Effect of sulfamethazine on anaerobic digestion of manure mediated by biochar

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Abstract

Antibiotic contamination from animal production and wastewater treatment process will release antibiotic resistant genes to the environment and potentially threaten human health. Meanwhile, the residual antibiotic in manure could have inactive impacts on anaerobic digestion (AD). This study explores the effect of sulfamethazine on manure AD mediated by biochar. The results show that biochar weakens the adverse effects of sulfamethazine on AD by adsorption sulfamethazine during the initial stage (0-3 days) of AD and promoting the growth of hydrolytic bacteria (especially Firmicutes and Bacteroidetes) and methanogens (especially Methanothrix and Methanosarcina). Besides, the presence of biochar improves the biogas production capacity of AD and promotes microbial diversity and community richness. Thus, the addition of biochar greatly reduces sulfamethazine and is testified to be a desirable strategy to mitigate the inhibition of sulfamethazine on AD.

Keywords: Biochar; Anaerobic digestion; Microbial diversity; Antibiotic.

1. Introduction

With the development of animal husbandry, the amount of livestock manure is increasing in China (Bai *et al*., 2021; Wang *et al*., 2020a). Meanwhile, antibiotics such as sulfamethazine (SMZ) have been widely used to prevent livestock and poultry diseases and promote their growth (Chauhan *et al*., 2022; Song *et al*., 2017; Kaur *et al*., 2022). As the biggest consumer and producer of antibiotics, China was estimated to consume 162,000 tons of antibiotics, of which about 52% were used for animal production (Zhang

et al., 2015a). However, the absorption and utilization rates of the antibiotics by animals are low and about 30% ~ 90% of them enter the ecological systems through animal excreta in the forms of original drugs and primary metabolites (Li *et al*., 2013a). Antibiotics cause serious antibiotic pollution around the farm and soil environment (Kantiani *et al*., 2010; Hurtaud-Pessel *et al*., 2011). AD technology, a typically used biological treatment of manure from livestock and poultry, can degrade a great number of organic matters in livestock and poultry manure and subsequentially produce biogas for energy recovery. Researchers have shown that the presence of antibiotics inhibited biogas production during AD, and a high concentration of antibiotics even led to the collapse of the digestion system, which could be difficult to recover (Cetecioglu *et al*.,2013; Zhang, *et al*., 2019a). Therefore, the existence of biochar restricts the progress of AD, and measures should be taken to eliminate antibiotics in the AD system.

Biochar is prepared by pyrolysis of organic matter (Deng *et al*., 2021a and 2022; Sun *et al*., 2021a; Thota *et al*., 2022; Xu *et al*., 2021;). It is a carbon-rich material with adsorption capacity for high-level organic pollutants (including pharmaceuticals) (Lehmann *et al*., 2015) and dyes (Deng *et al*., 2021b; Sun *et al*., 2021b) and absorption of electormagneitc waves (Lu *et al*., 2021; Qi *et al*., 2021). The unique carbon sequestration function and multifunctional characteristics made biochar a research hotspot in the fields of soil improvement, functional material preparation, environmental remediation, biological waste management (Si *et al*., 2022; Liu *et al*., 2019; Palansooriya *et al*., 2019; Wan *et al*., 2020). At present, biochar has reportedly removed antibiotics

(Rajapaksha *et al*., 2014). Hoslett *et al*. (2020) prepared biochar from food and plant residues at the pyrolysis temperature of 300 ℃ to study the removal effect on tetracycline in the aqueous solution. The results showed that the biochar can remove tetracycline in the aqueous solution well, with the maximum adsorption capacity reaching 15.5208 mg g^{-1}. However, few researches have focused on the adsorption of antibiotics in the AD system containing antibiotics by biochar. In addition, the organic functional groups contained in alkali metal salts and biochar show strong buffering capacity, which was helpful to balance pH reduction caused by VFA accumulation in AD system. At the same time, the electrochemical properties enable biochar as a mediator to accelerate the interspecific electron transfer in anabolism(Wang *et al*., 2020b).

Therefore, it is necessary to explore the adsorption of biochar on antibiotics and the relationship between antibiotics, biochar, environmental factors, and microorganisms in the process of AD. Sulfamethazine is a broad-spectrum bacteriostatic agent, which is widely used in veterinary treatment (Shim *et al*., 2013). At present, studies have shown that the content of SMZ in livestock and poultry feces is high. Pan *et al*. (2011) also detected that the concentration of SMZ in manure was 28.7 mg kg^{-1}, and the content of SMZ in manure was 67.1 mg kg^{-1} (Ji *et al*., 2011), and the detection rate of sulfamethazine was the highest. Other studies showed that the content of SMZ in manure reached 100 mg L^{-1} (Christian *et al*., 2003). In short, SMZ widely exists in livestock manure, causing environmental pollution. This study, explored the AD of manure with four concentrations of SMZ (0, 20, 60, and 120 mg kg^{-1}, dry weight) mediated by biochar. The results are

helpful to reduce the presence of SMZ in manure, thereby providing effective strategies to control SMZ pollution in the environment.

2. Materials and methods

2.1 Materials

2.1.1 Biochar preparation

Biochar was prepared from corn stalks collected from an experimental plot at Shenyang Agricultural University. The corn stalk was first dried, crushed, and then passed the 30-mesh sieve. Next, the corn straw was pyrolyzed in a muffle furnace (SX2-4-10TP, Shanghai Yiheng Technology Co., Ltd, Shanghai China), then carbonized at 100 ℃ for 1 hour, and finally heated to 500 ℃ for complete carbonizations for 2 hours.

2.1.2 Digestion raw materials

Manure and corn stalk gathered from a village near Shenyang Agricultural University and the experimental field of Shenyang Agricultural University respectively, were used as the substrate for the AD process. Sludge with high activity from a well-operated anaerobic reactor in "comprehensive energy demonstration base in cold areas of Northeast China" of Shenyang Agricultural University was used as the inoculum of the anaerobic digesters. All raw materials used in this study did not contain antibiotics, which was determined by tests. The characteristics of the raw material are listed in Table 1.

Table 1. Chemical characteristics of the raw material used in this study.

