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Effect of Substrate to Inoculum Ratio on Biogas Production and Microbial Community During Hemi-Solid-State Batch Anaerobic Co-digestion of Rape Straw and Dairy Manure

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Abstract

The substrate to inoculum (S/I) ratio is crucial for the rapid start-up of solid-state anaerobic digestion (SS-AD) systems. In this study, the performance of methane production and microbial community structure were evaluated during co-digestion of rape straw (RS) and dairy manure (DM) at different S/I ratios (2:3, 1:1, 2:1, 3:1, and 4:1) in batch hemi-solid-state anaerobic digestion (HSS-AD) tests. The highest methane yield of 209.1 mL/g VS_{added} and highest volumetric methane production of 0.4 L/(L·d) were achieved at S/I ratios of 2:3 and 2:1, respectively. Lower S/I ratios (1:2, 1:1, and 2:1) steadily produced biogas throughout the AD period, while higher S/I ratios (3:1 and 4:1) failed to produce biogas during the initial stage of AD because of excess accumulation of volatile fatty acids and low pH. The predominant bacteria and archaea in stable digesters were *Firmicutes* and acetoclastic *Methanosaeta*, respectively, while *Bacteroidetes* predominated and the relative abundance of hydrogenotrophic *Methanobacterium* increased significantly in acidic digesters. Amounts of bacteria and archaea were inhibited in acidic digesters. Our results provide useful information for enhancing efficient methane production and advancing the understanding of the microbiome in HSS-AD of RS and DM at different S/I ratios.

Keywords Substrate to inoculum ratio · Hemi-solid-state digestion · Rape straw · Methane production · Microbial community

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Introduction

Anaerobic digestion (AD) by a complex bacterial consortium in oxygen-free environments plays a key role in converting organic wastes to methane-rich biogas, a renewable energy that may help solve the problem of fossil energy shortage and reduce environmental pollution [1]. Based on the total solids (TS) content of the feedstock, AD has been developed as wet (< 10% TS), hemi-solid (10–15% TS), and solid (> 15% TS) state technologies [2]. Wet anaerobic digestion (W-AD) is typically applied to substrates with high moisture contents, including domestic and industrial wastewater [3]. However, this approach is not suitable for wastes with high solid contents such as agricultural crop straws, as it requires much greater water and digester volumes for decomposing low moisture feedstocks and generates large amounts of wastewater. Compared with conventional W-AD, solid-state anaerobic digestion (SS-AD) has several advantages, including higher volumetric methane productivity, lower wastewater generation, no floating substrates, and positive energy balances [4]. In recent years, SS-AD has received attention for managing agricultural wastes. This technology was used in > 60% of recently built anaerobic digesters in European countries [5]. Currently, most medium and large-scale biogas plants use W-AD technology with animal manure (< 10% TS) as a feedstock in China. Additionally, manure has limited availability, making it unusable as a feedstock supply for large-scale biogas production in many regions of China. Therefore, alternative feedstocks such as crop residues and the development of an efficient methane production process are needed.

Agricultural crop straws are abundantly available feedstocks for SS-AD. In China, an estimated 30 million tons of rape straw (RS) are generated every year, over 50% of which is dumped or burned as waste, resulting in serious environmental pollution [6]. Many studies of SS-AD have evaluated various crop straws such as corn stover, wheat straw, and rice straw as feedstock and confirmed its economic feasibility [7–9]; however, few studies have been focused on RS. The use of agricultural RS as an SS-AD feedstock for biogas production has advantages such as its low operating cost because of geographically concentrated cultivation; low sulfur content, which is beneficial for producing electricity and purifying methane to produce compressed bio-natural gas; and high cellulose content with excellent biochemical methane potential [10, 11]. However, RS as an SS-AD feedstock has limitations, including its low methane production yield per unit mass and unstable processing during digestion because of an imbalanced nutrient content for microbial growth due to its high carbon to nitrogen (C/N) ratio (> 40) at high TS (> 20%) [12, 13]. Many studies have confirmed that co-digestion of crop straws and animal manures can balance the C/N ratio, improve buffer capacity, and achieve synergistic effects beneficial to the SS-AD process [2, 14]. Considering the complementary nature of the C/N ratio between RS and animal manures, mixing both substrates at proper C/N ratios may be useful for methane production.

The inoculation dose is considered as the most important factor for improving high methane yield and digester stability in batch SS-AD using lignocellulosic feedstocks. The inoculation dose affects not only the microbial population, but also the physical and chemical properties of fermentation during SS-AD [15, 16]. Larger inoculation doses in SS-AD shorten the start-up time and increase the specific methane production rate (SMPR) based on weight by providing greater buffering capacity and more methanogens [7, 17]. However, excessive inoculum requires more space and decreases the volumetric methane production rate (VMPR). In contrast, very low inoculum doses can induce SS-AD failure [2, 17]. To achieve a proper SMPR and VMPR balance, the substrate to inoculum (S/I) ratio is often optimized for SS-AD

lignocellulosic biomass. The optimal S/I ratio for SS-AD reactors with different crop straws including corn stover, rice straw, and wheat straw at 15–25% TS was reported to be in the range of 1–30 [3, 8, 18]. The wide range of optimal S/I ratios, except for the S/I ratio in batch SS-AD, results from variations in reactor performance, in combination with other factors such as lignocellulosic characteristics, substrate TS content, and inoculum source. According to Motte et al. [19], the S/I ratio effects only the start-up phase, TS content is the main parameter governing methane production during the growing phase of SS-AD, and the methane production rate of wheat straw at 15% TS is higher than that of feedstock at 20% and 25% TS. The highest MVPR from co-digestion of corn stover and dairy manure (DM) is achieved at 15% TS in a continuous reactor [20]. Similar results were observed by Suksong et al. [21] and Xu et al. [22]. Furthermore, in large-scale digesters, the continuous and intensive mixing of 10–15% TS of feedstock is easier to maintain compared to that of 20–25% TS [23]. These results indicated that hemi-solid-state AD (HSS-AD) might have thresholds for efficient methane production using lignocellulosic feedstocks. No previous studies have focused on evaluating methane production, digestion stability, and the microbial community structure during co-digestion of RS and DM at different S/I ratios in an HSS-AD system.

