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Comparison of Anaerobic Co-Digestion of Buffalo Manure and Excess Sludge with Different Mixing Ratios under Thermophilic and Mesophilic Conditions

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Abstract: In this study, the main aim is to evaluate the mixing ratio of co-digestion of buffalo manure (BM) and excess sludge (ES) influenced for methane yield and digestate dewaterability. Five batch experiments with different BM and ES mixing ratios were carried out under thermophilic and mesophilic conditions. The methane yield of co-digestion of BM and ES increased by 10.1–73.5% under thermophilic conditions and 87.9–153.3% under mesophilic conditions, compared with the mono-anaerobic digestion of ES under the same conditions. Shannon and Chao1 indices showed that the bacterial species of the mesophilic digesters were more abundant than that of the thermophilic digesters. With the increase in the BM proportion in the substrate, the normalized capillary suction time (NCST) and total solids (TS) of sediment (centrifugal dewatering) increased. The NCST at thermophilic temperature (8.98–12.54 $s \cdot g^{-1}$-TS) was greater than that at the mesophilic temperatures (5.45–12.32 $s \cdot g^{-1}$-TS). However, the TS of sediment was not directly related to the digestion temperature. This study has shown that anaerobic co-digestion of BM and ES at the appropriate ratio (BM/ES = 1:1.5) has a significant meaning in a high methane yield.

Keywords: methane yield; bacterial communities; digestate; normalized capillary suction time; TS of sediment

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1. Introduction

With the continuous increase in industrial structure adjustment in Chinese agricultural industry, the proportion of intensive and large-scale breeding has increased rapidly. China has an estimated 23.5 million buffalo, and the annual production of buffalo manure (BM) is 172.1 million tons (9.3 tons per water buffalo per year) [1,2]. At present, the storage and treatment capacity of manure in most of the farms in our country is insufficient [3]. It is estimated that only 30% of the livestock and poultry waste in the intensive farms has been preliminarily treated and used, and the remaining 70% is directly discharged to the environment. In addition, the extensive use of antibiotics in the livestock industry leads to a variety of antibiotic residues in the feces of livestock and poultry [4]. Therefore, the direct discharge of a large number of livestock manure seriously pollutes water sources and the rural ecological environment, posing a serious threat to soil, water, air, and the safety of people and animals [1]. At present, the processing methods of BM mainly include composting and production of bedding materials [5]. Anaerobic digestion is the most common treatment for BM in China [5].

The annual production of municipal excess sludge (ES) in China is 39 million tons (80% water content) [6]. Due to the use of antibiotics, harmful substances, such as heavy metals, resistance genes, and pathogens, can also accumulate in ES [7]. Therefore, improper handling of ES will cause harmful gases, resistance genes, heavy metals, and other pollutants to return to the environment and may spread pathogens [8]. Currently, the main

methods to treat sludge are landfilling, composting, natural drying, and incineration, and considering waste characteristics, energy recovery and land use. Anaerobic digestion is considered as a sustainable option to treat ES [9].

The mono-digestion of ES usually results in a lower hydrolysis rate and methane yield due to its low volatile solids (VS), total solids (TS), carbon/nitrogen (C/N), and nutrient imbalance [10]. The mono-digestion of BM is difficult to hydrolyze and has a poor digestion rate due to its high VS, TS, C/N, and cellulose [11]. Moreover, the accumulation of organic acids and ammonia due to the higher VS and TS in BM can inhibit the methane yield, and the digestate of BM has a poor fertility capacity. In general, the digestion of a single substrate reduces the economic viability of its digested products [12]. The co-digestion helps to improve digestion performance [13]. Sewage treatment plants and buffalo farms situated in suburbs offer the possibility of synergistic treatment between the two substrates. ES as a product of sewage treatment plants and BM as a by-product of buffalo farms are complementary in some characteristics, for example, BM has a higher C/N ratio while ES has a lower C/N ratio; microbial communities were more abundant in ES and less in BM; and ES has a higher water content, while BM has a lower water content [14]. In addition, correspondingly, the nitrogen content of digestate produced by co-digestion is high, which can also be used for fertilization. Therefore, the appropriate proportion of BM and ES co-digestion has the suitable water content, pH, C/N, and high volumetric loading rate, which also dilutes toxic compounds, balances microbial nutrients, and increases microbial diversity [15]. Co-digestion of BM and ES significantly increased the methane yield at 35 ± 1 °C compared with the mono-digestion of ES (50.64% improvement) or BM (79.48% improvement) [16].

The operating temperature and substrate of anaerobic digestion are the main determinants of digestion performance and digestate dewaterability [17]. Anaerobic temperature is generally kept within the range of thermophilic temperature (50~57 °C) or mesophilic temperature (30~38 °C) [18]. During the anaerobic co-digestion process of pig manure and sludge, the reduction rate of volatile solids (VS) decreased with increasing TS, and the maximum cumulative methane yield was 342 mL g^{-1}-volatile suspended solids $_{added}$ (VSS_{added}) when TS was 6% [12]. Thermophilic conditions can promote the solubility of organic matter, the biochemical reaction rate, the degradation rate of organic matter, and the methane yield, as well as the reduction in the residence time and destruction of pathogens more effectively [19]. Finally, the temperature also has an effect on the characteristics of digestate [20]. Anaerobic co-digestion of cow manure with cabbage waste revealed maximum methane yields of 184.4 and 321.7 mL g^{-1} VSS_{added} at thermophilic and mesophilic temperatures, respectively [15]. The optimal mixing ratio of pig manure and sludge for anaerobic co-digestion under thermophilic conditions was 2:1, and the cumulative methane production was 315.8 mL g^{-1}, which was 82.4% higher than that of sludge-only digestion [14]. However, little information is available on anaerobic co-digestion of BM and ES at thermophilic temperatures. Different substrate mixing ratios also lead to different physical and chemical properties, as well as microbial communities, which can affect the effectiveness of anaerobic digestion and dewatering performance [17]. The optimal condition for increasing methane production from anaerobic co-digestion of sewage sludge and cattle manure was a VS ratio of 3/7 [16]. The difference in anaerobic co-digestion between BM and ES at varied mixing ratios under thermophilic or mesophilic conditions is not clear. As well, the temperature and mixing ratio for the dewatering performance of digestate are not known. The digestate produced by the anaerobic digestion process needs to be dewatered for further disposal or use. In addition to the faster digestion rate and higher methane production, an adequate dewatering capacity of digestate is essential for the sustainable anaerobic digestion processes [21]. The results of anaerobic co-digestion of pig manure with sludge showed that the minimum NCST was 2.91 and 8.01 $s \cdot g^{-1}$-TS at mesophilic and thermophilic temperatures, respectively [17].