Type	TS(total solids, %)	VS (volatile solids, %)	TC (total carbon, g kg^{-1})	TN (total nitrogen, g kg^{-1})

Manure	45.0	30.7	426.6	30.5
Corn stalk	88.4	75.2	632.4	8.4
Inoculum	14.8	6.3	307.5	15.6

2.2 Set-up of anaerobic digestion reactors

0, 20, 60, 120 mg kg^{-1} of SMZ (based on TS of manure) were added to the digestion system as additives respectively (i.e., S0, S20, S60, S120). A series of identical brown glass bottles with a working volume of 700 mL were used as triplicated batch AD reactors. There were 64 g of manure, 7 g of corn stalk, 140 mL of inoculum, and 5 g L^{-1} of biochar contained in each reactor and the rest was water. The systems with different SMZ dosages were named as S0, S20, S60, and S120, respectively. Reactors without biochar and SMZ were set up as blank, which was named as CK. Each condition was performed in triplicate. All the reactors were placed in water baths and incubated at 37 ℃. The batch experiments lasted for 40 days and the produced biogas were collected every day, while biomass samples were collected every three days. The samples were divided into two parts and stored in centrifuge tubes. One part of the sample was centrifuged at 5000 rpm for 15 min, of which supernatant was used to determine volatile fatty acids (VFAs), pH, ammonia nitrogen (AN), and soluble chemical oxygen demand (SCOD). The other part of the samples was stored in a refrigerator at -80 ℃ for DNA extraction.

2.3. Analytical methods

Standard methods (APHA, 2005) were used to determine TS and VS data. Total carbon (TC) and total nitrogen content (TN) were analyzed by an elemental analyzer

(EA3000, Euro Vector, Italy). SCOD was analyzed according to International Organization for Standardization (ISO 6060:1989). pH was determined using a portable pH meter (HQ40d-pHC101, HACH, Loveland, USA). VFAs concentrations were determined by gas chromatograph (6890N, Agilent, California, USA) (Zhang *et al*., 2019b). 240 and 250 ℃ were set as the temperature of the injector and detector, respectively. The initial oven temperature was 80 ℃ for 5 min and then increased to 220 ℃ at a rate of 10℃ min^{-1}. The injection volume of each sample was 2 ul. The details for the determination of AN was referred to International Organization for Standardization (ISO 11732:2005). SMZ was measured by high-performance liquid chromatography-tandem mass spectrograph with the internal standard method (Waters Quattro Premier XE, Waters, Massachusetts, USA). Firstly, Na_2EDTA and 1% formic acid acetonitrile aqueous solution were added to the sample for oscillation, then anhydrous sodium sulfate and NaCl were added for vortex mixing, centrifugation after an ultrasound, and the supernatant was taken. Anhydrous sodium sulfate and C18 adsorbent were added into the supernatant, whiche was centrifuged after horizontal oscillation, then settled the protein after standing, and the supernatant was taken into the test tube. The test tube under nitrogen was concentrated until it was dry, then 0.1% formic acid acetonitrile was added at a constant volume solution, and then vortex evenly to mix through 0.22 μM after filtering membrane, standby. The conditions of the Chromatographic column were as follows: ACQUITY UPLC™ BEH C18 (100mm×2.1mmi.d.1.7μm). The column temperature was 40 ℃ and the injection volume was 10 μL with a flow rate of 0.2 mL min^{-1}. The mobile phase was

methanol and 0.1% formic acid aqueous solution. The mass spectrum conditions were as follows: Ion source: ESI+, Ion source temperature: 550 ℃; Ion spray voltage: 5500 V; Inlet voltage: 10 V; Atomized gas 50 psi; Auxiliary heating gas: 50 psi; Air curtain: 30 psi; Monitoring mode: MRM mode is adopted (Perisa and Babic, 2014).

2.4 Microbial community analysis

DNA in triplicate was extracted from the biomass obtained on days 3, 21, and 39. NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, USA) was used to determine the concentration and purity of DNA. The DNAs were amplified using the primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) targeting V3-V4 region. Methanogens were amplified using the methanogens mcrA gene MLfF (GGTGGTGTMGGATTCACACARTAYGCWACAGC) and MLrR (TTCATTGCRTAGTTWGGRTAGTT). The 16S rRNA gene high-throughput sequencing procedure was performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) using the Illumina Hiseq PE300 platform. The sequences were clustered to generate operational taxonomic units (OTUs) at 97% similarity.

2.5 Chemicals

SMZ (N 98% purity) was obtained from Chemical Science and Technology for Western Asia Limited (Shandong, China). The other chemicals, such as NaOH and HCl, were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shenyang, China). The reagents used in HPLC and GC were chromatographic grade while other reagents were

analytical grade.

2.6 Statistical analysis

Origin 2021 (Origin Lab, Massachusetts, USA) was used to analysis data and prepare figure. The relative abundance was obtained by dividing the specific gene copy in each sample by the corresponding 16S rRNA gene copy.

3. Results and discussion

3.1 Changes in sulfamethazine

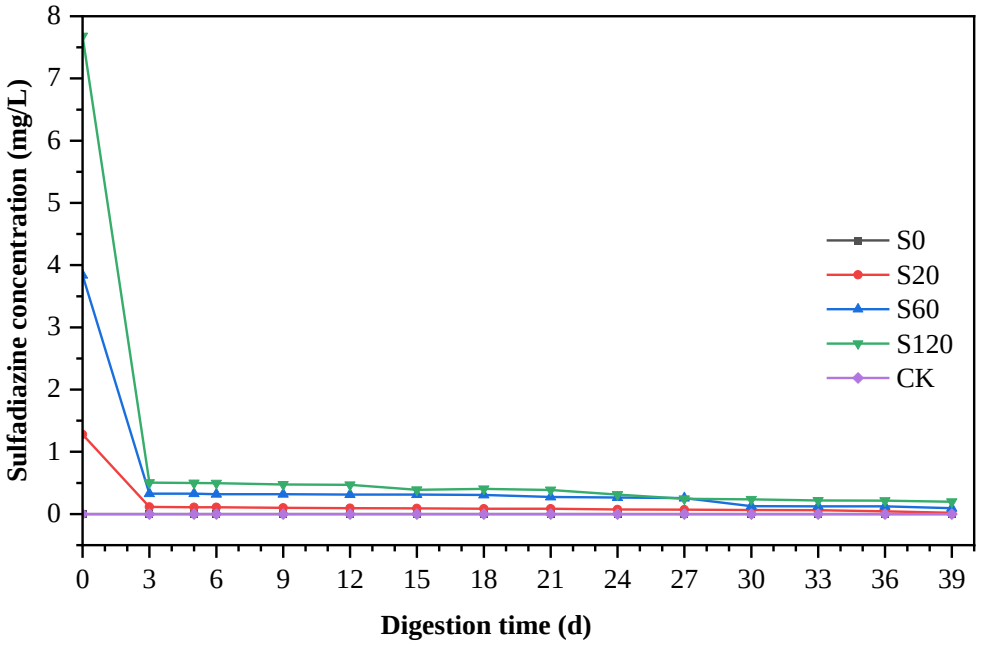


Fig. 1 Changes of sulfamethazine content.