The objectives of the current study were to (1) determine the proper S/I ratios required to achieve high SMPR and VMPR from RS and DM co-digestion in batch HSS-AD (15% TS), (2) at different S/I ratios, evaluate the digestion stability of digesters with dynamic changes in biogas production, pH, and volatile fatty acids (VFAs) concentration during AD, and (3) monitor the bacterial and archaeal communities in representative digesters using Illumina sequencing and quantitative real-time polymerase chain reaction (qPCR) to identify correlations between microbes and digester performance.

Materials and Methods

Feedstock and Inoculum

RS was collected from a crop straw processing station (Jingyan Country, Leshan City, Sichuan, China), oven-dried at 60 °C for 24 h in a circulation oven to a dry mass content of > 90%, smashed with a hammer mill to a particle size of 1-mm, and stored in ziplock bags. DM was obtained from a dairy farm (Suqi Town, Leshan City, Sichuan, China) and stored at − 4 °C. DM was thawed at 4 °C before use. The inoculum was collected from a 55-L well-run anaerobic digester in our laboratory operated under mesophilic conditions and fed with RS and DM. Prior to use, the inoculum was acclimated and degassed at 37 °C for 30 days to minimize background biogas production and then concentrated using a refrigerated centrifuge to a TS content > 15%. The characteristics of the RS, DM, and inoculum are shown in Table 1.

Batch Anaerobic Digestion Tests

Batch tests were performed in triplicate using 1-L homemade glass bottles with sample outlets at the bottom. The working volume of each reactor was approximately 0.7 L. The S/I ratios were 2:3, 1:1, 2:1, 3:1, and 4:1 based on the volatile solid (VS) content. Three reactors containing equal amounts of inoculum and water but without added substrate were used as controls. The initial TS content was 15% and the RS to DM mixture was adjusted to a C/N ratio of 34:1, in accordance with our previously optimized conditions [24]. All reactors were purged with N_2 for 5 min to

Table 1 Characteristics of rape straw (RS), dairy manure (DM), and inoculum

Parameter	RS	DM	Inoculum
Total solids (%)[a]	96.2 ± 0.0	12.8 ± 0.1	14.2 ± 0.1
Volatile solids (%)[a]	91.6 ± 0.1	11.4 ± 0.2	7.4 ± 0.2
Total carbon (%)[b]	52.3 ± 0.3	47.2 ± 0.4	9.8 ± 0.2
Total nitrogen (%)[b]	0.7 ± 0.3	2.1 ± 0.2	0.8 ± 0.1
C/N	70.7 ± 2.5	23.8 ± 2.1	12.2 ± 1.4
Soluble matters (%) [b]	15.1 ± 1.1	40.3 ± 2.3	ND
Cellulose (%) [b]	45.7 ± 1.9	22.8 ± 3.1	ND
Hemicellulose (%) [b]	25.9 ± 1.2	24.1 ± 2.3	ND
Lignin (%) [b]	12.7 ± 0.5	6.7 ± 1.0	ND
pH	ND	8.2 ± 0.1	7.8 ± 0.1
VFAs content (HAc g /kg) [a]	ND	2.8 ± 0.3	ND
Total alkalinity ($CaCO_3$ g /kg) [a]	7.2 ± 1.8	15.3 ± 1.5	16.2 ± 1.6

The letter a and b represents the value based on total weight of sample and total solid of sample, respectively; ND means not determined

remove oxygen, sealed using silicone stoppers, and incubated in a biochemical incubator for 30–60 days at 37 ± 1 °C. The biogas produced in each reactor was collected in 2-L gas aluminum foil bags. The composition and volumes of the biogas were measured every 1–3 days and all reactors were manually shaken twice per day for approximately 1 min. Approximately 6–7 g aliquots of digestion substrate were collected from the reactors and frozen at − 20 °C. The sampling times depended on the daily biogas production of each digester. For analysis, all samples were thawed at 25 °C and then centrifuged at 10,000 rpm for 10 min. The pH and VFA concentration of the supernatant were measured, the pellet was extracted to obtain the total genomic DNA, and the VS content was monitored to determine the biomass for DNA extraction.

Analytical Methods

Biogas volume was measured at ambient temperature using the water displacement method and corrected according to standard temperature (0 °C) and pressure (101.325 kPa). Methane and CO_2 content were determined using a biogas analyzer (Model Biogas 5000, Geotech, Coventry, UK). SMPR and VMPR were expressed according to formulas (1) and (2), respectively:

$$\mathrm{SMPR}\left(\mathrm{mL}\cdot\mathrm{g\ VS_{added}}^{-1}\right) = V_1/W \tag{1}$$

$$\mathrm{VMPR}\left(\mathrm{mL}\cdot\mathrm{mL}^{-1}\cdot\mathrm{d}^{-1}\right) = V_1/(V_2\times T_{80}) \tag{2}$$

where V_1 was the cumulative methane volume during the entire digestion period (mL), W was the weight (g) of VS substrate added to the digester (VS_{added}), V_2 was the reactor volume (mL), and T_{80} was the shortest technical digestion time (d) calculated according to the time for the cumulative methane volume to achieve 80% of V_1.

The TS, VS, total nitrogen, total carbon, and alkalinity of the samples were measured according to the standard methods of the American Public Health Association [25]. The pH was measured using a portable pH meter (Model SX-610, Sanxin, Shanghai, China). The lignocellulosic compositions (soluble substance, cellulose, hemicellulose, and lignin) of the feedstocks and inoculum were determined using a fiber analyzer (Model 2000, ANKOM,

Macedon, NY, USA) as previously reported [2]. The VFA sample concentrations, including formic acid (HFo), acetic acid (HAc), propionic acid (HPr), lactic acid (HLa), and butyric acid (HBu), were estimated by high-performance liquid chromatography (Model LC-20A, Shimadzu, Kyoto, Japan) according to Zhao et al. [26].