In this study, the effects of the BM and ES mixtures ratio on anaerobic co-digestion performance, microbial communities, and digestate dewatering performance were investi-

gated. The main research objective of this study is to obtain better dewatering performance under the premise of higher methane production and faster digestion rate.

2. Materials and Methods

2.1. Materials

Both ES and BM were from Jiujiang City, China. The ES was collected from the concentration tank of a sewage treatment plant, and the treatment process was CAST on a scale of 100,000 tons per day. BM was obtained from a cattle breeding farm near the wastewater treatment plant. The scale of the livestock farm was about 100 buffalos, and the yield of buffalo manure was about 500 kg per day. ES was concentrated by gravity precipitation overnight and then stored with the collected BM at 4 °C before the experiment. The main characteristics of different substrates are shown in Table 1.

Table 1. Characteristics of different substrates.

	BM	ES
TS (%)	20.27 ± 0.16	6.78 ± 0.03
VS (%)	16.62 ± 0.28	2.36 ± 0.08
VS/TS (%)	81.99	34.80
C (%)	43.04 ± 0.78	16.74 ± 0.20
N (%)	1.34 ± 0.21	2.57 ± 0.88
H (%)	5.27 ± 0.16	2.87 ± 0.01
S (%)	0.20 ± 0.02	0.46 ± 0.01
pH	7.44	7.04
C/N	32.01	6.51

Total Solids, TS. Volatile Solids, VS. Total Solids/Total Solids, VS/TS. Carbon, C. Hydrogen, H. Sulphur, S. Carbon/Nitrogen, C/N.

2.2. Experimental Setup

Five different ratios of BM and ES were used in the experiments, which included ES only, BM/ES = 1:3, BM/ES = 1:1.5, BM/ES = 1.5:1, and BM/ES = 3:1, respectively (Shown in Table 2). Additionally, the total mass of the sample within each digester was 1.6 kg. According to the references of similar studies, the ratio of BM to ES set in the experiment can regulate the C/N ratio, microbial community structure, and other properties [15,22]. The five different mixing ratios are denoted by M1, M2, M3, M4, and M5 under mesophilic conditions and by T1, T2, T3, T4, and T5 under thermophilic conditions. The C/N ratios of substrates from T1 to T5 (from M1 to M5) were 6.51, 8.28, 9.81, 12.90, and 16.73, respectively. The TS of co-substrates from T1 to T5 (from M1 to M5) were (6.78 ± 0.03)%, (7.39 ± 0.04)%, (7.94 ± 0.04)%, (9.15 ± 0.05)%, and (10.81 ± 0.07)%, respectively. High TS of BM led to sampling difficulty, so the experiment was not set up for mono-digestion of BM. For further practical use, no additional water was added to the digester to regulate the TS. The mixed preparation of BM and ES was configured according to weight without inoculum.

Table 2. Experience setup.

BM/ES (Based on VS)	ES Only	1:3	1:1.5	1.5:1	3:1
thermophilic conditions	T1	T2	T3	T4	T5
mesophilic conditions	M1	M2	M3	M4	M5

Co-substrate in different proportions were placed in anaerobic digesters, which have a 2 L working volume and were sealed with a rubber plug with two 4 mm inner diameter holes. One hole was used to gain the digestate by a peristaltic pump, while the other was connected to the airbag via a rubber hose to collect gas samples. To ensure an anaerobic environment for the experiment, each digestion digester was inflated with nitrogen for two minutes, replacing the digester's air. Additionally, the prepared digesters were placed in a

water bath at 55 ± 1 °C or 37 ± 1 °C, and then the digesters were shaken by hand once a day (1 min). All experiments were repeated 3 times.

2.3. Analytical Methods

The soluble extracellular polymers (EPS) in the digestate were extracted by using heat-centrifugation extraction [23]. The sample mixture was centrifuged at 2000× *g* for 5 min, and the supernate was used as a liquid sample through a 0.45 µm polyethersulfone superfine fiber filter. The specific operating steps are the same as that of the previous study [17].

TS, VS, and pH of the original substrate were determined using standard techniques for water and wastewater detection [24]. An elemental analyzer (Vario ELcube, Elementar, Frankfurt, Germany) was used to detect the percentage of elements in air-dried materials. The dissolved organic carbon (DOC) in the liquid samples was analyzed using a total carbon analyzer (TOC-V, Shimadzu, Kyoto, Japan). Based on standard methods, the determination of total ammonia nitrogen (TAN) in liquid samples were completed [24]. Free ammonia nitrogen (FAN) in liquid samples was calculated using the formula described in previous studies [25]. The polysaccharide content and protein content of EPS was determined in each part according to the anthrone method [26] and the modified Lowry method [27].

Capillary suction time (CST) and TS of the thickened digestate were the main parameters used to evaluate the dewaterability of anaerobic digestion digestate. TS of the thickened digestate was obtained by centrifugation of an anaerobic digestion solution at 2000× *g* for 10 min. Measurement of capillary suction time (CST) with a dedicated timer (304 M, Triton Electronics, Essex, UK) were completed. NCST was corrected from CST to eliminate the effect of different solid particles, that is, the ratio of CST to TS of the digestate. Gas chromatography (6890 N, Agilent, Palo Alto, CA, USA) was used to measure the methane concentration in each gas sample. Gas chromatography is equipped with a flame ionization detector and J&W 123–1730 column (320-µm internal diameter, 30-m length, and 0.50-µm film). Biogas volume was measured using a wet gas flowmeter (LML-2, Alpha, Changchun, China). The analysis was repeated three times for each sample.

