# Effects of cow manure ratios on methane production and microbial community evolution in anaerobic co-digestion with different crop wastes

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**Abstract:** The present study investigated the effects of cow manure ratios mixed with maize stover, rice straw, and wheat stalk at 3, 2, 1 (total solid based, TS-based), respectively, on methane production and microbial community structure during the anaerobic co-digestion process. Results showed cow manure co-digested with maize stover, wheat stalk, and rice straw at ratios of 2, 1, and 3 had the highest cumulative methane yields (272.99, 153.22 167.73 mL/g volatile solid (VS), respectively) and better stability (e.g. pH, volatile fatty acids (VFAs) and their component). The main microbe evolution had a similar trend which was *Petrimonas* and *Methanosaeta* in the early digestion process (Days 0-7) and then evolved into *Longilinea*, *Ruminofilibacter*, and *Methanosarcina* with the progress of digestion, but the relative abundance of these microbes in each reactor was different. It was worth noting that *Caldicoprobacter* in cow manure to maize stover ratio of 2, and to rice straw ratio of three reactors had a relatively higher proportion than reactor of cow manure to wheat stalk ratio of 1, and *Hydrogenophaga* was the specific bacterium in cow manure to wheat stalk ratio of 1 reactor. In addition, *Petrimonas* showed positive relationship with VFAs and *Longilinea* was the opposite. *Methanosaeta* and *Methanobacterium* contributed the most during the peak period of methane production in cow manure and maize stover co-digested reactor, and showed positive relationship with acetic acid. However, *Methanosarcina* and *Methanospirillum* made a great contribution during the peak period of methane production in cow manure co-digested with wheat stalk and rice straw reactors. These findings could provide further information on the application of cow manure co-digested with crop wastes.

**Keywords:** cow manure ratio, methane production, microbial community evolution, crop wastes, anaerobic co-digestion
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## 1 Introduction

China is an agricultural country that has extremely rich crop wastes and livestock manure resources[1]. Approximately 900 million t of crop straw among which maize stover, rice straw, and wheat stalk were the main crop wastes produced in 2017, and only approximately 30% of this amount was used[2]. Cow manure (CM) holds a great share standing for more than half of the total amount of manure currently and will further reach around 75% in one decade[3]. CM may contain traces of antibiotics, heavy metals, and pathogens, which not only affect the plants through salt toxicity through direct application but also humans via the food chain of accumulated toxins[4]. Hence, the inappropriate disposal of these wastes has been identified as an important reason for environmental problems on a global (climate change, ozone depletion) and regional (soil acidification, aquatic eutrophication, particulate matter formation) scale[5]. Different from the traditional treatment method, anaerobic digestion (AD) is a cost-effective process for treating biowastes because it has many advantages, such as reduction of greenhouse gas emissions, production of renewable energy, and production of biofertilizers[6,7]. Anaerobic digestion (AD) acts as a viable technology to extract the

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residual value of agricultural wastes (i.e., crop residues and manure) generated by farm sectors[8].

Monodigestion often suffers some drawbacks such as unstable processes[9,10] and low gas production rate[11,12] furthermore undermining its associated environmental benefits and economic revenue[13,14]. Therefore, anaerobic co-digestion (AcoD) has drawn great attention in recent years as it can supply a more balanced nutrient media for the growth of microorganisms[15,16]; thereby, the biological process benefits from the optimized microbial community structure and improved metabolic intensity[17,18]. In addition, under AcoD strategies, some inhibitors, such as ammonia nitrogen and longchain fatty acids (LCFA), are diluted to a secure level below threshold points, thereby their toxic effects on methanogens are reduced or totally eliminated[19,20]. Materials with a high carbon-to-nitrogen (C/N) ratio are usually mixed with those having a low C/N ratio so that the average ratio could be brought to a desirable level[21]. Manure has a low C/N ratio, which increases the likelihood of process failure or inhibition when used as a single feedstock[22]. In addition, manure has a high moisture content which acts as a solvent for dry biomass making it useful as a base substrate for co-digestion[23]. Mixing manure with other high C/N and dry biomass wastes (i.e., crop straw) in a certain proportion can adjust the C/N of raw materials and balance the nutrients, so as to increase the methane yields and reduce the start-up time[24,25]. Many studies were about the co-digestion of manure and crop residues[26-29]. In addition, anaerobic co-digestion of manure with straw has been shown to promote methanogenesis making the methanogens community have strong ecological functions and vigorous methanogens[30-32]. Also, many researches were about improving methane production by pretreatment or adding some additive.

However, there are few studies about the comparison of the effects of CM adding ratios on methane production during anaerobic co-digestion with maize stover, wheat straw, and rice straw, respectively. Though the three straws are mainly composed of cellulose, lignin, and hemicellulose, there are still some different aspects due to their distinction in growth habits. Hence, it was very essential to investigate the suitable CM ratios in each digestion system for their further application in biogas plants. Moreover, the characteristics and the comparison of microorganisms' evolution in different CM-adding ratios co-digestion systems were not investigated. Therefore, it was very essential to clarify how the CM adding ratios promote methanogenesis by affecting the methanogens community, and this could push its application in biogas engineering. Therefore, the purposes of this study were to 1) Analyze the methane yield characteristics of the anaerobic reaction system under different CM ratios and comprehensively evaluate the influence of cow manure adding ratios on the stability of each mixed digestion system; 2) Clarify the evolution of microbial characteristics of different CM ratio systems during the co-digestion process.

## 2 Materials and methods

### 2.1 Feedstocks and inoculum

Cow manure (CM), maize stover (CS), wheat stalk (WS), and rice straw (RS) were used as the co-substrates for the anaerobic digestion, respectively. CM was obtained from a farm at Hebei Agricultural University of China. CS, WS, and RS were obtained from a local farm (Lianyungang, Jiangsu, China). The CS, WS, and RS were dried naturally after being collected and then crushed and sifted through a 30-mesh sieve. The inoculum was obtained from a biogas station that operates at a mesophilic condition by using CM and CS as co-substrates (Dingzhou, Hebei, China). The key physicochemical properties of these feedstocks and inoculum were summarized in Table 1.