Microbiological Analysis

Total DNA was extracted from the inoculum and samples collected from batch tests using different S/I ratios. A DNeasy PowerSoil Kit (Model 12888-50, Qiagen, Hilden, Germany) was used to extract the DNA and qualitatively evaluated by 1% agarose gel electrophoresis. The DNA concentrations were determined using a NanoDrop spectrophotometer (Model 1000, Thermo Fisher Scientific, Waltham, MA, USA).

Sequence Analysis The community structures of bacteria and archaea of the samples were analyzed by sequencing the V3-V4 hypervariable region of 16S rRNA gene. The V3-V4 region was amplified using the universal primer pairs 343F (5′–TACGGRAGGCAGCAG–3′) and 798R (5′–AGGGTATCTAATCCT–3′), and 344F (5′–TGYCAGCCGCCGCGGTAA–3′) and 915R (5′–YCCGGCGTTGAVTCCAATT–3′), which specifically target bacteria and archaea, respectively. The primers included Illumina barcode sequences for each sample for multiplexing. The amplicon libraries were sequenced with the Illumina Miseq system using the pair-ended 2 × 250 and 2 × 300 base pair protocol for bacteria and archaea, respectively [26, 27]. Raw data were trimmed using Trimmomatic (v.0.35) software and the fragments were assembled using FLASH (v.1.2.11). Sequences shorter than 200 base pairs were excluded from analysis. After demultiplexing, valid reads were assigned species-equivalent operational taxonomic units (OTUs) at 97% sequence similarity using VSEARCH (v.2.4.2). Taxonomic classification of the remaining OTUs and calculation of the alpha diversity metric were performed using Mothur (v.1.30.1) software and phylum and genus level designations were determined according to the Silva database (v.123) (http://www.arb-silva.de).

Quantitative PCR Analysis The qPCR was performed using an ABI 7500 system (Life Technologies, Carlsbad, CA, USA). Total bacterial content of the samples was determined by qPCR using primers 63F (5′–GCAGGCCTAACACATGCAAGTC–3′) and 335R (5′–CTGCTGCCTCCCGTAGGAGT–3′) as previously described [28]. Reagents for the qPCR system were purchased from Takara Biotechnology (Shiga, Japan). The amplification in 20-μL volumes was performed and standard curves were produced as presented by Zheng et al. [29] and Hua et al. [30], respectively.

The group-specific qPCR primers and 5′-nuclease probes (TaqMan) used to detect methanogenic archaea microorganisms were previously described Yu et al. [31] and DNA was amplified from two orders, *Methanobacteriale* (MBT) and *Methanomicrobiales* (MMB), and two families, *Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst). The TaqMan probes were labeled with fluorescent dyes FAM (reporter) and BHQ-1 (quencher). The reagents for qPCR mixtures were purchased from Takara, and the two-phase amplification protocol and standard curves were conducted in 20-μL volumes as previously described [32]. The DNA volume-based concentration (copies/μL) was converted to the digestion substrate biomass-based concentration (copies/g VS) according to the VS concentration of each sample used for DNA extraction. The DNA concentrations in all reactor samples were quantified in triplicate.

Statistical Analysis

Experimental data were standardized using Excel 2010 software (Microsoft, Redmond, WA, USA). Statistical data were plotted using Sigmaplot version 10.0 (Systat, San Jose, CA, USA). Differences between tested parameters were assessed for significance at α levels of 1 and 5% using SPSS version 17.0 statistical software (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

Substrate and Inoculum

Characteristics of the substrates and inoculum are shown in Table 1. Compared to DM, RS had higher carbon content and lower nitrogen content. In consideration of the nutrient to anaerobic microbe balance, solid-state co-digestion of RS and DM demonstrated the potential to obtain a proper C/N ratio (20.0-35.0) and improve methane yield. RS contained higher amounts of structural carbohydrates including cellulose, hemicellulose, and lignin compared to that in DM. Similar results were found by Tian et al. [13]. However, the content of soluble matter such as free sugars, oligomers, and organic acids was higher in DM than in RS. In general, non-structural carbohydrates are easily degraded and may contribute to improved biogas production rates [29, 33]. Notably, total alkalinity may increase the buffering capacity in digesters and maintain a stable pH, preventing the accumulation of excess VFAs and possible AD failure [3]. The alkalinity of the inoculum was significantly ($p < 0.01$) higher than that of RS. This suggests that adding optimal amount inoculum size can improve methane yield by increasing the organic loading capacity of HSS co-digestion.

Digester Performance

Daily Methane Production and Content Daily methane production and methane content from digesters using RS and DM at different S/I ratios are shown in Fig. 1. Different trends were observed between digesters with lower and higher S/I ratios (Fig. 1A). The digesters with lower S/I ratios of 2:3, 1:1, and 2:1 steadily produced methane throughout the AD digestion period, while digesters with higher S/I ratios of 3:1 and 4:1 ceased methane production at approximately day 7 and then resumed this activity on days 15 and 20, respectively. This may be because digesters with strong buffering capacity can maintain a balance between acidification and methanation at low S/I ratios. Yang et al. [34] reported that the failed SS-AD digesters were efficiently recovered by addition of a suitable inoculum. Our results showed that the peak time and duration of methane production were delayed, and the highest daily methane production was decreased relative to increasing S/I ratios from 2:3 to 4:1. These results suggested that the S/I ratio played an important role during the start-up phase and in determining the rate of methane production during AD when using solid-state feedstocks. The highest daily methane production of the digesters was 19.7 mL/g VS_{added} at an S/I ratio of 2:3, compared to only 5.7 mL/g VS_{added} at an S/I ratio of 4:1. Notably, two successive peaks of methane production were observed during AD in digesters with S/I ratios 1:1 and 2:1. This may be because at certain S/I ratios, the easily digestible components of feedstocks were converted into methane sooner than the structural carbohydrates, such as cellulose and hemicellulose, resulting in the two peaks [19, 29]. As shown in Fig. 1 A and B, variations in the trends of methane content from digesters with different S/I ratios were similar to