During the digestion process, the SMZ concentrations in the system were monitored every three days. As shown in Fig. 1, SMZ was removed faster in each treatment at the initial stage of digestion, while then became slow. In the first three days of AD, the removal efficiencies of SMZ in the system were 91.2%, 91.5%, and 93.4% for S120, S60, and S20, respectively. In this study, the removal of antibiotics in the anaerobic digestion

system was mainly divided into two parts: biochar adsorption and AD degradation. According to other studies, the adsorption of antibiotics by biochar occurred rapidly, and the adsorption equilibrium was usually reached in more than ten hours. (Zhang *et al*., 2019c; Zhang *et al*., 2020). The degradation of antibiotics by AD was a slow and continuous process. Therefore, the rapid decline of antibiotic concentration in 0-3 days was mainly due to the adsorption of biochar. When the biochar reached the adsorption equilibrium, the adsorption rate of biochar for antibiotics was equal to the desorption rate. At this time, the reduction of antibiotics mainly depended on anaerobic digestion and degradation, and the degradation rate became slower. This was the reason for the rapid degradation of biochar in 0-3 days. On day 39 of AD, the SMZ contented in S20, S60 and S120 systems were: 0.0206 mg L^{-1}, 0.0931 mg L^{-1}, and 0.1970 mg L^{-1}, respectively. The final removal rates of SMZ for S20, S60, and S120 were 98.4%, 97.6%, and 96.8%, respectively. However, Cheng *et al*. (2018) reported that there was only 8.3% ~ 31% of removal efficiency of sulfonamides during digestion in farm wastewater. And in another study, Chen *et al*.(2005) reported that there was only 23.7% of SMZ removed during AD. SMZ was rarely removed during conventional AD of manure (Mohring *et al*., 2009; Mitchell *et al*., 2013). However, in this study, it seemed that the biochar in the AD system removed most of SMZ by adsorption at a fairly quick pace, which occurred in an early stage and greatly reduced the negative effects of SMZ. This indicated that biochar reduced SMZ in the AD system through adsorption.

3.2 Biogas production during anaerobic digestion

The cumulative biogas production and daily biogas production during AD of different treatments are shown in Fig. 2A and B. The cumulative biogas productions on day 40 in CK, S0, S20, S60 and S120 were 4115 mL,4467 mL,4090 mL,3820 mL and 3756 mL, respectively (Fig. 2A). The peak biogas production of S0 appeared on day 3, while S20 appeared on day 2, and S60, S120, and CK appeared on day 4 (Fig. 2B). Compared to CK, the cumulative biogas yield was increased by 8.6% in S0, while decreasing by 0.6%,7.2%, and 8.7% in S20, S60, and S120, respectively. The addition of biochar enhanced the anaerobic digestion as there was an increase in total biogas yield while adverse effects were found in the reactors with SMZ dosage, where the cumulative biogas yield was decreased by approximately 8.4%,14.5%, and 15.9% in S20, S60, and S120 compared to S0, respectively. It seemed that as the dosage of SMZ was increased, the inhibitory effect on AD was increased, which also agreed with the results reported by Zhang *et al*. (2019a), who observed that the addition of 20 mg kg^{-1} SMZ reduced the biogas production of anaerobic digestion of cow dung by 16%, and the addition of 50 mg kg^{-1} SMZ even seriously destroyed the performance of the anaerobic digestive system of cow dung. In another study, Ma *et al*. (2021a) found that biogas production was decreased by 18.5% in the anaerobic digestion of cow dung supplemented with 1 mM sulfamethazine. Compared with their results, adding biochar seemed to depress the inhibitory effects of SMZ on biogas production to a certain extent. This further confirmed that the adsorption of SMZ by biochar in reactors during the initial stage of AD reduced the concentration of SMZ and weakened its negative influence on biogas production.

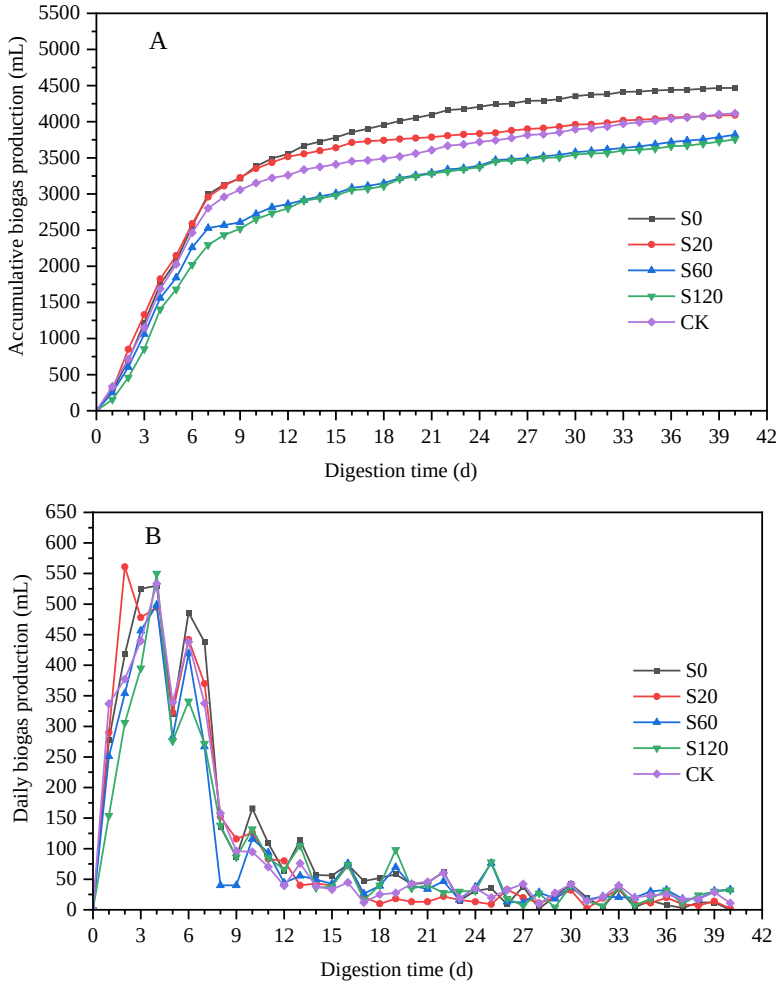


Fig. 2 Cumulative biogas production (A) and daily biogas production(B).