In order to clarify the relationship between microbial communities and different mixing ratios of co-substrates, high-throughput sequencing technology was used to analyze microbial communities and extract DNA at the end of the co-digestion process. The Illumina MiSeq platform (TruSeqTM DNA Sample Prep kit, Illumina, CA, USA) and soil DNA kit (E.Z.N.A.® Soil DNA kit, Omega Bio-tek, Winooski, VT, USA) were used to extract microbial DNA from the solid phase of anaerobic digestion samples for amplicon sequencing. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified by Primers 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and 338F (5′-ACTCCTACGGGAGGCAGCAG-3′). Finally, the species information classification and the integration of the original data were completed by referring to relevant websites, and the OTU (operational taxonomic unit) table was completed. Data preprocessing methods and detailed experimental procedures are shown in reference [17].

2.4. Statistical Analysis

SPSS 22.0 software was used for correlation analysis. The smaller the p value, the more reasonable it is to assume that there was a difference between the things compared, and it is generally believed that $p < 0.05$ is considered to be statistically significant. Two-way ANOVA (significance level $\alpha = 0.05$) was adopted to analyze whether different temperatures and different mixing ratios had significant effects on pH, DOC, FA, TAN, and methane yield and other indicators (Please see the Supplementary Material) [28]. In addition, further methane yield analysis using the modified Gompertz model was required to fit the methane yield. Additionally, the modified Gompertz equation in the hydrogen production kinetic model used in this study is as follows (P: ultimate methane yield, mL; P_{max}: maximum hydrogen production potential, mL; R_{max}: maximum hydrogen production rate, mL H_2/h;

e: the base of the natural logarithm, taking the value 2.718; λ: lag phase of hydrogen production; t: hydrogen production time, h):

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

3. Results

3.1. Digestion Performance

The pH of each digester ranged from 6.5 to 8.5 throughout the anaerobic digestion process (Figure 1), which is a suitable pH range for anaerobic bacteria metabolic reproduction [29]. The pH of the thermophilic digester increased more quickly than that of the mesophilic digester. This is because a high concentration of TAN in the thermophilic digester will result in a rise in pH and alkalinity [30].

DOC levels declined substantially during the initial stage of anaerobic digestion under thermophilic and mesophilic conditions and then gradually tended to be stable (Figure 1). The accumulation of soluble organic compounds and insoluble organic compounds hydrolyzed to soluble organic compounds and small molecule organic acids in raw materials is the main source of DOC [12]. As a result, the high DOC level during the initial stage could be explained by rapid hydrolysis and acidification of the co-substrate [31].

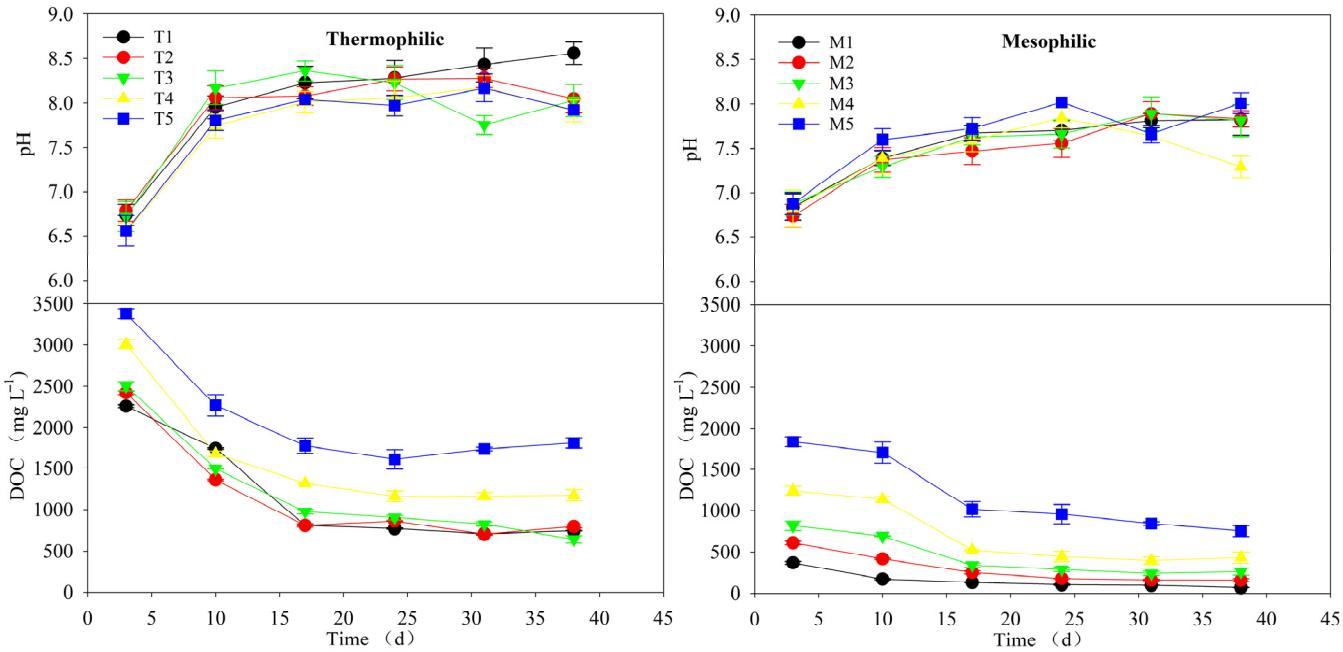

Figure 1. Changes in DOC and pH during co-digestion.

TAN increased with the evolution of the digestion process (Figure 2). The TAN level of thermophilic digesters was higher than that of mesophilic digesters. The variation trend of FAN concentration is basically consistent with that of TAN, and the FAN level was much higher in the thermophilic digesters than in the mesophilic digesters.

The results of the cumulative methane yield were fitted using a modified Gompertz model (Figure 3 and Table 3). The experimental results are in good agreement with the fitting results ($R^2 \geq 0.9258$). The ultimate methane yield (UMY) of T1–T5 was 77.4, 123.4, 134.3, 88.2, and 85.3 mL g^{-1}-VSS$_{added}$, respectively. The UMY of M1–M5 was 53.1, 112.9, 134.6, 99.9, and 99.9 mL g^{-1}-VSS$_{added}$, respectively. When the ratio of BM to ES was lower than 1:1.5, UMY increased with the increase in the proportion of BM. Until the ratio of BM to ES reached 1:1.5 (T3 and M3), the UMY of thermophilic and mesophilic reached its highest point (Figure 3). This finding suggests that co-digestion of ES and BM under T3 and M3 conditions had a beneficial synergistic impact, which provided adequate TS, C/N,

nutrient balance, and microbial community [32]. When the ratio of BM to ES exceeded 1:1.5, UMY decreased with the increase in BM. High proportion of BM had an adverse effect on mass transfer, microbial species richness, and microbial activity, which reduced microbial consumption of the substrate [33].