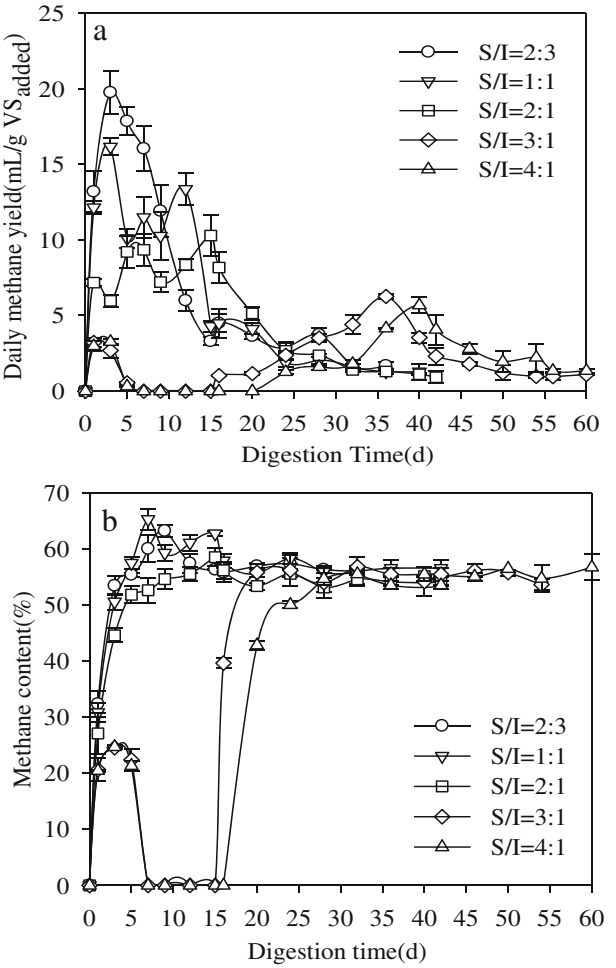

Fig. 1 (A) Daily methane production and (B) methane content of biogas from digesters with rape straw and dairy manure as substrate at different substrate/inoculum (S/I) ratios over time in days (d)

the daily methane production. At S/I ratios of 2:3, 1:1, and 2:1, the methane content of the biogas sharply increased after the first 2 days during the initial phase of AD, and was then maintained in a stable range, suggesting that the digesters achieved quick start-up. In contrast, at S/I ratios of 3:1 and 4:1, the methane contents dramatically decreased after the first 3 days of beginning AD, stopped producing methane for an interval of approximately 10 days, and then significantly increased and maintained elevated levels of methane production during a stable phase until approximately days 20 and 26, respectively. The average methane contents from digesters at S/I ratios of 2:3, 1:1, and 2:1 were 57.4%, 58.2%, and 54.8%, respectively, showing a stable range of methane content that did not significantly differ ($p > 0.05$) from the digesters at S/I ratios of 3:1 and 4:1 with methane contents of 54.9% and 54.4%, respectively. This indicated that the low methane content and the prolonged lag phase during the initial stage of AD at a high S/I ratio were caused by an imbalance between acidification and methanogenesis. To avoid this problem, it is necessary to increase the inoculum dose during the adaptation phase to improve the buffering capacity and shorten the start-up time in batch SS-AD [19, 35].

Methane Production Rate The SMPR and VMPR of digesters at different S/I ratios are shown in Fig. 2. The highest SMPR observed was 209.1 mL/g VS_{added} obtained at an S/I ratio of 2:3, which was significantly higher ($p < 0.05$) than those of 195.8, 177.5, 121.7, and 103.6 mL/g VS_{added} at S/I ratios of 1:1, 2:1, 3:1, and 4:1, respectively (Fig. 2A). Previous results showed that methane yields for RS with different particle sizes ranged from 188 to 243 mL/g VS_{added} in biochemical methane potential tests at an S/I ratio of 1 [13], which agree with the results obtained in the present study. Notably, VMPR decreased with an increase in S/I ratios from 2:3 to 4:1. Higher methane yields were obtained in batch SS-AD at lower S/I ratios because of the increase in methanogens provided by the increasing inoculum, which contributed to the efficient conversion of VFAs to methane [16, 36]. As shown in Fig. 2 B, comparison of VMPRs among digesters with different S/I ratios was quite different than those of SMPRs. The highest VMPR at 0.42 mL/

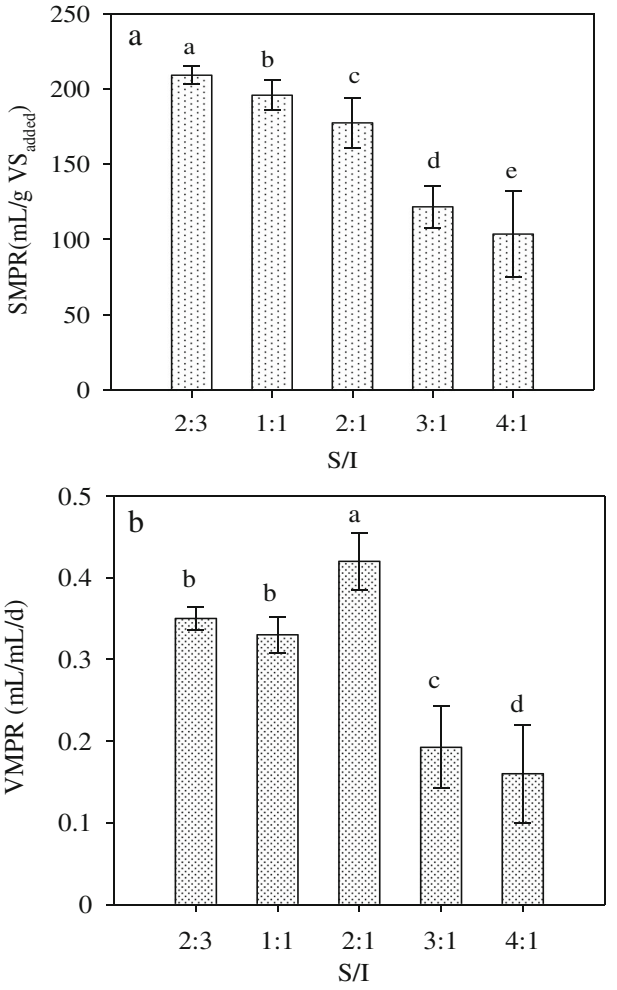

Fig. 2 (A) Comparison of specific methane production rate (SMPR) and (B) volumetric methane production rate (VMPR) from digesters with rape straw and dairy manure as substrate at different substrate/inoculum (S/I) ratios. Different lower-case letters indicate significant differences among the different digesters according to LSD pairwise comparison method at $\alpha = 5\%$