3.3 Characteristics of manure digestive liquid

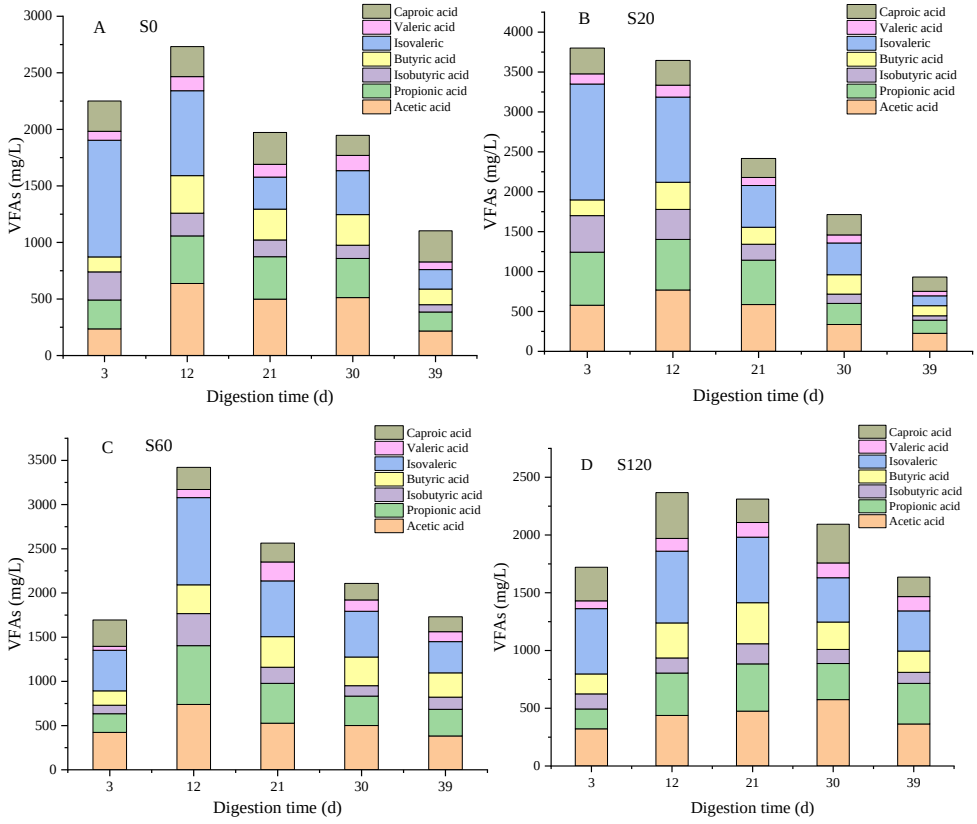
VFAs were produced by hydrolysis and acidification of raw materials under the action of microorganisms. VFAs were the raw material in the methanogenic stage (Zhao *et al*., 2020). However, excessive VFAs will reduce the metabolic rate of anaerobic microorganisms and thus affect AD performance. Fig.3A-E show the change in VFAs concentration in each treatment group. The VFAs concentration was increased at the initial stage of AD but decreased after reaching the maximum. Compared with CK, VFAs were less variable in S0 during the whole AD process, and the overall VFAs concentration

remained low, which suggested that biochar can alleviate the over-acidification of the AD system and maintain stability of AD. The VFAs concentration in S20 was increased dramatically since the early stage and the peak value of VFAs concentration was the highest among all the reactors. Combined with the peak biogas production of S20 on the second day, it could be seen that under the action of biochar and 20 mg L^{-1} SMZ, the hydrolysis stage of AD was accelerated and the rapid progress of AD was promoted. On the other side, the peak values of VFAs in S60 and S120 were smaller and the concentrations remained high level at the end of AD, indicating that the digestion process of the two groups was inhibited. Although the hydrolysis stage of S20 was accelerated, the biogas yield of S20 was lower than that of S0, indicating that SMZ had a certain adverse impact on methane production.

In general, the optimum pH range of AD methane production is 6.8-7.5 (Ma *et al*., 2021b). At the initial stage, with the production of acid in the digesters, the pH of all treatments was decreased (Fig. 3F). It was notable that pH of CK was decreased most significantly at the initial stage. The common difference between CK and other groups was caused by the addition of biochar, which suggested that biochar alleviated the acid inhibition in AD reactors and reduced pH variation of the reactors. With the progress of AD, pH of each group showed an upward trend. pH of the groups added with SMZ was the lowest on the 30^{th} day. This might because that sulfanilamide antibiotics have a certain impact on the metabolism of microorganisms in AD and further reduced the pH. Zhang (2019a) reported that 50 mg kg^{-1} SMZ reduced the pH of the

anaerobic digestion system to 5.9, resulting in the brief collapse of the anaerobic system. In this experiment, pH of the AD reactors was kept within the appropriate range of AD, indicating that biochar can be added to alleviate the adverse impact of SMZ on pH.

SCOD, an important parameter reflecting the operation of the AD system and the activity of the microbial community, can indirectly reflect the changes in organic matter in the AD system (Badia *et al*., 2019). Fig. 3G shows that SCOD was decreased gradually and finally fluctuated at a low concentration level. It showed that a large number of organic matters were degraded during the early stage of digestion. After day 21, degradable organic matter was decreased with decreasing SCOD value slowly, before finally reaching stable. Compared with S0, the concentration of SCOD in groups added with SMZ was lower, which was not conducive to AD.