The results for the volumetric methane yield (Table 4) are consistent with those for the cumulative methane yield just at the beginning and in the middle. After statistical analysis of the data, the overall change trend of the volumetric methane yield was firstly increased, then decreased, and finally increased again.

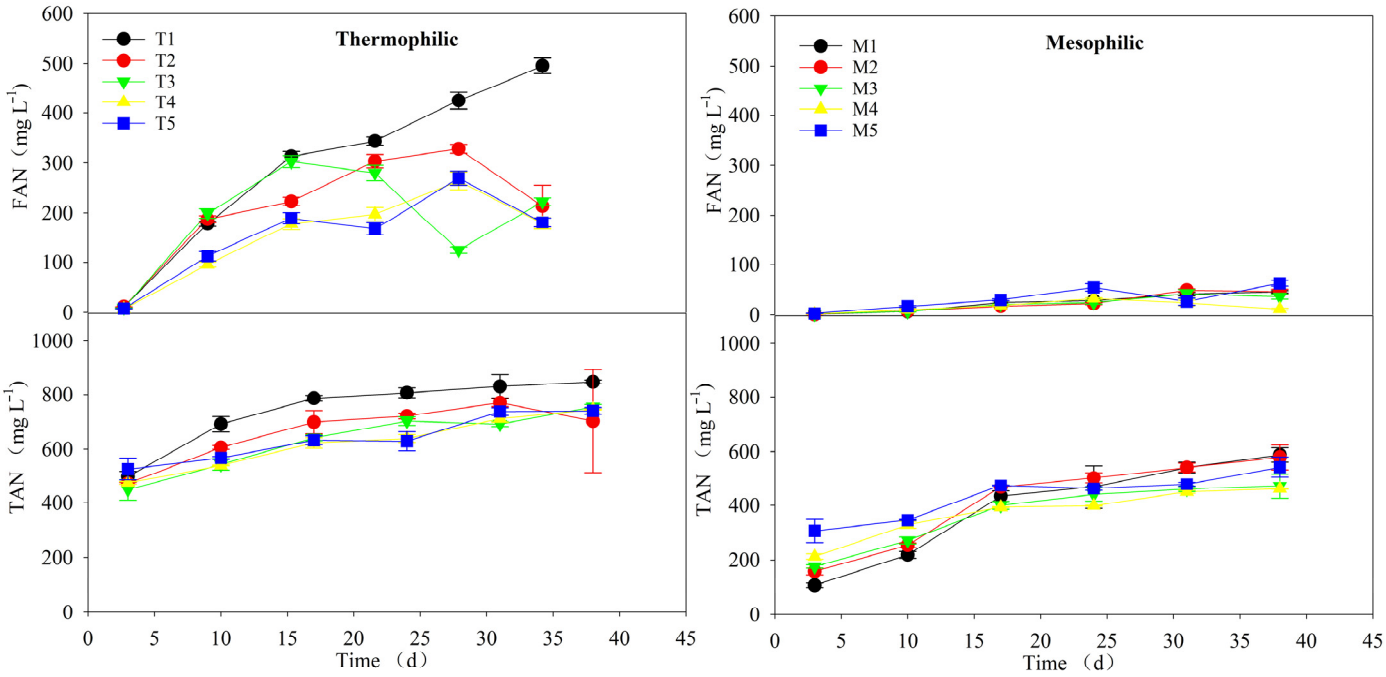

Figure 2. Changes in FAN and TAN during co-digestion.

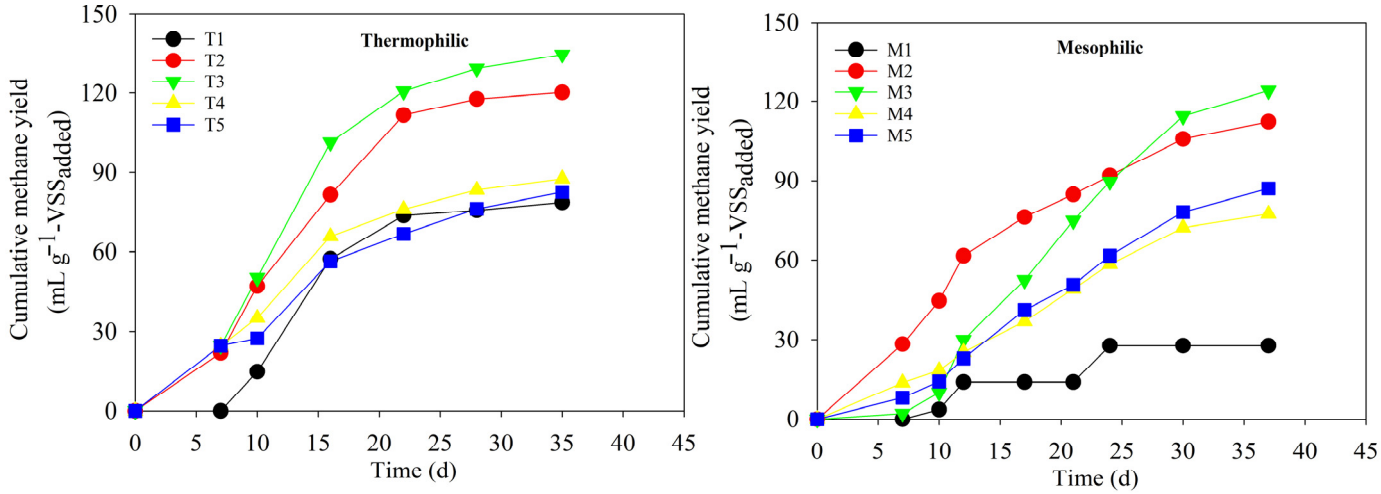

Figure 3. Cumulative methane yield of different experiments.

Table 3. Parameters of modified Gompertz model.