(mL·d) was observed in the digester at an S/I ratio of 2:1, which was 1.3-, 1.2-, 2.2-, and 2.6-fold ($p < 0.05$) higher than those for digesters with S/I ratios of 2:3, 1:1, 3:1, and 4:1, respectively. Similar to the results for SMPRs, the VMPRs were also much lower for digesters at S/I ratios of 3:1 and 4:1. The S/I ratio of the highest SMPR did not always correspond to the highest VMPR, possibly because the large inoculum may involve a larger volume, which decreased organic loading of the digester. Therefore, an effective method for improving methane production of SS-AD from lignocellulosic substrates may be to optimize the S/I ratio.

An extensive range of S/I ratios as previously studied in SS-AD of lignocellulosic feedstocks, but the results were variable. The highest methane yield of corn stover was obtained with an S/I ratio of 2.4 [18], while the highest production of yard trimmings was 1 (based on VS) in mesophilic SS-AD [37]. Under buffered conditions, higher S/I ratios of 28–47 were shown to be effective for SS-AD of wheat straw [19]. By varying the TS and particle size of the feedstock, the AD duration (approximately 300 days) was 5- to10-fold longer than that of digesters with low S/I ratios [19]. The differences may be related to both the heterogeneity of the feedstocks and activity of the inoculum.

Evaluation of the Stability of the AD Process

Many studies using lignocellulosic feedstocks in batch SS-AD have been conducted to improve methane production and assessing the stability of the AD process, but only for the initial and final phases [2, 5, 37]. Few studies have focused on the stability of SS-AD during the intermediate stage.

Digestion process failure and low methane yield may be caused by imbalances in the methanogenic archaea and hydrolytic, fermentative, and acetogenic bacteria [26]. Typically, these imbalances result from unfavorable conditions such as insufficient buffering capacity of the AD system and accumulation of VFAs [3], causing a dramatic fluctuation in pH. This inhibits the activity of microbes with different metabolic functions, particularly methanogenic archea, which are more sensitive to pH, and ultimately disrupts the stability of the AD process and reduces methane production [29]. Therefore, pH and VFAs are important factors useful for estimating the performance of AD.

Changes in pH, VFAs, and daily biogas production during AD are shown in Fig. 3. An initial peak of daily biogas production occurred in all digesters on the first day and daily biogas production decreased with increasing S/I ratios. This suggests that the microorganisms in the inoculum, through acclimation of adaptability, were highly active and that the inoculating dose is a limiting factor during the initial production of biogas from transformation of the soluble matter. As shown in Fig. 3 A, B, and C, digesters at S/I ratios of 1:2, 1:1, and 2:1 steadily produced biogas, the pH of the three digesters remained above 6.9 throughout the entire AD period, and the total VFA content was < 20.0 g/kg. In comparison, digesters at S/I ratios of 3:1 and 4:1 failed to produce biogas when the pH decreased to less than 6.2 and the VFA content was > 20.0 g/kg (Fig. 3D, E). The subsequent peak in daily biogas production was not observed until the pH recovered to > 6.4 and the VFA content was reduced to < 20.0 g/kg. The optimal pH range of 6.5–8.2 was reported by Lee et al. [38] for efficient AD, while Zheng et al. [29] found that stable production of biogas was maintained at total VFA concentrations of < 18 g/L, which agrees with the results of our study. Additionally, as the substrate concentration increased in digesters at S/I ratios from 1:1 to 4:1, the second peak period of biogas