**Table 1 Characteristics of raw materials and inoculum**

| Parameter | CM | CS | RS | WS | Inoculum |
|---|---|---|---|---|---|
| Total solids (TS)/% | 16.61± 0.01 | 91.67± 0.00 | 94.22± 0.02 | 93.31± 0.01 | 4.43± 0.03 |
| Volatile solids (VS)/% | 13.29± 0.02 | 73.34± 0.01 | 75.38± 0.01 | 74.65± 0.03 | 3.54± 0.03 |
| Total Carbon (TC)/%[a] | 38.47± 3.09 | 43.65± 0.16 | 43.54± 0.25 | 44.02± 0.66 | ND |
| Total Nitrogen (TN)/%[a] | 5.15± 0.66 | 1.42± 0.12 | 1.03± 0.30 | 0.88± 0.41 | ND |
| Carbon to nitrogen (C/N) ratio[a] | 7.47± 1.57 | 30.74± 2.51 | 42.77± 1.63 | 50.02± 0.94 | ND |
| pH | 7.29±0.17 | ND | ND | ND | 7.51±0.28 |
| $NH_4^+$-N/mg·L$^{-1}$ | 29.81±5.98 | ND | ND | ND | ND |

Note: CM: Cow manure; CS: Maize stover; RS: Rice straw; WS: Wheat stalk; ND: Not determined. [a] TS-based, others without the mark are wet weight-based. The same as below.

### 2.2 Reactor setups

In this study, the CMs were completely mixed with each crop waste (i.e., CS, WS, and RS) at ratios of 3:1, 2:1, and 1:1 (total solid based, TS-based) and then thoroughly mixed with inoculum to obtain a TS of 10% at feedstock to inoculum (F/I) ratios of 2 (TS based), respectively. In this study, a 650 g mixture was added to a series of 1-L anaerobic reactors that had been flushed with $N_2$ for 5 min to create an anaerobic environment prior to the beginning of each digestion experiment. Inoculum without any feedstock was used as a control. All treatments were conducted in triplicate. Reactors were placed in an incubator with a temperature constant of 35°C for 45 d. Biogas generated from each reactor was collected using Tedlar gas bags on a daily basis for production and composition analysis. In this study, the experimental group of CM co-digested with CS at a ratio of 3:1, 2:1, and 1:1 was expressed as $CSCMT_3$, $CSCMT_2$, and $CSCMT_1$, respectively. Similarly, treatments of CM co-digested with WS and RS at a ratio of 3:1, 2:1, and 1:1 was $WSCMT_3$, $WSCMT_2$, $WSCMT_1$, $RSCMT_3$, $RSCMT_2$, and $RSCMT_1$, respectively.

### 2.3 Analytical methods

The methane content and production were measured by a portable biogas meter (BIOGAS5000, Geotech, UK). The physicochemical properties of samples collected from the reactors during the digestion process were analyzed (i.e., total solid content (TS), volatile solid content (VS), total nitrogen (TN), total carbon (TC), pH, $NH_4^+$-N content, and volatile fatty acids (VFAs) content). Samples without any treatment were used for the measurement of the TS, VS, TN, and TC. TS and VS contents were analyzed based on standard methods. TN and TC contents were measured by the elemental analyzer (Hanau, Germany). Samples were centrifuged at 5000 r/min for 10 min to separate the liquid from the solid. The liquid was used to determine the pH, $NH_4^+$-N content, and VFA contents. The pH of samples was determined with a pH meter. The VFA contents (i.e., formic, acetic, propionic, and butyric acids) were analyzed using a Liquid Chromatograph with a capillary column (HPX-87H 300.0 mm×7.8 mm, Aminex, Shimadzu, Japan,) and detector (SPD-S20A, Shimadzu, Japan). The column temperature was 40°C and the column pressure was 1.3 MPa. The injection temperature was 90°C, and the injection volume was 10 $\mu$L. The flow rate of the mobile phase was 0.6 mL/min with an absorption wavelength of 205 nm, and the

sample measurement time was 30 min. Dilutions of the corresponding compounds were injected as standards to quantify the compounds and confirm the peak positions. The content of $NH_4^+$-N was measured by a flow injection analyzer (AA3, SEAL, Germany).

### 2.4 Model application

The experimental data were validated using a modified Gompertz model (GM) and a First-order (FO) model. Methane yield was calculated, and the cumulative methane yield was simulated with the modified Gompertz model shown in Equation (1), which was developed by Lay et al.[33]

$$M = P \cdot \exp\left\{-\exp\left[\frac{e \times R_{\max}(\lambda - t)}{P}\right] + 1\right\} \quad (1)$$

where, $M$ is the cumulative methane yields, mL/g VS; $P$ is the maximum methane potential, mL/g VS; $R_{\max}$ is the maximum methane yields rate, mL/d; $e$ is Euler's number (≈2.71828); $\lambda$ is the lag phase, d; $t$ is the time, d.

$K_h$ reflects the rate of the hydrolysis stage and can be calculated using the net cumulative biogas yield by applying Equation (2)[34,35].

$$\ln\left(\frac{P-M}{P}\right) = -K_h t \quad (2)$$

The non-linear fit of methane yields based on Equation (1) and the linear regression of $\ln\left(\frac{P-M}{P}\right)$ against time ($t$) based on Equation (2) were performed using Origin 8.6 and SPSS 19.0 software, respectively. All data are expressed as the mean ± standard deviation, and the threshold for significance is $p<0.05$.