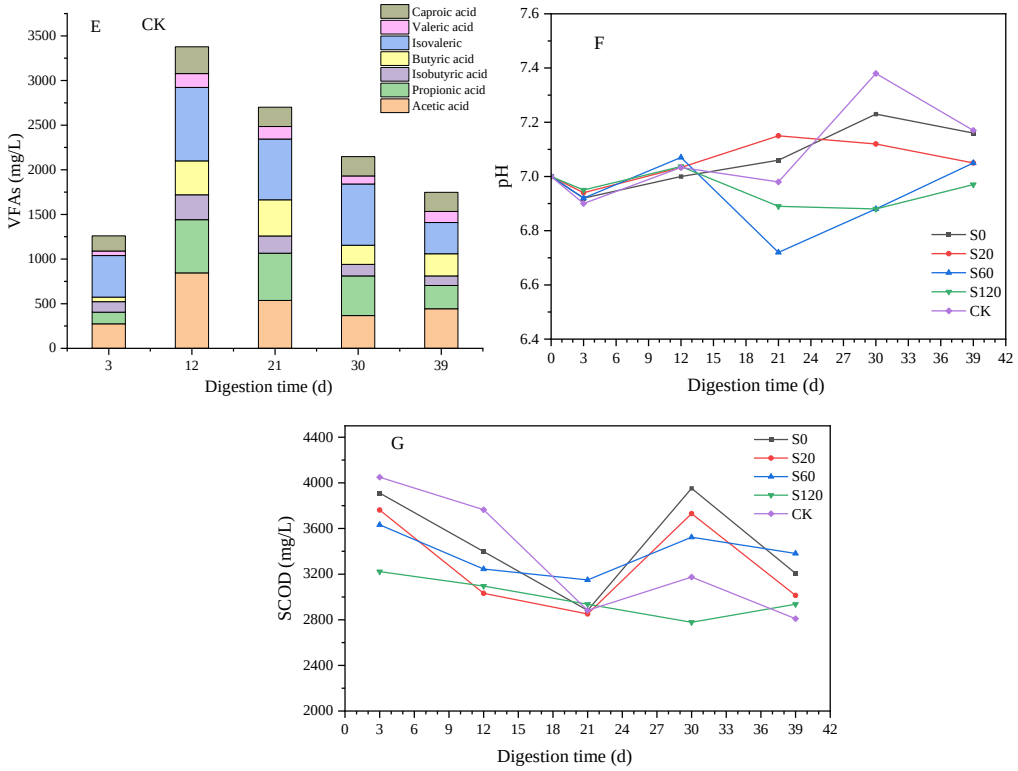


Fig. 3 Changes of VFAs (acetic, propionic, isobutyric, butyric, isovaleric, valeric, and caproic acid) concentrations(A-E); Changes of pH (F), and SCOD (G).

3.4 Microbial community

3.4.1 Alpha diversity of the bacterial community

Table 2. Bacterial community diversity and richness index.

Sample ID	Sobs	ACE	Chao1	Shannon	Simpson	Coverage
S0_3	1022	1847.282	1519.537	4.424	0.040	0.984
S0_21	1153	1539.593	1518.147	4.612	0.040	0.987
S0_39	1533	1887.386	1883.127	4.915	0.029	0.993
S20_3	1179	1664.183	1645.323	4.739	0.027	0.986
S20_21	1182	1645.967	1611.195	4.783	0.030	0.986

S20_39	1441	1845.429	1852.000	4.995	0.025	0.990
S60_3	1243	1692.104	1656.050	4.890	0.024	0.987
S60_21	1407	1820.933	1840.448	4.917	0.027	0.989
S60_39	1235	1633.665	1650.112	4.605	0.041	0.989
S120_3	1054	1489.463	1479.311	4.667	0.028	0.986
S120_21	1454	1902.316	1914.111	4.981	0.025	0.989
S120_39	1282	1679.686	1652.121	4.624	0.037	0.990
CK_3	1154	1648.640	1673.480	4.642	0.034	0.984
CK_21	1502	1890.736	1855.542	5.037	0.024	0.991
CK_39	1212	1628.811	1639.377	4.562	0.041	0.988

All samples were statistically analyzed for OTU at a 97% similarity level. Species diversity and richness index are shown in Table 2. The coverage of more than 0.98 indicated that the sequencing results well represent the real situation of microorganisms in the sample (Shu *et al*., 2015). Sobs is the actual observed value of species richness, while ACE and Chao1 were species richness indexes. Shannon index and Simpson index can reflect the diversity of the microbial community. The greater the Shannon index, the higher the community diversity, while the greater the Simpson index, the lower the community diversity. As shown in Table 2, the Sobs index of S0_39 and S20_39 were 1533 and 1441, respectively, and the Shannon index of S0_39 and S20_39 were 4.915 and 4.995, respectively. The richness and diversity indexes of S0 and S20 were higher

than those of other groups, while S60 and S120 were the lowest, which showed that low concentration of SMZ did not show adverse effects on community diversity and richness, but high concentration showed obvious adverse effects.

3.4.2 Bacterial community structures

The results of microbial community structure are shown in Fig. 4. Hierarchical cluster analysis classifies samples according to their similarity and difference (Zhu *et al*., 2021). The bacterial communities of the samples were divided into two groups according to the digestion stage (Fig. 4A). The digested samples on day 3 were classified into one cluster, whose classification position was far away from the others. At day 3 of AD, they were classified into two clusters according to the presence and absence of SMZ. The digested samples on days 21 and 39 were gathered together. With the continuation of the reaction, the results showed that the distance between S0 treatment and S20 treatment was close, while the distance of CK, S60, and S120 treatments was close, which might because the microbial activity in S20 treatment recovered with the continuous degradation of SMZ in S20. The higher concentration of SMZ in S60 and S120 still has a certain adverse impact on the microbial community structures. At the same time, it also demonstrated the difference between S0 and CK, suggesting that the addition of biochar has affected the microbial communities. The differences and similarities of microbial communities were investigated by Principal coordinate analysis (PCoA) in this study. PCoA in (Fig. 4B) demonstrated that CK, S60, and S120 tended to gather together, indicating that biochar had a certain weakening effect on SMZ.