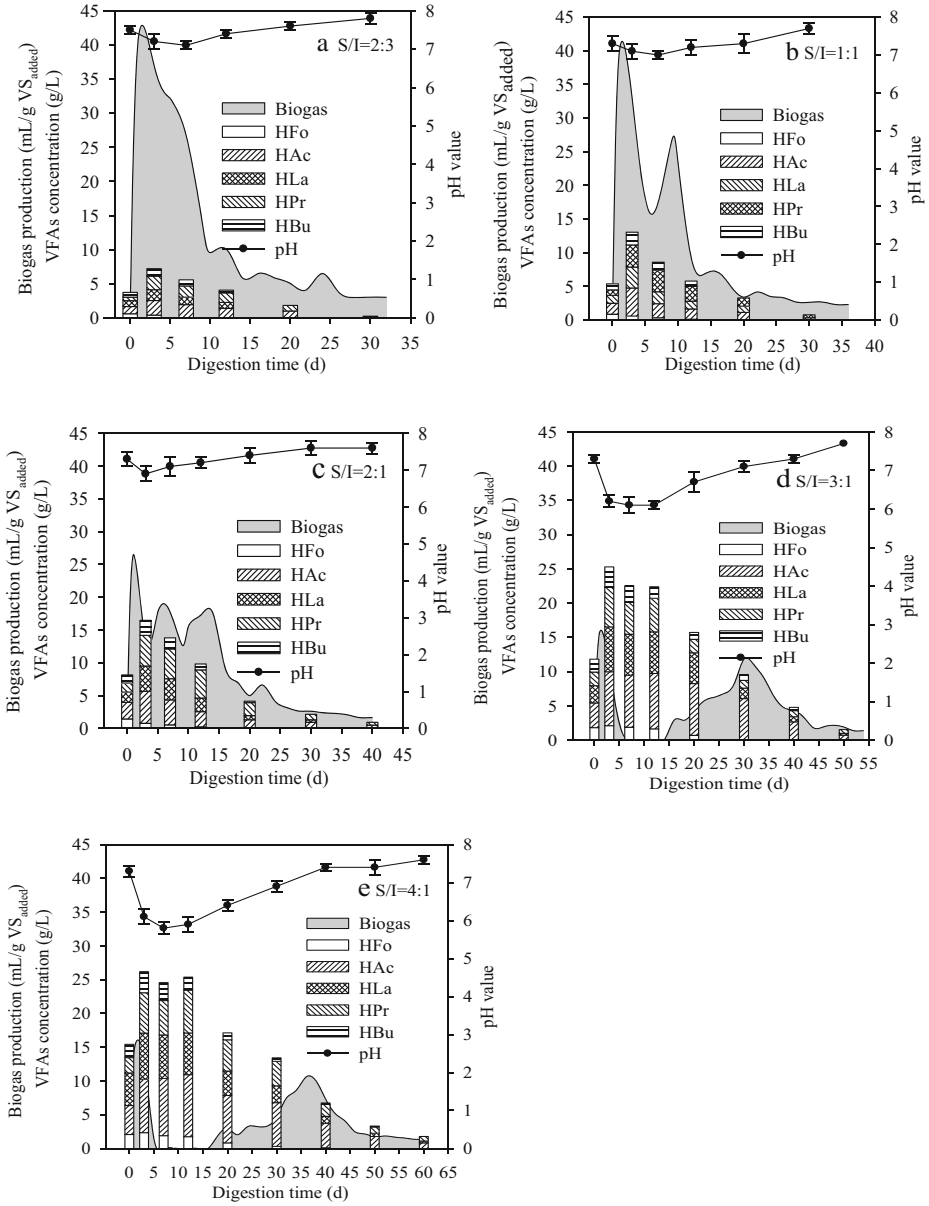

Fig. 3 Changes in pH, levels volatile fatty acids (VFAs), and daily biogas production of digesters with rape straw and dairy manure as substrates at different substrate/inoculum (S/I) ratios over time in days (d). HFo:formic acid; HAc: acetic acid; HPr: propionic acid; HLa: acid; HBu: butyric acid

production was prolonged from day 9 to day 36. A similar phenomenon was reported by Zheng et al. [29]. At the higher S/I, this delay is likely related to the low pH caused by the extensive degradation of easily degradable compounds into VFAs during the phases of hydrolysis and fermentation. Furthermore, excess accumulation of VFAs may initially inhibit methanogenesis.

Interestingly, the total VFA content was closely associated with daily biogas production. In digesters with S/I ratios of 2:3, 1:1, and 2:1, the secondary peak of daily biogas production appeared as the VFA content decreased. The highest amounts of VFAs were obtained on day 3 with individual VFA concentrations in the three digesters of 2.1–4.8 g/kg for HAc, 2.0–4.6 g/kg for Hpr, 1.6–3.9 g/kg for HLa, 1.1–2.3 g/kg for HBu, and 0.4–0.8 g/kg HFo. HFo was preferentially depleted with increasing fermentation time with the relative reduction rates of the other four VFAs being HAc > HLa > HBu > HPr from days 3–20. These results suggest that the anaerobic microorganisms preferred to metabolize formic acid and lactic acid rather than butyrate and propionate acid. Although HLa, HBu and HPr could be converted to HAc through acetogenesis, it was either favorable or unfavorable in degradation reactions depending on the standard Gibbs free energy (ΔG). Under standard thermodynamic conditions, $\Delta G0 = -4.2$, $+48.1$, and $+76.1$ kJ/mol for HLa, HBu, and HPr, respectively [39, 40]. Generally, in an efficient AD system with proper S/I ratios, these acetogenesis reactions are coupled with methanogenesis. This may be because acetogenesis and methanogenesis were intense in digesters with high biogas production at S/I ratios of 2:3, 1:1, and 2:1 during the first 20-day period. However, this phenomenon was not observed in digesters with low biogas production at S/I ratios of 3:1 or 4:1 because their accumulated VFA concentrations on days 3–12 were 22.4–25.3 and 24.6–26.2 g/kg, respectively. This was particularly the case for HPr, which is more inhibitory to methanogens than HAc [41], with concentrations reaching 4.6–5.8 g/kg (S/I ratio of 3:1) and 5.1–6.4 g/kg (S/I ratio of 4:1). Chen et al. [42] found that an HPr concentration of 1 g/L inhibits methane production, which is lower than the levels observed in the present study. These differences may be related to the buffer capacity of the AD under different operational parameters such as the feedstock and inoculum.

Analysis of the Microbial Community

To date, few studies have examined the dynamics of the microbial community in SS-AD compared to the numerous studies in W-AD. Moreover, inconsistent conclusions were reported regarding the SS-AD microbial communities. Microbes in SS-AD are highly sensitive to multiple digestion factors, including substrate type and size, inoculum source, and operating conditions, and thereby the structure of the microbial community may be affected by these factors. To elucidate the plausible correlations between the bacteria and archaea communities with the performance of digesters at different S/I ratios, high-throughput analyses and qPCR were performed. The samples for microbiological analysis were collected from digesters with S/I ratios of 2:3, 2:1, and 4:1 on day 12 for two reasons. First, the highest SMPR and VMPR were achieved at S/I ratios of 2:3 and 2:1, respectively, and opposite values were found for an S/I ratio of 4:1. Second, the digestion status and daily biogas production differed in digesters at these three S/I ratios on day 12.