	Ultimate Methane Yield, UMY ($mL\ g^{-1}\ VSS_{added}$)	Maximum Methane Production Rate, Rm ($mL\ g^{-1}\ VSS_{added}\ d^{-1}$)	Lag Phase, λ (d)	R^2
T1	77.4113	8.2826	8.3674	0.9995
T2	123.4127	7.6679	4.1721	0.9975
T3	134.3303	9.5828	4.6437	0.9994
T4	88.1772	5.1276	2.6361	0.9967
T5	85.2538	3.9214	1.8739	0.9899
M1	53.1468	1.4273	11.4014	0.9258
M2	112.8605	5.1341	1.2674	0.9898
M3	134.6415	6.1726	8.4226	0.9955
M4	99.8927	2.5425	1.7529	0.9579
M5	99.8772	3.5892	6.0988	0.9980

Table 4. Results of volumetric methane yield.

Mixing Ratio	Volumetric Methane Yield ($L\ L^{-1}$)
T1	1.83
T2	3.71
T3	5.00
T4	4.29
T5	5.64
M1	1.25
M2	3.39
M3	5.01
M4	4.86
M5	6.61

3.2. Bacterial Communities

There were differences in the Chao1, Shannon, and Simpson indices of samples at different temperatures and BM/ES ratios (Table 5). The results showed that both the temperature and BM/ES ratio influenced the microbial diversity during anaerobic digestion. Alpha diversity of the bacteria index showed that the diversity of the microbial community was more abundant in ES than in BM, which is in accordance with the previous research [34].

Table 5. Alpha diversity of bacteria.

	OTUs	Chao1	Shannon	Simpson
ES	618	619.0	7.92	0.9908
BM	544	545.6	7.18	0.9749
M1	543	546.0	6.91	0.9652
M2	662	662.1	7.49	0.9777
M3	655	655.3	7.74	0.9871
M4	702	702.1	7.83	0.9875
M5	618	618.2	7.26	0.9771
T1	457	457.0	6.08	0.9180
T2	534	534.0	7.03	0.9716
T3	454	454.1	6.39	0.9401
T4	549	549.0	7.35	0.9812
T5	420	420.0	6.41	0.9534

Under thermophilic conditions, the bacterial community (Figure 4) was mainly composed of Firmicutes, Chloroflexi, Actinobacteria, Proteobacteria, and Thermotogae. Under mesophilic conditions, the bacterial community (Figure 4) was mainly composed of Firmicutes, Chloroflexi, Actinobacteria, Proteobacteria, Bacteroidetes, and Armatimonadetes.

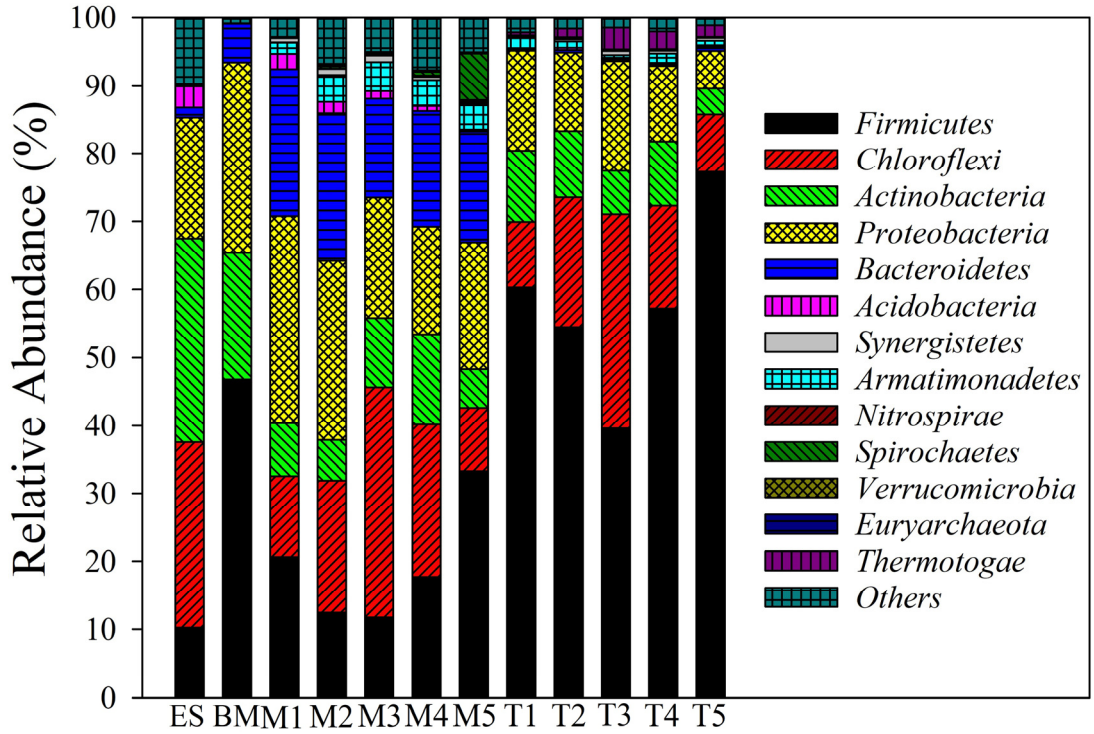

Figure 4. Relative abundance of the bacterial community at the phylum level.

Under thermophilic and mesophilic conditions (Figure 4), the relative abundance (RA) of Firmicutes first increased with the increasing proportion of BM in the substrate (T1–T3 and M1–M3), reached a minimum at T3 and M3, and then increased again with the increasing proportion of BM (T3–T5 and M3–M5).

Proteobacteria are bacteria responsible for hydrolysis and acidification, which produce acetic acid and metabolize fermentation [35,36]. Under thermophilic conditions, the RA of Proteobacteria at T2 decreased due to the addition of BM compared with T1, and the RA of Proteobacteria at T3 increased with the addition of BM compared with T2. The RA of Proteobacteria decreased with increasing proportion of BM (T3–T5) (Figure 4). The RA of Proteobacteria decreased under mesophilic conditions as the proportion of BM in the co-substrate increased, and the RA of Proteobacteria was lowest at M4. However, compared with M4, the RA of Proteobacteria at M5 increased with the increase in BM proportion.