### 2.5 Microbiological analysis

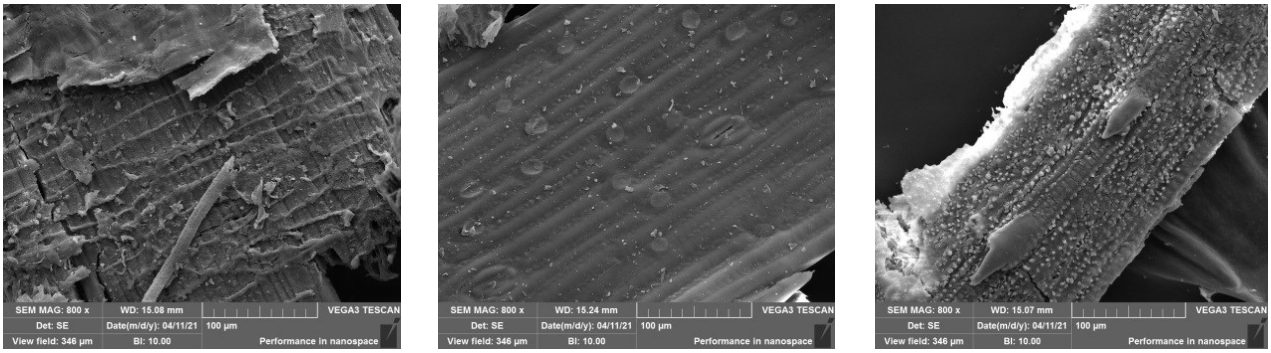
The functional microorganisms contributing to methane production and the microbial community evolution during different CM ratios and crop waste co-digestion processes were analyzed by using high-throughput sequencing. Microbial community structure of pure inoculum and treatments with the highest methane yield at the different co-digestion reactors of CM with crop wastes (i.e., $CSCMT_2$ (2:1), $WSCMT_1$ (1:1), $RSCMT_3$ (3:1) were analyzed on Days 1, 3, 7, 15, 30, and 45. The samples of treatments on Days 1, 3, and 7 were chosen because of their notable difference in daily and cumulative methane yields which can reflect the functional microbe contributing to the methane production in the acidification and hydrolysis stage. Similarly, the samples on Days 15 and 30 were selected for their stable methane contents which can represent the main microorganisms in the methanogenic stage. The samples on Day 45 indicated the microbial community structure at end of the digestion process. Among them, bacteria were shown as B. That is to say, the bacterial community structure on Days 1, 3, 7, 15, 30, and 45 were expressed as 1B, 3B, 7B, 15B, 30B, and 45B. Archaea were shown as A and the samples on Days 1, 3, 7, 15, 30, and 45 were shown as 1A, 3A, 7A, 15A, and 30A.

The main process of microbial gene sequencing included DNA extraction, polymerase chain reaction (PCR) amplification, library construction, and sequencing. The DNA of samples was extracted by the FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) and amplicon sequencing was done through an Illumina MiSeq® PE300 platform (Allwegene Technology, China). The amplified sequence used for microbe was in the V3-V4 hypervariable region using the following universal primer sets: for bacteria, 338F, and 806R (5'-ACTCC TACGGGAGGCAGCA-3' and 5'-GGACTACHVGGGTWTCTAAT-3')[36]; for archaea, 344F and 806R (5'-ACGGGGYGCAGCAGGCGCGA-3' and 5'-GGACTACVSGGGTATCTAAT-3')[37]. Raw fastq files obtained through the Miseq platform were demultiplexed, and quality-filtered using QIIME (version 1.17) with the following criteria as Cai et al.[38] reported. After demultiplexing, the reads were assigned operational taxonomic units (OTUs) at 97% sequence similarity cutting off using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The RDP classifier (http://rdp.cme.msu.edu/) was used to analyze the taxonomy of each 16S rRNA gene sequence against the silva 16S rRNA database using a confidence threshold of 70%[39] and obtain the species classification information corresponding to each OTU. The OTUs were classified using the alpha diversity metric. The alpha diversity metrics were determined according to compared the smallest libraries for each sample. The alpha diversity metrics included Chao 1, Shannon index, and phylogenetic diversity (i.e., Kingdom, phylum, class, order, family, genus, species)[40,41].

## 3 Results and discussion

### 3.1 Electron microscopy (SEM) of each crop wastes

The scanning electron microscopy (SEM) results showed the three kinds of straw microstructure had significantly different structures (Figure 1). The structure of CS is tight, and the fiber bundles are arranged clearly and slightly convex (Figure 1a). However, the surface structure of WS is relatively dense and smooth with almost no convex point but there are bubbles, and the veins can be seen (Figure 1b), also the specific surface area is relatively small which is not conducive to the attachment of microorganisms. The surface of RS is rough and more convex with a rod-like structure and an obvious fiber bundle skeleton structure (Figure 1c). The structure of CS and RS is relatively loose, soft texture, and has strong ventilation compared with WS.



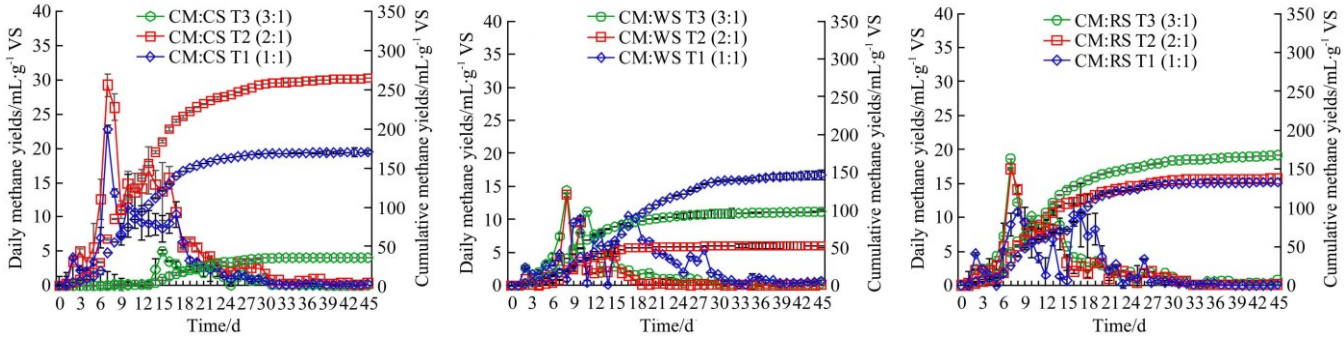

a. CS b. WS c. RS

Note: CS: Maize stover; WS: Wheat stalk; RS: Rice straw. The same as below.