Bacterial Community A total of 238,470 sequences (an average of 29,809 sequences per sample) were obtained for the four samples analyzed after the quality check, with 110,487 sequences identified as bacteria. The alpha diversity of the microbial communities (bacteria and archaea) in the inoculum and samples on day 12 from digesters with the three representatives S/I ratios of 2:3, 2:1, and 4:1 is presented in Table 2. The index of coverage was > 99% for all samples, confirming the representativeness of the OTU set. A total of 540–700 OTUs of bacteria were identified. The greatest number of OTUs was obtained from the inoculum samples followed by samples from digesters at S/I ratios of 2:3, 2:1, and 4:1. Similar trends were found for Simpson and Shannon diversity indices. The results indicated that the seed

Table 2 Alpha diversity of bacterial and archaeal communities on day 12 in digesters with different substrate/inoculum (S/I) ratios

Microbes	Digesters	Reads	Observed OTUs	Coverage (%)	Simpson	Shannon
Bacteria	Inoculum	25,461	700	99.36	0.97	6.68
	S/I = 2:3	27,853	668	99.14	0.96	6.19
	S/I = 2:1	30,381	570	99.28	0.95	6.10
	S/I = 4:1	26,792	540	99.30	0.93	5.85
Archaea	Inoculum	32,117	44	99.98	0.69	2.61
	S/I = 2:3	31,016	47	99.99	0.71	2.66
	S/I = 2:1	32,759	48	99.98	0.71	2.57
	S/I = 4:1	32,091	51	99.98	0.73	2.60

sludge used in this study was high quality and activated through acclimation, and that a strong acidic/low pH environment may be detrimental to the growth of hydrolytic and acidifying bacteria during the HSS-AD process.

Based on the classification results of the total OTUs at a 97% sequence similarity in the Silva database, the relative abundance of 13 major bacterial phyla identified from the inoculum and different S/I ratios is shown in Fig. 4 A. Approximately 98% of the total sequences from the inoculum were classified into known bacterial phyla, with *Firmicutes* accounting for 38.3% of the sequences and representing the most prevalent phylum, followed by *Bacteroidetes* (27.5%), *Proteobacteria* (11.6%), *Spirochaetae* (9.1%), *Tenericutes* (2.8%), *Synergistetes* (1.8%), *Chloroflexi* (1.4%), and *Planctomycetes* (1.3%). Other phyla were each represented by < 1% of the total sequences. These major bacterial phyla have also been found in other studies [26, 29, 43] and are likely to universal in anaerobic digesters fed with lignocellulosic feedstocks. Considering that the inoculum was collected from a well-run digester with high methane production from RS and DM, these identified bacteria may play an important role in degrading lignocellulosic substrates during AD. After 12 days of digestion, the proportions of the major phyla in three digesters with increasing S/I ratios showed marked variations as follows: *Firmicutes* gradually decreased (55.8%, 45.0%, and 32.9%); *Bacteroidetes* significantly increased (25.2%, 32.8%, and 43.9%); *Spirochaetae* initially decreased and then increased (3.0%, 2.2%, and 3.3%); *Fibrobacteres* initially increased and then decreased (1.2%, 2.3%, and 1.1%); and *Synergistetes*, *Tenericutes*, *Cyanobacteria*, and *Actinobacteria* each increased slightly. These results indicate that *Firmicutes* and *Bacteroidetes* were the two most predominant phyla in all digesters, which is consistent with a study on SS-AD digesters fed different ratios of corn stover feedstock to liquid anaerobic digestion effluent [43]. Studies have reported that *Firmicutes* and *Bacteroidetes* contain many known bacteria that can hydrolyze and ferment fiber into organic acids, with some species being positively correlated to VFA concentration [29, 43]. Therefore, a greater abundance of *Firmicutes* and *Bacteroidetes* in digesters fed RS and DM at optimal S/I ratios resulted in greater degradation of the cellulose and hemicellulose components and higher production of VFAs during hydrolysis and acidification. This provided more carbon nutrients for the archaea and correspondingly improved methane production. Interestingly, during the initial phase of HSS-AD the most prevalent phylum gradually shifted from *Firmicutes* in digesters with S/I ratios of 2:3 and 2:1 to *Bacteroidetes* in digesters with an S/I ratio of 4:1. This may be because some *Firmicutes* species were suppressed at higher VFA concentrations and lower pH in digesters with an S/I ratio of 4:1, and some *Bacteroidetes* species showed greater resistance to the acidic environment.