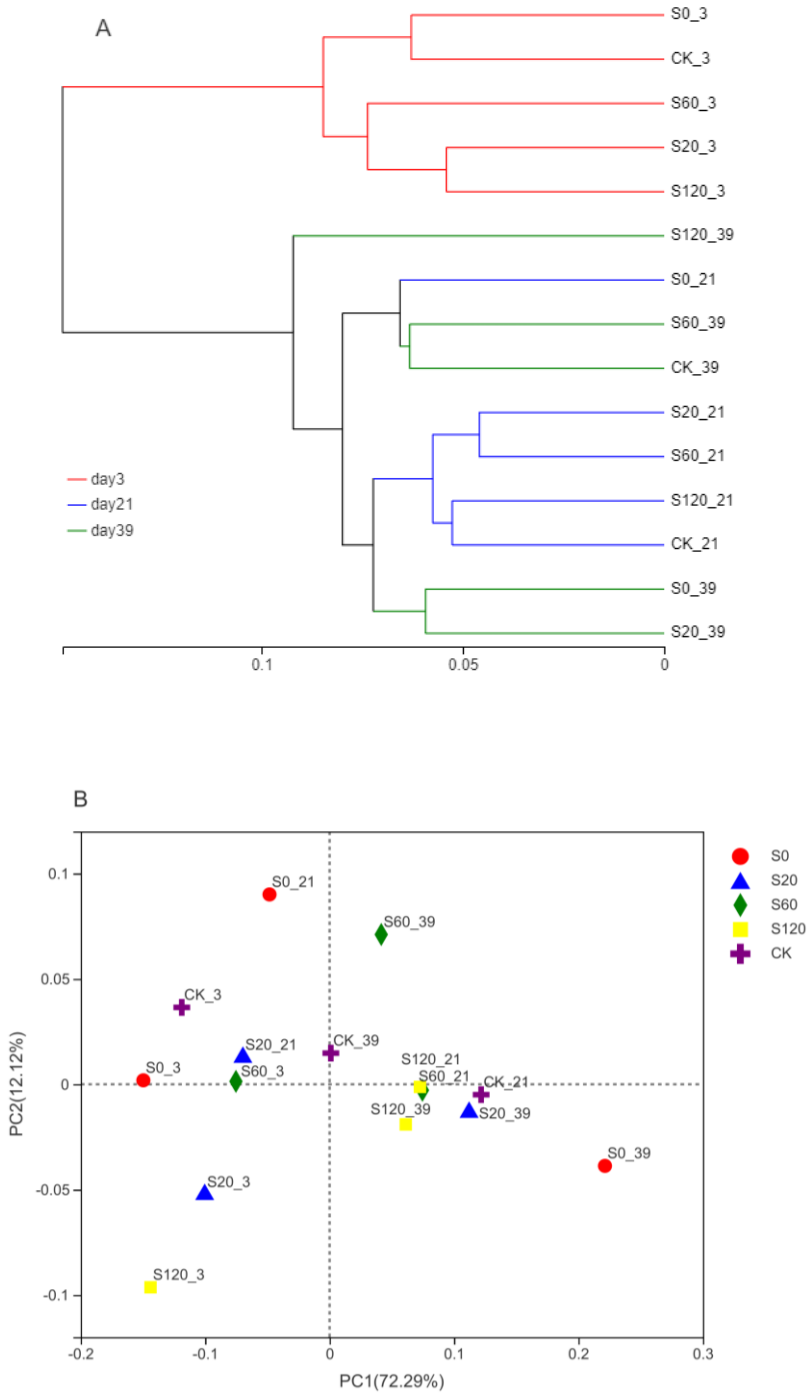


Fig. 4 Hierarchical clustering analysis of bacterial community (A); PCoAs for bacterial community at genus level (B).