Figure 1 Scanning electron microscope (SEM) with CS, WS, RS

### 3.2 Methane yields in anaerobic co-digestion reactors

In co-digestion reactors of CM and CS, $CSCMT_3$ had a long lag phase to produce methane (on Day 13) and reached the production peak (4.95 mL/g VS) on Day 15, then gradually decreased (Figure 2). This was due to the higher CM ratio indicating high nitrogen content and inappropriate C/N ratio which contributed to the low activity of microorganisms[42]. However, $CSCMT_2$ and $CSCMT_1$ started on the first day and reached the highest daily methane yield (29.25 and 22.77 mL/g VS, respectively) on Day 7. Also, their cumulative methane yields were higher than $CMCST_3$ which were 6.68 and 5.24 times, respectively. The daily methane yields in CM and WS co-digested reactors showed $WSCMT_3$ and $WSCMT_2$ generally increased and reached the peak of daily methane yields on the 8th day (14.38, 13.71 mL/g VS, respectively) (Figure 2b). However, the daily methane yields of $WSCMT_1$ were extremely unstable in the first 15 d, and this was due to the low CM ratio implying low nitrogen content and buffering capacity which was consistent with its lower pH and $NH_4^+$-N content (Figure 3b). In addition, the $WSCMT_1$ had the largest cumulative methane yields, then $WSCMT_3$ and $WSCMT_2$ followed. In co-digestion reactors of CM and RS, the daily methane yields for $RSCMT_3$ and $RSCMT_2$ started on the first day and reached the daily methane peak on the 7th day (18.66 and 17.14 mL/g VS, respectively) (Figure 2c). On the contrary, the daily methane yields of $RSCMT_1$ fluctuated greatly in the whole digestion duration with multiple daily production peaks which was consistent with its lower pH and $NH_4^+$-N content (Figure 3c). $RSCMT_3$ had the highest cumulative methane yields, then followed by $RSCMT_2$ and $RSCMT_1$, besides, $RSCMT_2$, and $RSCMT_1$ had similar values. In addition, a higher CM ratio could be beneficial to the stability of the reactor for co-digestion with RS. However, the appropriate ratio of CM helped to achieve the highest methane yields for co-digestion with CS and WS, respectively. Hence, the proper CM ratio was determined by the species of crop wastes used in the co-digestion reactor which may be due to their particular microstructure (Figure 1).




a. CSCM b. WSCM c. RSCM

Note: CM: Cow manure. CS: Maize stover. WS: Wheat stalk. RS: Rice straw.

Figure 2 Changes in daily and cumulative methane yields with CSCM, WSCM, and RSCM

### 3.3 Dynamic characteristics of co-digestion reactor

For the modified GM model, the fitting index ($R^2$) values were above 99% (Table 2). In co-digestion of CM with CS reactors, $CSCMT_2$ had the largest maximum methane yield rate ($R_{max}$) which was 4.65 and 1.06 times of $CSCMT_3$ and $CSCMT_1$, respectively. Meanwhile, $CSCMT_2$ had the shortest lag phase ($\lambda$) and the $\lambda$ of $CSCMT_3$ and $CSCMT_1$ were 3.16 and 1.07 times of $CSCMT_2$, respectively. For CM and WS co-digestion reactors, $WSCMT_1$ had the highest $R_{max}$ and the longest $\lambda$, then $WSCMT_2$ and $WSCMT_3$ followed. These results showed that decreasing of adding the dosage of CM could increase the $R_{max}$ of the CM with WS co-digestion system, and also prolong the $\lambda$ of the system. In addition, in co-digestion reactor of CM and RS, $RSCMT_3$ had the highest $R_{max}$ and shortest $\lambda$, then $RSCMT_2$ and $RSCMT_1$ followed indicating higher CM ratio was beneficial for the co-digestion system. Generally, $R_{max}$ and $\lambda$ of each treatment are consistent with daily and cumulative methane yields (Figure 2). For the FO model, the fitting index ($R^2$) values were above 90% (Table 2). The $CSCMT_2$ had higher $K_h$ of co-digestion reactors which was corresponding to the daily methane yields, and cumulative methane yields (Figure 2). Also, this was consistent with the pH (Figure 3) and VFAs (Figure 4) during the digestion process. $K_h$ of CM co-digested with WS and RS reactors increased with the CM ratio and this was due to more easily degradable substance in CM[43,44] contributing to the intermediate macromolecular products. Hence, the CM ratio affected the co-digestion process and the proper ratio of CM could increase $R_{max}$ and shorten the $\lambda$ in the co-digestion reactor of CM and CS, also keeping the stability of reactors.