The RA of Chloroflexi was the highest at T3 and M3. Under thermophilic and mesophilic conditions, when the BM/ES ratio is less than the BM/ES = 1:1.5, the RA of Chloroflexi increased with the increasing BM proportion. On the contrary, when the BM/ES ratio was greater than the BM/ES = 1:1.5, the RA of Chloroflexi decreased with the increasing BM proportion (Figure 4). High organic loading rates, accumulation of VFAs, or low pH can lead to a reduction in the RA of Chloroflexi [37].

The RA of Bacteroidetes decreased as the proportion of BM increased, which reached the lowest at M3. The RA of Bacteroidetes increased with the increase in the BM proportion when the BM/ES ratio is greater than the BM/ES = 1:1.5 (Figure 4).

Armatimonadetes, Acidobacteria, Thermotogae, and Spirochaetes also appeared in thermophilic or mesophilic digesters. The RA of Armatimonadetes in thermophilic digesters was lower than that in mesophilic digesters (Figure 4). Armatimonadetes could increase the cellulolytic rates [38]. The RA of Acidobacteria in mesophilic digesters decreased with the addition of BM, and the RA of all Acidobacteria in mesophilic digesters was lower than that of Acidobacteria in ES. This may be the effect of the pH in each digester on the RA of Acidobacteria [39]. Thermotogae was present in all of the thermophilic digesters, which consist of thermophilic bacteria typical of carbohydrate degradation and may play a necessary role in the thermophilic process [40]. The RA of Spirochaetes

was high at M5 and extremely low in other experimental groups. Spirochaetes may rely primarily on monosaccharides but also involve in the degradation of disaccharides or polysaccharides [41].

3.3. Dewaterability of Digestate

EPS is usually composed of microorganisms and their metabolites, which are mainly organic substances, such as proteins and polysaccharides [42]. During the whole process of anaerobic digestion, polysaccharides and proteins extracted from the slime layer were different at different times under either thermophilic or thermophilic conditions (Figures S1–S4 in Supplementary Material). This may be caused by the different solubility of organic matter at different times in the anaerobic digester [22]. Polysaccharides and proteins in loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) were less abundant than those in mucus at thermophilic and mesophilic temperatures. The protein concentration was higher than the polysaccharides concentration under all conditions. These findings are similar to those of previous studies [17,22].

NCST is an indispensable parameter for evaluating the dewatering performance of digestate after digestion. NCST is modified from capillary suction time (CST), and dewatering performance is inversely proportional to the NCST value [43]. The CST index measures the filterability of sludge and reflects the characteristics of the dewatering process [44]. At the end of thermophilic anaerobic digestion, NCST values (Figure 5) of T1–T5 were 8.98, 9.73, 12.24, 10.10, and 12.54 s·g^{-1}-TS, respectively. At the end of mesophilic anaerobic digestion, NCST values (Figure 5) of M1–M5 were 5.45, 6.09, 5.84, 7.97, and 12.32 s·g^{-1}-TS, respectively. The fluctuation of NCST under mesophilic conditions was lower than that under thermophilic conditions.

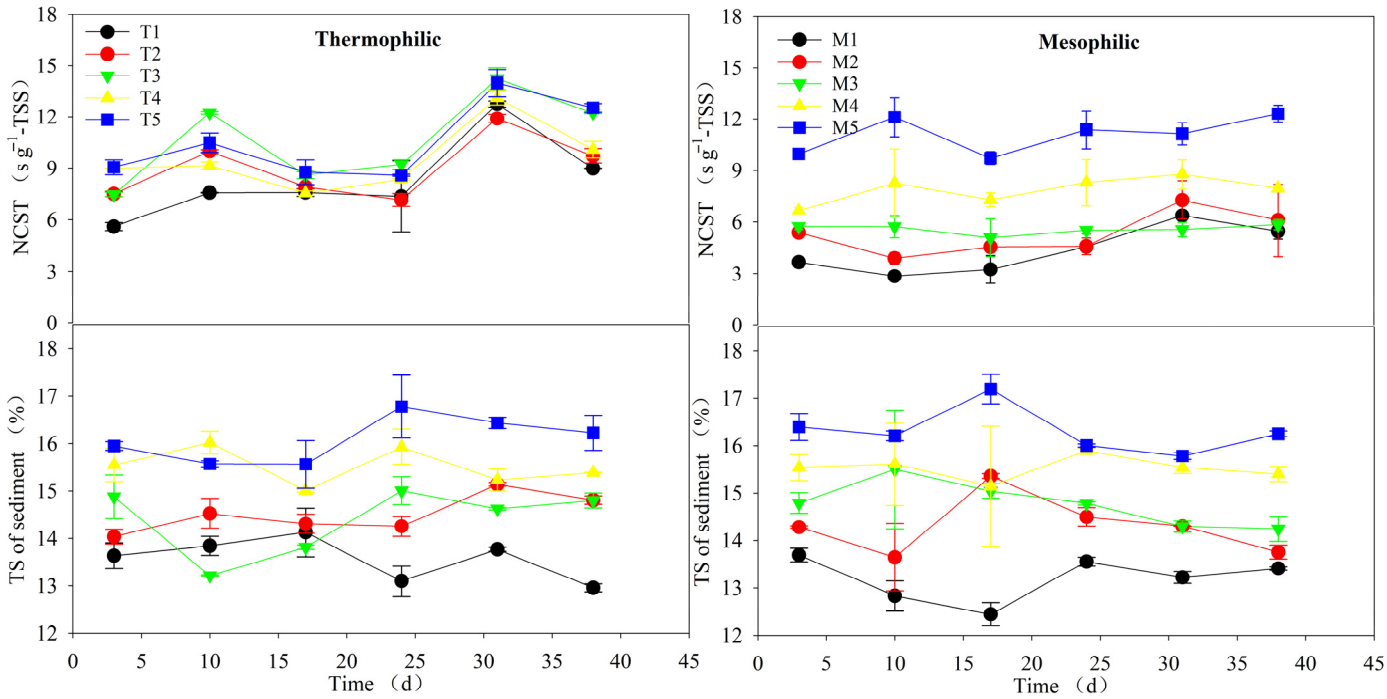

Figure 5. TS of sediment and NCST during the co-digestion process.