3.4.3 Changes of bacterial community and methanogenic archaea community

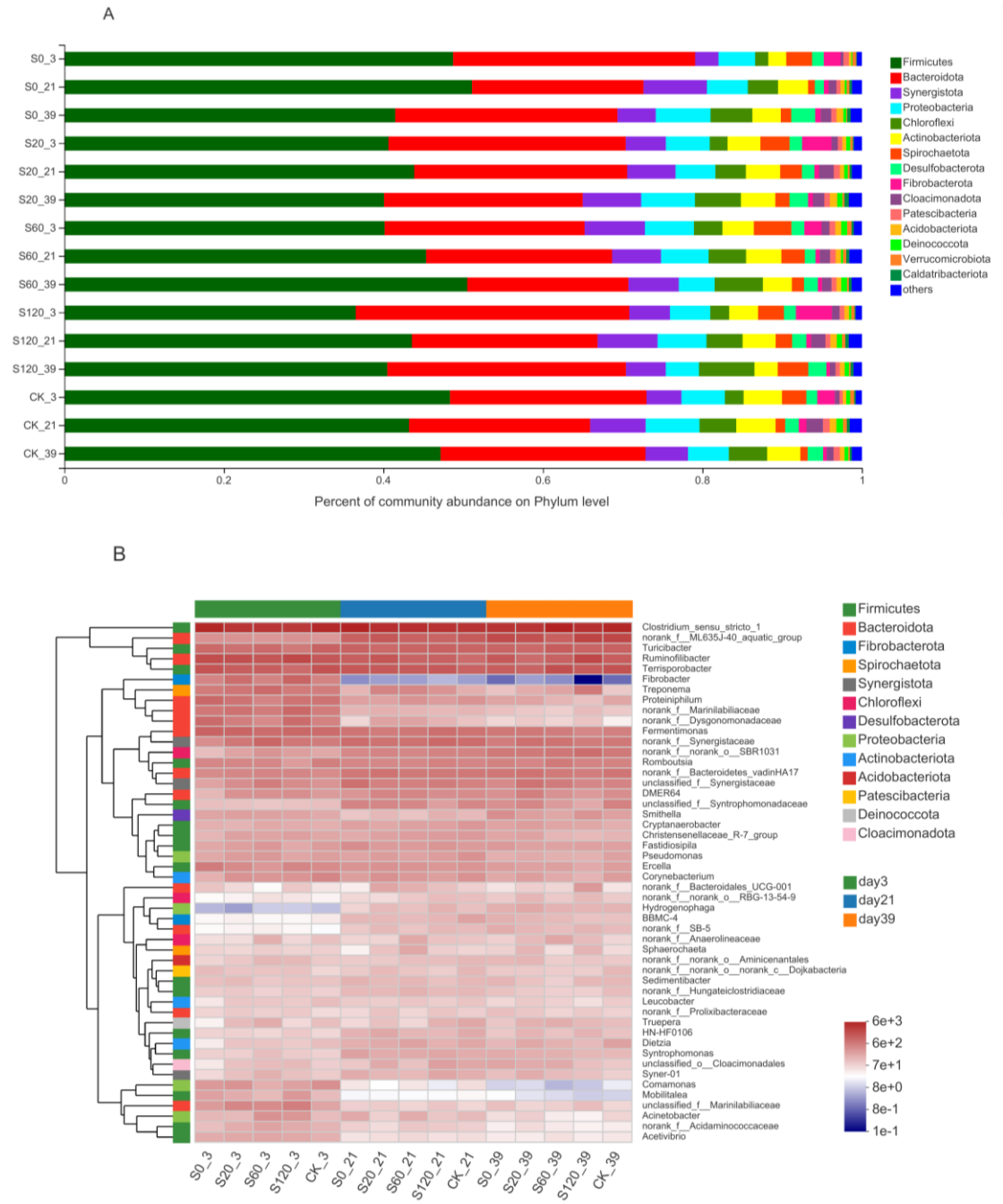


Fig. 5 Relative abundance of bacterial phyla (A); The heatmap based on relative abundance of bacteria at genus level (B).

Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Synergistetes were the main phyla in the AD system (Fig. 5A), which was consistent with the results reported

by Tian *et al*. (2015). The proportion of Firmicutes and Bacteroidetes was more than 70%, which were the most predominant phyla during the AD. The researchers have pointed out that Firmicutes and Bacteroidetes played an active role in the hydrolysis and acetogenesis during AD (Yang *et al*., 2021). Firmicutes dominated the reactors on day 3, with a relative abundance of 48.7%, 40.6%,40.2%, 36.5%, and 48.4% in S0, S20, S60, S120, and CK, respectively. Compared to CK, Firmicutes were increased by 0.62% in S0 but declined by 19.2%,16.9%, and 24.6% in S20, S60, and S120, respectively. Then Firmicutes increased in the middle of the digestion process and it was more abundant in all the biochar-supplemented reactors than CK. Meanwhile, the heatmap (Fig. 5B) shows that Clostridisum_sense_stricto_1, ROMBOUTSIA, Turicibacter, Terrisporobacter decreased SMZ-dosed reactors in the early stage of digestion, all of which belong to Firmicutes. Therefore, the growth of Firmicutes benefited from the addition of biochar, while the addition of SMZ repressed the growth of Firmicutes during the early period of digestion. The decreased abundance of Firmicutes with the increased concentration of SMZ indicated that Firmicutes were sensitive to SMZ. Firmicutes, constituted by syntrophic bacteria, play a crucial role in cellulose degradation and can degrade various VFAs (Li *et al*. 2013b). Besides, extracellular enzymes (such as cellulase, lipase, and protease) produced by members of Firmicutes, played a role in the metabolism of cellulose, protein, lignin, and lipid. This may explain why the SMZ treatments produced fewer biogas. The increase of Firmicutes in SMZ-dosed groups during the middle stage of digestion suggested the elimination of SMZ by biochar adsorption.

On day 3, Bacteroidetes sequences counted for 30.3%, 29.8%,25.1% ,34.3%, and 24.6% in S0, S20, S60, S120, and CK, respectively. Compared to CK, Bacteroidetes were increased by 23.2%, 21.1%,1.2%, and 38.3% in S0, S20, S60, and S120, respectively, which indicated that biochar supplementary increased the Bacteroidetes during the anaerobic digestion process. Bacteroidetes microorganisms play a crucial role in cellulose degradation. They are strictly anaerobic bacteria, which usually degrade carbohydrates into acetate, ethanol, and trace propionate during digestion (Zhang *et al*., 2015b). Typically, various lytic enzymes and acetic acid were produced by most of the bacteria belonging to Bacteroidetes during the degradation of organic materials (Chen and Dong, 2005; Riviere *et al*., 2009). Thus, the increase of Bacteroidetes would help to improve the hydrolysis and acid production capacity of the digestive system and provide raw materials for the methanogenic stage, which helped to speed up the process of anaerobic fermentation.

The bacteria of the Proteobacteria phylum are known for their utilization of glucose, propionate, butyrate, and acetate (Ariesyady *et al*., 2007). The little change of Proteobacteria in each group, ranging from 4.2% to 6.9%, suggested that the digestion and acidogenesis carried out by Proteobacteria remained stable. Synergistetes were identified at a comparatively low level on day 3, at 2.9%, 5.0%,7.6%, 5.1% and 4.4% in S0, S20, S60, S120, and CK, respectively, which indicated that Synergistetes were less affected by biochar and SMZ Compared to day 3, the amount of Synergistetes generally increased on day 21, while decreasing in the later stage of digestion. Silvestre *et al*. (2015)

have reported that Synergistetes establish a syngenetic metabolism with hydrogen-utilizing methanogens, and participate in the interspecies electron transfer as electrochemically active bacteria. This indicated that methane production might benefit from the stimulation of Synergistetes enrichment, so the increased of Synergistetes in the middle and later stages of AD was conducive to methane enrichment.

Actinobacteria and Chloroflexi had little change in the digestion reactor. They are related to the decomposition of polysaccharides or phenolic compounds and the degradation of glucose (Ariesyady *et al*., 2007). They were less affected by SMZ and biochar.

The AD process mediated the conversion of organic wastes to biogas through four metabolic stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Zhang *et al*., 2017). As a result, it is important to understand the microbial community at a lower level. The Heatmap (Fig. 5B) illustrates the bacterial community structure at the genus level. The predominant bacteria at genus level during AD included Clostridium_sensu_stricto (12.5-22.7%), Ruminofilibacte (3.7-9.1%), Terrisporobacter (3.7-7.4%), norank_f_ML635J-40_aquatic_group (1.2-9.4%), Turicibacter (2.7-5.4%), Fermentimonas (1.9-4.4%), and norank_f_Synergistaceae (1.6-4.2%), within which Clostridium_sensu_stricto, Ruminofilibacterand Terrisporobacterwere well-recognized for the hydrolysis and acidogenesis and were often observed in microbial communities treating manure (Zhang *et al*., 2017). The resilience of these populations suggested that the AD reactors were well-functioned despite the interference of SMZ.