**Table 2 Parameters of the GM and FO model at different CM ratios in co-digestion reactors**

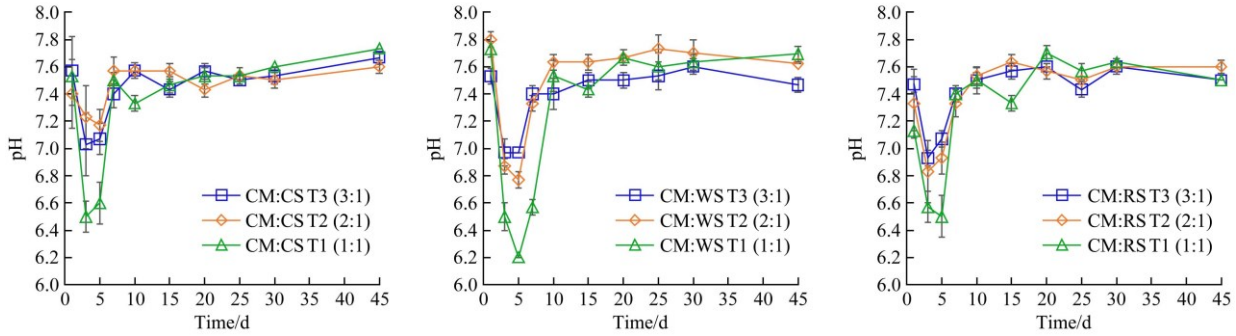
| Experimental group | | Modified Gompertz model (GM) | | | | | First-order (FO) model | |
|---|---|---|---|---|---|---|---|---|
| | | Ultimate specific methane yield/mL·g$^{-1}$ VS | $P$/mL | $R_{max}$/mL·d | $\lambda$/d | $R^2$ | $K_h$ | $R^2$ |
| CM:CS | $T_3$(3:1) | 40.89 | 41.12±0.11 | 3.70±0.08 | 12.98±0.17 | 0.99974 | 0.01773 | 0.90138 |
| | $T_2$(2:1) | 272.99 | 264.02±0.25 | 17.21±0.27 | 4.11±0.20 | 0.99998 | 0.08108 | 0.96965 |
| | $T_1$(1:1) | 214.47 | 214.16±0.11 | 16.19±0.17 | 4.38±0.12 | 0.99999 | 0.07969 | 0.96314 |
| CM:WS | $T_3$(3:1) | 100.35 | 99.10±0.18 | 7.36±0.16 | 3.58±0.24 | 0.99984 | 0.12374 | 0.96285 |
| | $T_2$(2:1) | 68.06 | 67.54±0.10 | 7.84±0.21 | 5.44±0.19 | 0.99984 | 0.11972 | 0.91423 |
| | $T_1$(1:1) | 153.22 | 154.39±0.39 | 8.04±0.13 | 5.91±0.21 | 0.99992 | 0.03398 | 0.94347 |
| CM:RS | $T_3$(3:1) | 167.73 | 146.41±0.32 | 9.22±0.12 | 3.46±0.32 | 0.99986 | 0.09513 | 0.97744 |
| | $T_2$(2:1) | 137.25 | 137.34±0.24 | 9.17±0.21 | 3.63±0.27 | 0.99989 | 0.09131 | 0.96851 |
| | $T_1$(1:1) | 132.61 | 133.24±0.27 | 9.07±0.23 | 4.67±0.27 | 0.99986 | 0.05082 | 0.92794 |

Note: $P$ is the maximum methane potential, mL/g VS; $R_{max}$ is the largest maximum methane yield rate; $\lambda$ is the lag phase, d; $K_h$ is the rate of the hydrolysis stage; $T_1$ means the treatment of CM to crop wastes ratios of 1:1; $T_2$ means the treatment of CM to crop wastes ratios of 2:1; $T_3$ means the treatment of CM to crop wastes ratios of 3:1.

### 3.4 pH, VFAs, and $NH_4^+$-N contents in anaerobic co-digestion reactors

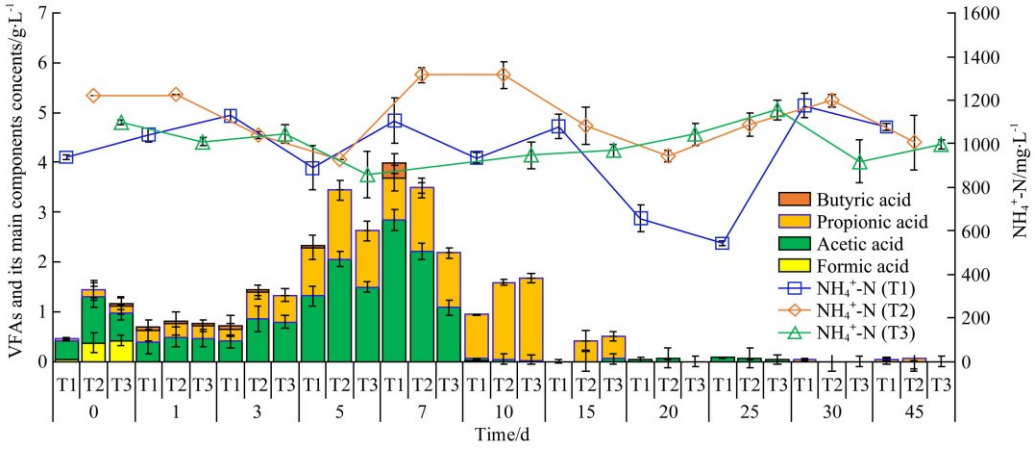
The pH values in all co-digestion reactors were in the range of 6.0-7.8 indicating adding CM was beneficial to maintaining a suitable pH environment during the digestion process and this contributed to higher daily and cumulative methane yields[45,46] (Figure 3). In addition, a higher CM ratio was corresponding to a more stable pH in a co-digestion reactor and this was due to lower CM ratios caused by the higher C/N ratio which implied the buffer capacity was worse than the higher CM ratio co-digestion reactor and the intermediate products such as VFAs was easy to accumulate (Figure 4)[47]. Hence, higher CM ratios could be beneficial for the stability of pH in the co-digestion process.

In CM and CS co-digestion reactors, the VFAs content of $CSCMT_3$ was higher in the first 7 d of the digestion (3.985 g/L) and then decreased rapidly (Figure 4a), which was consistent with the trend of $NH_4^+$-N concentration and lower daily and cumulative methane yields (Figure 3a). $CSCMT_2$ and $CSCMT_1$ have similar changes in VFAs concentration and were consistent with changes in pH (Figure 3). In addition, the VFA contents had similar trends which showed higher CM ratios corresponding to higher VFAs contents when co-digested with WS and RS (Figure 4b and 4c) and this was due to the higher CM ratio implying more easy degradation matters which accumulated easily as to untimely consumption. Acetic acid was the main component of VFAs (accounting for more than 50%) in the first 7 d and was mostly consumed on the 10th day during the digestion process, and this result is in agreement with the findings of Chi et al.[48] and Goswami et al.[49] In addition, propionic acid accounted for a large proportion of VFAs especially, in the first 7 d exceeding 1 g/L, but it has no obvious effect on the anaerobic reaction system[50-52]. All the VFA concentrations in each reactor did not reach the inhibition concentration of VFA (all below 13 g/L)[53] which indicated there was no VFA inhibition in the digestion process.