Centrifugal dewatering is also a method to evaluate the dewatering performance of the digestate [45]. Centrifugal dewatering, which is different from the CST determination technique, makes use of a powerful driving force (2000× *g*). The adhesion between water and digested particles is virtually completely destroyed by centrifugal dewatering. Under mesophilic or thermophilic conditions, the TS of the sediment of the digestate increased first and then decreased with the evolution of the digestion process (Figure 5). Correspondingly,

centrifugal dewatering efficiency of T1–T5 was 62.1%, 61.2%, 54.4%, 51.8%, and 45.3%, respectively. The centrifugal dewatering efficiency of M1–M5 was 61.0%, 59.7%, 57.9%, 53.8%, and 45.1%. Regardless of the thermophilic condition or the mesophilic condition, the TS of the sediment increased with the increase in the initial TS of the substrate, and the centrifugal dewatering efficiency of the digestate decreased.

4. Discussion

4.1. Digestion Performance

The decrease in the pH value of the digesters is related to the decomposition of organic matter, and during anaerobic digestion, the pH value gradually decreases and remains above 6.5, indicating the stability of the digestive system [46]. In this study, the pH value first increased and then decreased, but all digesters remained above 6.5, indicating that the stability of the anaerobic digestion system in this study was good. The decreasing trend of the pH value occurred earlier in the thermophilic digesters than in the mesophilic digesters, which may be due to the thermophilic temperature favoring the decomposition of organic matter [46]. The pH variation in this experiment was similar to previous studies, but the minimum pH value (7.1) was greater than that of this experiment (6.5) [46]. This may be caused by the different properties of the substrate and digestion temperature [17]. Additionally, high VS/TS in this study may have resulted in the formation of organic acids, thus reducing the pH value [47].

The DOC level of thermophilic digesters was higher than that of the mesophilic digesters, which could be explained by the fact that the increment of temperature increases the hydrolysis rate and solubility of organic matter [19]. By comparing different experiments at the same temperature, DOC levels were found to increase with the proportion of BM in the co-substrate, as the proportion of BM increased in the co-substrate, which was similar to the previous studies [17,22]. In the hydrolysis phase, the main components of COD were volatile fatty acids [21]. With the evolution of the digestion process, the DOC level gradually decreased, which is to enter the stage of acetic acid methanation, because DOC was converted into inorganic matter and biogas by methanogenic bacteria [48]. The temperature had a significant effect on both pH value and DOC, while mixing ratios had a significant effect only on DOC.

Thermophilic conditions increase the degradation of nitrogen-rich substances and solubility of organic matter in co-substrates, so TAN levels increased under thermophilic conditions [19,49,50]. However, under the mesophilic condition, the TAN concentration in all reactors increased gradually with the increase in TS. This suggested that it was difficult for manure to release ammonia under strong alkaline conditions [46]. When TAN concentration is greater than 1500 mg L^{-1}, ammonia could impede methanogen growth [48]. The concentration of TAN in all digesters was less than 1500 mg L^{-1}, so the inhibitory effect by TAN in anaerobic digesters could be ignored in this study. Notice that the TAN of the digestate is meaningful for the utilization and development of the digestate as a fertilizer [51].

FAN can permeate cell membranes and reduce methanogen activity, which significantly decreases the methane yield at a specific concentration [52]. The high FAN concentration was due to the accumulation of VFA, and the high FAN concentration will increase pH and total alkalinity, which will subsequently lead to changes in the hydrolytic bacterial community [46]. FAN proportion in TAN is determined by temperature and pH [53]. Therefore, the FAN level of mesophilic digesters was significantly lower than that of thermophilic digesters (Figure 2). Under thermophilic conditions, the concentration of FAN after the fifth day was more than 90 mg L^{-1}. FAN has a substantial inhibitory effect on methanogens at concentrations more than 90 mg L^{-1} [21,50]. FAN and TAN levels in this study were relatively low compared to the related mesophilic anaerobic digestion study [21], especially FAN concentrations, which may be due to the nitrogen content of the substrate. In addition, according to the data analysis, both temperature and mixing ratios had significant effects on TAN and FAN.

The maximum UMY was lower than the cumulative methane yield described in the previous study (around 187.21 mL g^{-1}-VSS_{added}) [16]. This may be related to the low pH (<7.5) of the substrate in this study, with higher pH (around 9.0) being able to consume more organic matter to increase methane yield [16]. Despite this, the UMY of the ES and BM co-digestion was higher than that of ES mono-digestion. This is attributed to the co-digestion having suitable water content, pH, C/N, nutrient balance, and volume loading rate, which can dilute toxic compounds and improve the buffer capacity [17]. The trend of the UMY for the M3 treatment group changed after day 25, which may be due to the suitable C/N of M3. The low yield in the early stage may be due to a less microbial community, and the better degradation effect of lignocellulosic substances in the later stage leads to the increase in UMY. There were no significant differences in UMY between thermophilic and mesophilic anaerobic digestion. Under mesophilic conditions, it may be the restriction of the hydrolysis steps that caused the lower methane yield [22]. Under thermophilic conditions, FAN (higher than 90 mg L^{-1}) had a substantial inhibitory effect on methanogens resulting in a limited biomethane generation [21,50].

In addition, both temperature and mixing ratios had significant effects on the cumulative methane yield. Additionally, inocula were not used during the anaerobic digestion process in this study, resulting in a long lag phase of methane production. Overall, the higher ultimate methane yield (UMY) under thermophilic conditions (T1–T5) than under mesophilic conditions (M1–M5) in this study may be due to the fact that easily digestible organic matter in the substrate was produced through hydrolysis processes in a thermophilic environment, thus enhancing the methanation process [54].

Compared with similar studies, the overall methane yield in this experiment is low, which may be related to the properties of the ES, and the TS of ES is low, which may be due to the high content of inhibitors in the ES, which inhibits the methanogenesis. The abrupt change in volumetric methane yield in the later stage may be due to the large proportion of BM, which had a dilution effect on the harmful substances in ES, so that the concentration of the inhibitor was reduced, and the volumetric methane yield of T5 (M5) was increased.

