abundance levels ranging from 9.74% to 20% in each group. The data showed that SMX had little effect on the bacterial community structure, and a few genera fluctuated in relative abundance due to their different tolerance to SMX, and the groups worked together through synergy to maintain the stable operation of the anaerobic digestion system.

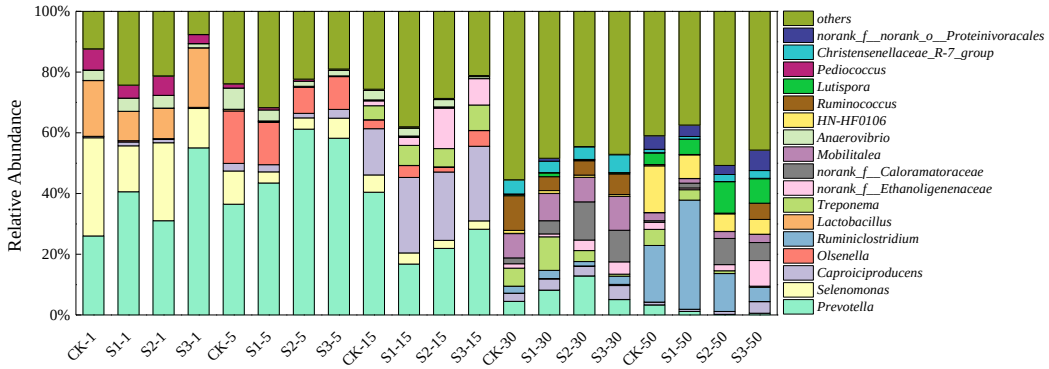

Figure 14. *Changes in bacterial community structure at the genus level during anaerobic digestion with different concentrations of SMX*

Conclusions

- 10 mg/kg·TS and 20 mg/kg·TS sulfamethoxazole had positive effects on anaerobic digestion, increasing biogas production by 14.16% and 6.62%, respectively, while 40 mg/kg·TS had no significant effect on gas production, presumably with a higher inhibition threshold than 40 mg/kg·TS.

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- Sulfamethoxazole enhanced hydrolysis by stimulating hydrolase activity, with the highest level of enzyme activity at 20 mg/kg·TS dosing concentration.
- The dosing concentration of 10 mg/kg·TS, 20 mg/kg·TS increased bacterial community diversity, while S3 was inhibited in the early stage of the experiment and species diversity recovered with the degradation of sulfamethoxazole; bacterial community structure was more stable and turnover was carried out with the change of substrate concentration, *Prevotella*, *Caproiciproducens* showed stronger tolerance to sulfamethoxazole at the genus level and *Selenomonas* was more sensitive.
- Sulfamethoxazole was readily degraded in the mesophilic anaerobic digestion system with a 5d degradation rate of 99.64%~99.89%. This study demonstrated that mesophilic anaerobic digestion is a feasible solution for the treatment of livestock manure containing sulfonamide drugs.

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