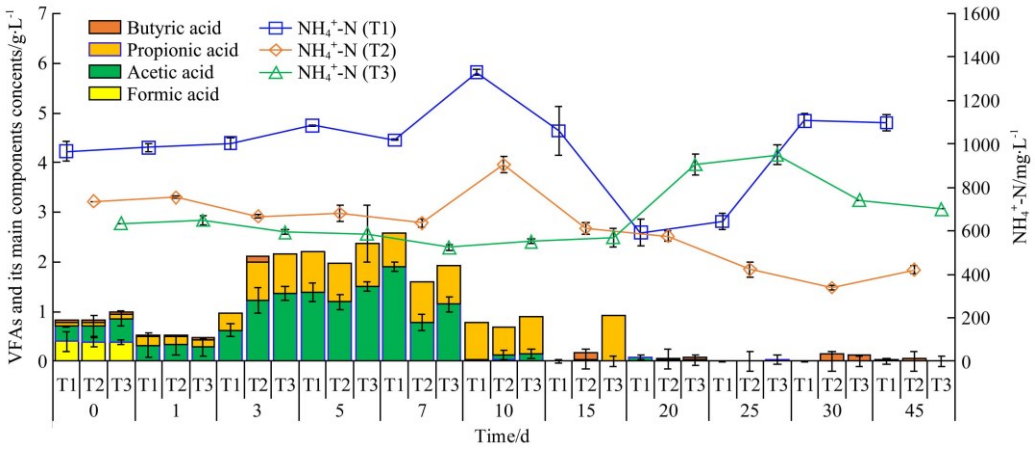



a. CSCM b. WSCM c. RSCM

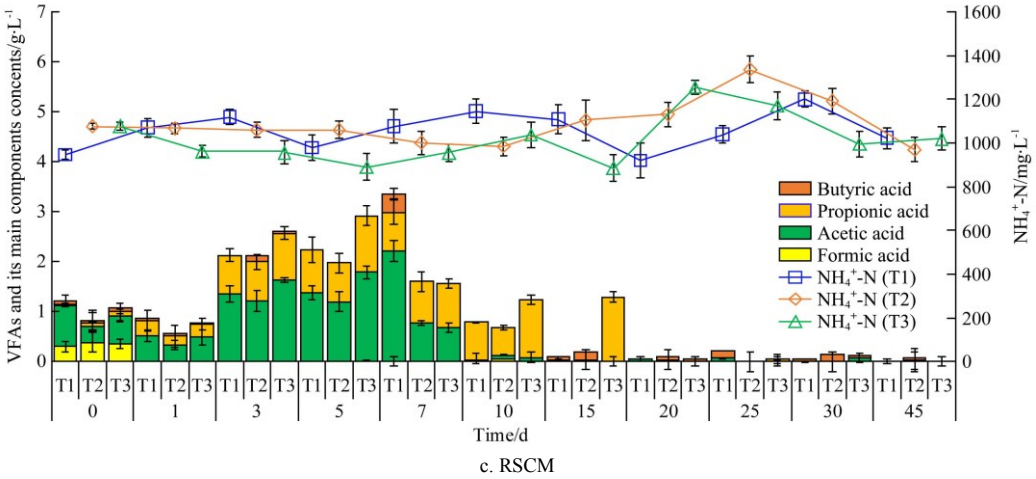
Figure 3 Changes in pH value of CSCM, WSCM and RSCM




a. CSCM




b. WSCM




c. RSCM

Figure 4 Changes in VFAs and $NH_4^+$-N contents with CSCM, WSCM, and RSCM

The $NH_4^+$-N content trend (Figure 4) was consistent with the pH (Figure 3) indicating reactors had enough buffering capacity to maintain the acid-base balance during the anaerobic digestion process. In addition, the $NH_4^+$-N contents in the co-digestion of CM with CS and RS had a relatively stable $NH_4^+$-N content maintained at about 1100 mg/L (Figures 4a and 4c). However, the co-digestion of CM with WS had large fluctuations of $NH_4^+$-N concentrations during 7-30th days which caused insufficient nutrients and inadequate buffering capacity resulting in an unstable system (Figure 4b). This was the main reason why the daily methane production of the co-digestion of CM with WS fluctuated during this period. The $NH_4^+$-N contents in all reactors were all below 3000 mg/L which had no inhibition of methanogens[54-56].