4.2. Bacterial Communities

Alpha diversity of bacteria showed that under thermophilic conditions, the microbial diversity of samples with different BM/ES mixing ratios was lower than that in sludge. This phenomenon indicates that adding BM under thermophilic conditions may lead to the reduction in substance abundance and diversity, thus affecting the stability of the anaerobic digestion process. Chao1 and Shannon indices under mesophilic conditions were larger than those under thermophilic conditions. This indicates that the diversity and abundance of bacteria under mesophilic conditions were greater than that under thermophilic conditions. This may be due to the inactivation of some bacterial communities that are not adapted to the thermophilic environment. A similar analysis was conducted, which found the microbial community of mesophilic anaerobic digestion shows greater diversity than that of thermophilic anaerobic digestion [17].

Firmicutes are thought to play an important role in the production of extracellular enzymes and the improvement of cellulose and protein hydrolysis [55]. Firmicutes have the potential to transfer electrons during anaerobic digestion, especially under alkaline conditions, showing a higher RA [56,57]. Therefore, the pH of thermophilic digesters was higher than that of mesophilic digesters, which results in a high RA of Firmicutes in thermophilic digesters.

The RA of Proteobacteria under thermophilic conditions was lower than that under mesophilic conditions (Figure 4), which can be interpreted as the effect of different reaction rates at different temperatures [58]. The RA of Proteobacteria with different BM/ES ratios may be related to the pH value in the reaction [31].

Chloroflexi is a common hydrolytic fermentation bacterium in the anaerobic digestion of organic solid waste, which can degrade complex organic compounds [59]. High organic loading rates, accumulation of VFAs or low pH can lead to a reduction in the RA of

Chloroflexi [37]. The RA of Chloroflexi and UMY were the highest in T3 and M3, which could be interpreted as the synergistic work of Chloroflexi and acetic acid methanogens to degrade organic matter [60].

Bacteroidetes play an important role in the hydrolysis and acidification of anaerobic digestion [61]. The substrate hydrolysis rate positively correlated with the RA of Bacteroidetes [62]. However, the RA of Bacteroidetes in thermophilic digesters was very low, which might be because Bacteroidetes are not heat-resistant bacteria [62].

4.3. Dewaterability of Digestate

Under mesophilic conditions, NCST increased obviously with the increase in TS. These phenomena are similar to those found in previous studies [17,22]. The NCST level of the thermophilic digestate was greater than that of the mesophilic digestate, which means that the mesophilic digestate has better dewaterability. The higher RA of *Firmicutes* and *Actinobacteria* resulted in higher hydrolysis of carbohydrates, proteins, and lipids during anaerobic digestion, which led to the dewaterability of digestate deterioration [63]. In addition, *Proteobacteria* with certain RA may improve the dewaterability of the digestate [63]. It may be due to the combined action of higher RA of Firmicutes and Actinobacteria under thermophilic conditions and higher RA of Proteobacteria under mesophilic conditions; the dewaterability of the mesophilic digestate was better than the thermophilic digestate [22]. The values of NCST in this study were higher than those in other studies of co-digestion [17], which may be related to the TS of the substrate. The TS of pig manure was higher than that of BM, resulting in a higher NCST of the digestate, which led to poor dewatering performance. In addition, through data analysis, it is known that both temperature and mixing ratio had significant effects on NCST.

The TS of sediment was used to characterize the centrifugal dewatering performance of the digestate. The variation trend of the centrifugal dewatering efficiency caused by different TS in this experiment is consistent with previous studies. [17,23]. The TS of sediment increased with the increase in TS [63]. There was a significant negative correlation between the average centrifugal dewatering efficiency and the average NCST (−0.975 **) during the whole mesophilic anaerobic digestion process. This phenomenon is similar to previous studies [17]. However, under thermophilic conditions, the NCST showed a poor correlation with centrifugal dewatering efficiency than that described by previous research [17]. It is possible that the different digestion substrates lead to a small range of NCST (8.98–12.54 $s \cdot g^{-1}$-TS) in the experiment under thermophilic conditions, so small deviations may reduce the correlation between NCST and centrifugal dewatering efficiency. Data analysis showed that only the mixture ratios had a significant effect on centrifugal dewatering efficiency.

Without the BM control group, it cannot be well seen that the harmful substances in ES can inhibit the anaerobic digestion process. Moreover, ES obtained in this study has a low content of organic matter, and a large number of inorganic substances in ES will cause a high concentration of inhibitors, thus affecting the stability of digestion. According to the chart of data analysis results, in terms of cumulative methane yield and volumetric methane yield, T3 (M3) has the largest yield; in terms of the dewatering performance of digestate, the NCST of T3 (M3) and the TS of sediment are above the medium level, which can reduce the subsequent cost of digestate disposal; in terms of the DOC change trend, T3 (M3) degrades organic matter more thoroughly, so a mesophilic anaerobic co-digestion reactor with an BM/ES = 1:1.5 has better prospects for an engineering application.

5. Conclusions

The difference in the ultimate methane yield between thermophilic and mesophilic conditions was not significant due to the special property of the co-substrate. However, the ultimate methane yield was significantly affected by mixing ratios, and the overall trend of ultimate methane yield was first increased and then decreased. Neither temperature nor mixing ratios had a significant effect on the digestion rate. As well, both temperature and

mixing ratios had significant effects on the dewatering performance of the digestate. The mesophilic conditions provided better digestate dewaterability and less energy demand. Therefore, a mesophilic anaerobic co-digestion reactor with an BM/ES = 1:1.5 has better prospects for an engineering application.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15086690/s1, Figure S1: protein of EPS under thermophilic conditions; Figure S2: polysaccharide of EPS under thermophilic conditions; Figure S3: protein of EPS under mesophilic conditions; Figure S4: polysaccharide of EPS under mesophilic conditions. Table S1: Results of Two-way ANOVA for DOC; Table S2: Results of Two-way ANOVA for pH; Table S3: Results of Two-way ANOVA for TAN; Table S4: Results of Two-way ANOVA for FAN; Table S5: Results of Two-way ANOVA for cumulative methane yield; Table S6: Results of Two-way ANOVA for cumulative methane yield; Table S7: Results of Two-way ANOVA for TS of sediment; Table S8: Results of Two-way ANOVA for ultimate methane yield; Table S9: Results of Two-way ANOVA for maximum methane production rate; Table S10: Results of Two-way ANOVA for lag phase.

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