Overall, the addition of biochar promoted the hydrolysis bacteria such as Firmicutes and Bacteroidetes, which was conducive to the methane production process while the treatments with SMZ led to the reduction of Firmicutes during the digestion to a certain extent, resulting in fewer raw materials of methanogenic bacteria and less methane production.

The primers 338F / 806R did not target the methanogenic archaea community, thus, the Methanogens community was further investigated using the primers MLfF (GGTGGTGTMGGATTCACACARTAYGCWACAGC) and MLrR (TTCATTGCRTAGTTWGGRTAGTT).

Euryarchaeota comprised the main Methanogens phyla in the AD reacter, which accounted for over 93.5% of the total Methanogens sequences in each treatment. The abundance of methanogens was high in S0 in the early stage (during the peak methane production period), and the decrease in the middle and the late stages may be due to the shortage of substrates (Fig. 6A). Heatmap shows that Methanothrix, Methanosarcina, Methanoculles, Methaocropusculum belonging to Euryarchaeota are abundant at the genus level (Fig. 6B). Acetate can be directly metabolized into CO_2 and CH_4 by Methanothrix, which is considered a strictly acetoclastic archaeon. Moreover, Methanothrix can reduce CO_2 to CH_4 by accepting electrons directly via direct interspecies electron transfer (DIET) (Rotaru *et al*., 2014). Therefore, more energy was saved and effectively used for microorganism growth in biochar addition reactors (Yang *et al*., 2021). The abundance of Mathanothrix was higher in the reactors supplemented

with biochar, especially during the early stage, which coincided with the higher biogas production during this period. Methanosarcina, a mixotrophic methanogen, can not only generates methane through acetoclastic and hydrogenotrophic pathways (Jo De *et al*., 2012) but also converts propionic acid to CH_4 through direct interspecific electron transfer (Barua *et al*., 2017). Methanosarcina was enriched in almost all treatments with biochar compared to CK. Therefore, the presence of biochar could improve methane production by promoting DIET. Besides, biochar changed and regulated the composition of the Archaea community and enhanced the environmental adaptability of Archaea during anaerobic digestion.

Therefore, the supplementary biochar might promote anaerobic digestion by promoting the DIET between microorganisms. Meanwhile, the complex pore structure and high specific surface area of biochar might provide a stable carrier for microorganisms to a certain extent, thus promoting the survival, growth, and reproduction of microorganisms. The dose of SMZ affected the activity of methanogens and reduced methane production during the anaerobic digestion process. Based on the antibacterial characteristics of SMZ, SMZ may potentially inhibit some microorganisms in the environment (Zhang *et al*., 2019a). Although the results of SMZ concentration explained that most of SMZ had been adopted by biochar at the early stage, the residue still had a slight toxic effect on methanogen activity according to the sequencing results.

Fig. 6 Relative abundance of methanogens genus (A); The heatmap based on relative abundance of methanogens at genus level (B).

3.5 Relationship between microbial community and environmental factors

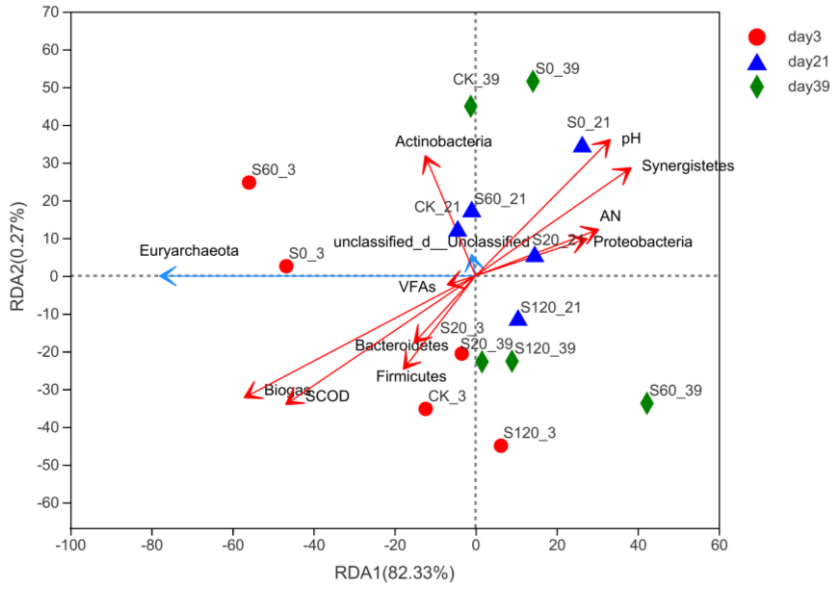


Fig. 7 Redundancy analysis of bacterial community, methanogenic microbial community, and environmental factors. Red arrows represent selected environmental factors and bacterial communities.

The relationships between the main bacterial community, methanogenic microbial community, and environmental factors were assessed by redundancy analysis (Fig. 7). The correlation of environmental variables is indicated by the angle between arrows, and the degree of interpretation of environmental factors on community structure and distribution is indicated by the length of arrows. Physicochemical parameters including SCOD, VFAs, pH, and AN were used as environmental factors to evaluate the AD performance (Zhang *et al*., 2015c; Song *et al*., 2017). 82.6% of the total variation of methanogens was explained by major bacterial communities and environmental factors. Biogas, SCOD, VFAs, and Euryarchaeota were positively correlated, while pH, AN, and Euryarchaeota were negatively correlated, which also further showed that the existence of biochar was conducive to the growth of methanogens. The two bacteria with the highest

relative abundance were Firmicutes and Bacteroidetes, which were positively correlated with Euryarchaeota. This further showed that they can promote the growth of biogas production and methanogens.

4. Conclusions

In the anaerobic digestion system supplemented with 5 g L^{-1} corn stalk biochar, the biogas production was increased and the microbial diversity was promoted during the AD process. The biochar mainly weakened the adverse effect of SMZ during the AD process through adsorption, which mainly occurred during the early period of digestion (0-3 days). The removal rate of SMZ was greater than 97.6%,. The result clearly suggested that the supplementary of biochar could be an effective strategy of removing the Sulfamethazine and enhancing the microbial activity during the anaerobic digestion process. This biochar can be used for other fields likc nanocomposites (Cai, et al., 2022; Wang, et al., 2022) and energy units (Gao, et al., 2022).

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