### 3.5 Diversity analysis of microbial community

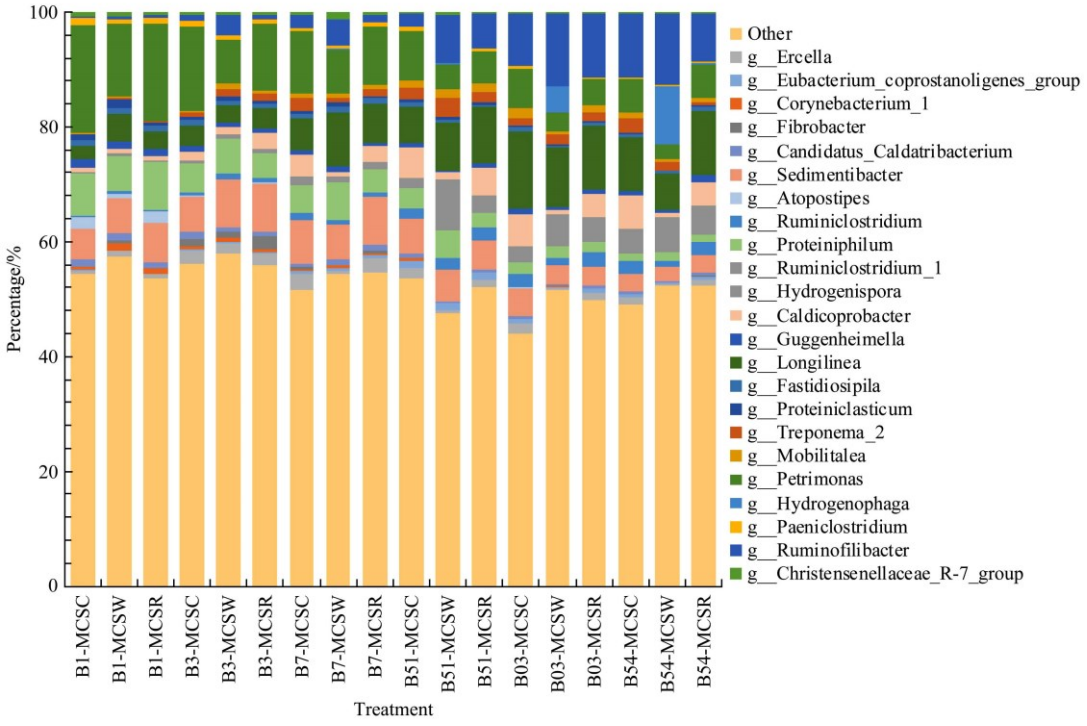
For all the reactors, the dominant bacteria in the early stage (0-7th day) of the co-digestion process were *Petrimonas*, and its relative abundance gradually decreased with the progress of the digestion process (Figure 5a). This was because a higher CM ratio implied more easily degradable organic matters and this contributed to the growth of *Petrimonas* which mainly decomposes simple sugars to produce acetic acid, $CO_2$, and hydrogen[57], then the relative abundance gradually decreased with the consumption of these degradable substances. These were consistent with the daily and cumulative methane yields (Figure 2). Also, this was consistent with the pH and VFAs changing trend (Figures 3 and 4). With the progress of the digestion process, the main bacteria evolved into *Longilinea* and *Ruminofilibacter* on the 15th-45th days, and their relative abundance increased (Figure 5a). Both of them can effectively hydrolyze macromolecular refractory organics as *Longilinea* can produce organic acids by decomposing macromolecular organics and *Ruminofilibacter* can produce hemicellulase[58,59]. Hence, these bacterium dominated on the 15th-45th days. In addition, it was noted that the relative abundance of *Caldicoprobacter* in $WSCMT_1$ was lower than in the other reactors during digesting process, which was consistent with the lower CM ratio as *Caldicoprobacter* is the main bacteria for protein hydrolysis[60]. In addition, the relative abundance of *Hydrogenophaga* in $WSCMT_1$ increased from 0.11% to 4.54% on the 15th-30th days of digestion and further increased to 10.35% on the 45th day of digestion, while the relative abundance of *Hydrogenophaga* in $CSCMT_2$, $RSCMT_3$ was lower than 0.3% during digestion. Since *Hydrogenophaga* is more sensitive to hydrogen, a large increase in its relative abundance indicated a large increase in hydrogen production in the reactor. Hence, every digestion stages had different microbial compositions as the bacteria in the early stage mainly consumed carbohydrate, fat, and protein from cow manure, and it degraded the refractory organic matter from straw in the middle and late stage which was consistent with the daily methane yield having several peaks in whole digestion process (Figure 2). Though the evolution of the main bacteria community was relatively similar which were mainly acid-producing bacteria, the relative abundance of each reactor was different and there were special bacteria in $WSCMT_1$, and this contributed to the final difference digestion process.

The dominant archaea were different in various periods, i.e., the main archaea in the 1st-7th days were *Methanosaeta*, and then *Methanosarcina* increased and transformed into the main archaea. This was consistent with the changes in pH, VFAs, and $NH_4^+$-N concentration (Figure 3 and 4) as *Methanosaeta* can decompose acetic acid to form methane which is more sensitive to the environment than *Methanosarcina*, and the relative abundance gradually decreased from Day 3 to Day 7 with the increase of acetic acid concentration[61]. The relative abundance of *Methanosarcina* increased with the progress of digestion as it is the only methanogenic microorganism that can utilize all known methanogenic pathways, and it is relatively insensitive to the environment and can survive in higher concentrations of VFAs, $NH_4^+$-N, and hydrogen sulfide, and its number increases with the increase of acetic acid concentration[62,63]. In the co-digestion of cow manure and different straws process, the methane production was mainly produced by the acetic acid nutrition pathway in the first 15 d, and hydrogen nutrition pathway and methyl nutrition pathway after 15 d (Figure 2) according to the variation trend of relative abundance of *Methanosaeta* and *Methanosarcina*. In addition, *Methanobacterium* also existed in each sample, which can produce methane through interspecies hydrogen transfer[64]. Therefore, combined with methane production (Figure 2), *Methanosaeta* and *Methanosarcina* were the main methanogens, among which *Methanosaeta* had a higher methane production rate.

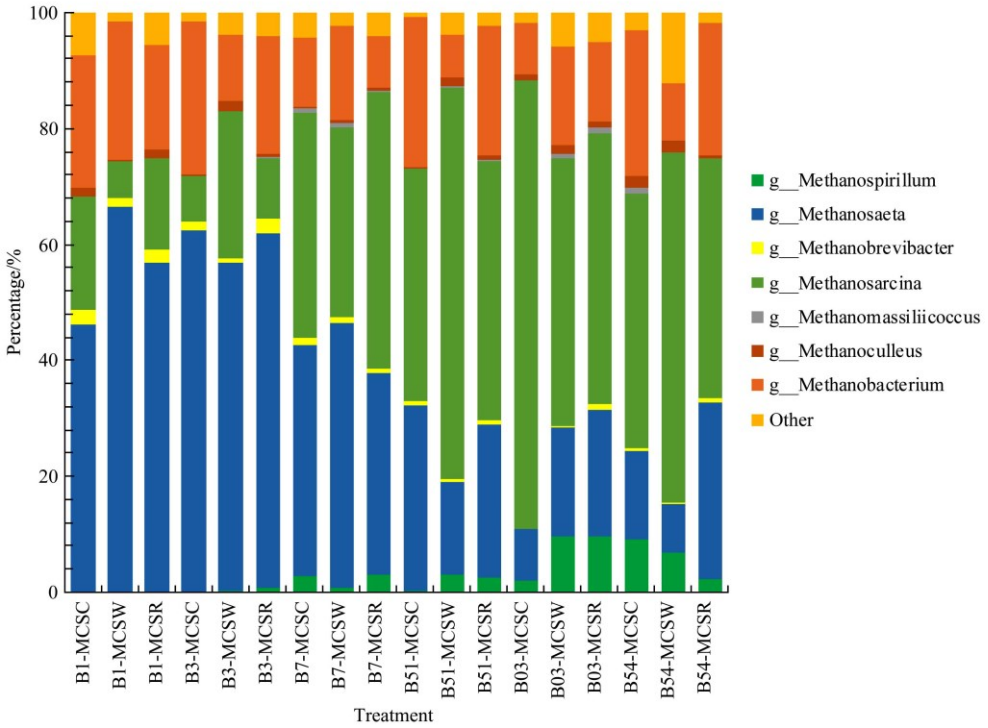
For bacteria, Redundancy Analysis (RDA) analyzed results showed *Petrimonas*, *Sedimentibacter*, and *Proteiniphilum* were highly positively correlated with the VFAs concentration and the three bacteria were close to the samples of the first 15 d (Figure 5c). This showed that they promoted the accumulation of VFAs in the hydrolytic acidification period, also this was consistent with the

VFAs contents (Figure 4). *Longilinea*, *Ruminofilibacter*, and *Ruminiclostridium*_1 contributed to the decomposition of organic matter in the middle and late stages (the 15th-45th days) of digestion. *Caldicoprobacter* was closer to the samples of $CSCMT_2$ and $RSCMT_3$ on the 15th-45th days, which showed a positive correlation with $NH_4^+$-N content and a negative correlation with VFAs content indicating *Caldicoprobacter* had a certain contribution to the accumulation of $NH_4^+$-N and the recovery of pH value in $CSCMT_2$, $RSCMT_3$ systems which were consistent with the $NH_4^+$-N content was more stable than in $WSCMT_2$ (Figure 4).

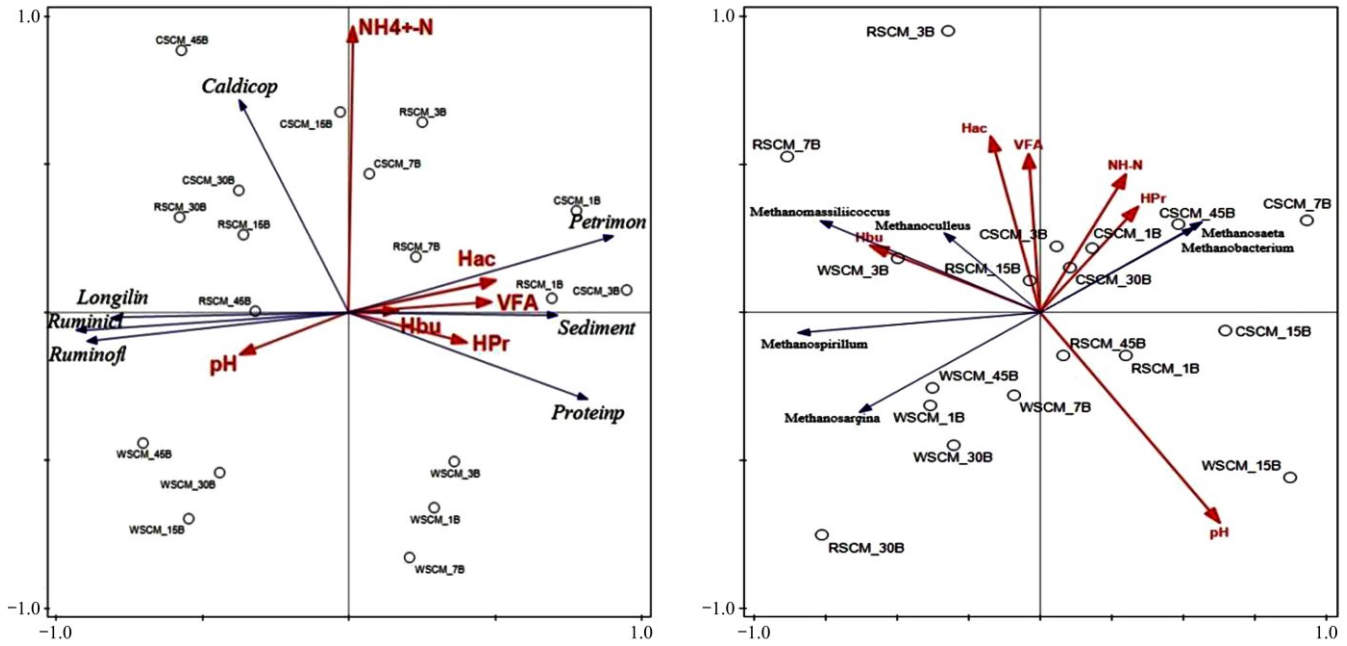
For archaea, RDA results showed that *Methanosaeta* and *Methanobacterium* promoted the consumption of acetic acid in $CSCMT_2$ (Figure 5d) and propionic acid concentration was the most important environmental factor. *Methanosarcina* and *Methanospirillum* were the main archaea at the peak period of gas production in $WSCMT_3$. All archaea were at right angles or obtuse angles with pH indicating that methanogens were negatively correlated with pH, that is, acidic pH would greatly inhibit the activity of methanogens and this also could find in methane production (Figure 2).




a. Bacteria




b. Archaea




c. RDA analysis of bacteria d. RDA analysis of archaea

Note: The red arrow means the environmental factors affecting the digestion process and the blue arrow means the main microbe in the reactors. The length of the arrow indicates the relevance between the environmental factors and microbial community.

Figure 5 Characteristics of microbes at genus level with bacteria, archaea, and RDA analysis of bacteria and archaea

## 4 Conclusions

Cow manure (CM) ratio affected the evolution of microbial community structure in co-digestion of different crop wastes which finally contributed to the different digestion processes. Hence, suitable CM ratio varied with the species of crop wastes as their special microstructure in terms of ensuring the normal start-up of the digestion system and improving methane production. The dominant bacteria was *Petrimonas* at the early stage of the co-digestion and then conversed with *Longilinea, Ruminofilibacter* until the end of the duration. To archaea, *Methanosaeta* accounted for a large proportion at the initial stage and then *Methanosarcina* became the dominant archaea. On the whole, the evolution of microbial community structure was similar in the $CSCMT_2$ and $CRCMT_3$. In addition, it was worth noting that the abundance of *Caldicoprobacter* in the $WSCMT_1$ was lower than that in the other two reactors, which was one of the important reasons for the instability of the reactor. Hence, for different crop wastes, a suitable ratio co-digested with CM could provide a stable digestion process and higher methane yields and this had an important guiding sense for biogas plants.

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