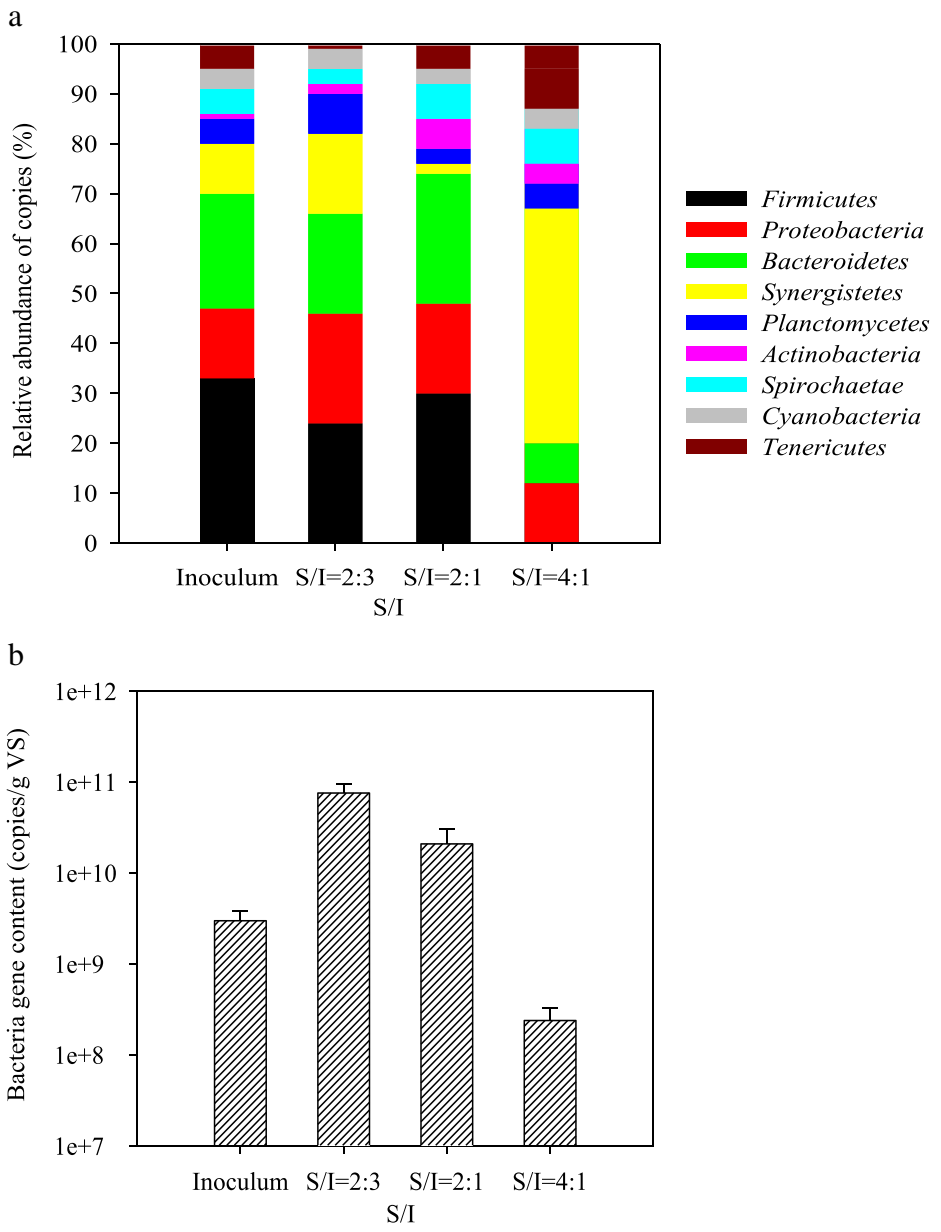

Fig. 4 (A) Relative abundance of different bacteria phyla and (B) total bacteria population numbers of inoculum and samples collected on day 12 of digestion substrates at different substrate/inoculum (S/I) ratios

The quantitative composition of the bacterial populations from the digesters with different S/I ratios is shown in Fig. 4 B. A strong correlation was found between the bacteria population and methane production rate. The quantity of bacteria in digesters at an S/I ratio of 2:3 reached 7.6×10^{10} copies/g VS, which was 3.6-fold higher than that in the digester with an S/I ratio of 2:1 and compared to only 2.4×10^{8} copies/g VS in the digester with an S/I ratio of 4:1. In

accordance with the sequencing data, the greater abundance of bacteria may have enhanced the decomposition of lignocellulosic feedstocks and improved SMPR and VMPR in the stable digesters. Moreover, the growth of bacteria was inhibited by excess VFA accumulation and low pH, which delayed the methane production period and resulted in a low methane production rate. Zheng et al. [29] and Li et al. [43] also found that the number of bacteria was positively correlated with methane yield and pH in healthy and sour digesters. Therefore, the amounts of bacteria using VFAs as a substrate under acidic pH may indicate the stability of HSS-AD.

Archaeal Community In total, 127,983 quality-checked archaeal sequences were identified, and 44–51 OTUs were detected in the samples from the inoculum and digesters at S/I ratios of 2:3, 2:1, and 4:1 on day 12. The Simpson and Shannon diversity indices were 0.69–0.73 and 2.57–2.66, respectively (Table 2). The similar diversity estimates among the samples revealed archaea community succession, but not advantageous changes in the species richness or evenness. Furthermore, the two diversity indices were lower than those of bacteria in the three digesters, indicating a more diversified population of bacteria degraded the complex components and carbohydrate structures of lignocellulosic biomass compared with the archaea population. However, the minor archaea constituents are thought to be responsible for the methanogenesis reaction [13]. As shown in Fig. 5 A, all OTUs were classified into 11 known genera (at 97% sequence similarity). Among them, the genus of *Methanosaeta* belongs to the *Methanosaetaceae* (Mst) family; the two genera of *Methanosarcina* and *Methanomicrococcus* belong to the *Methanosarcinaceae* (Msc) family; the three genera of *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera* belong to the *Methanobacteriaceae* family of the *Methanobacteriales* (MBT) order; and the five genera of *Methanoculleus*, *Methanocorpusculum*, *Methanofollis*, *Methanogenium*, and *Methanospirillum* belong to the *Methanomicrobiales* (MMB) order. The predominant archaeal genera in the inoculum and HSS-AD digesters were *Methanosaeta*, *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, and *Methanosphaera.* Although the primary types of archaea identified were similar among samples, as shown in Fig. 5 A, the relative abundances clearly differed between the healthy digesters (S/I ratios of 2:3 and 2:1) and sour digesters (S/I ratio of 4:1). After 12 days of digestion, the digesters with S/I ratios of 2:3 and 2:1, which steadily produced biogas, had similar microbial profiles as that of the inoculums. This suggests that the acclimated seed sludge used in our study had high methane-producing activity, which was important for efficient start-up of the SS-AD system and stable methane production [5, 43]. *Methanosaeta* was the predominant archaea genus in the samples from digesters at S/I ratios of 2:3 (55.6%) and 2:1 (53.2%), which was significantly higher ($p > 0.05$) than that of the digester at an S/I ratio of 4:1 (39.8%). Although the relative abundances of *Methanosarcina* and *Methanoculleus* in the three digesters were lower than that of *Methanosaeta*, the trend in variation of both the methanogens was similar with *Methanosaeta.* Li et al. [43] found that *Methanosarcina* dominates the methanogen community in SS-AD fed with corn stalk, which differs from the results of the current study. This apparent discrepancy may be related to differences in operational factors such as TS, feedstocks, and seeding, as it has been confirmed that these factors affect the microbiome community during AD [32, 44]. It is well known that *Methanosaeta* and *Methanosarcina* are acetoclastic methanogens that play an important role in acetoclastic methanogenesis. Zhao et al. [26] indicated that *Methanosaeta* improved methane production by digesters under conditions with high HAc content. In our study, digesters with S/I ratios of 2:3 and 2:1 on day 12 of fermentation contained high concentrations of acetic, lactic, and propionic acids (Fig. 3). Interestingly, in the digesters with an S/I ratio of 4:1, which showed excess VFA accumulation (25.4 g/L) and low pH (5.9), the relative abundance of

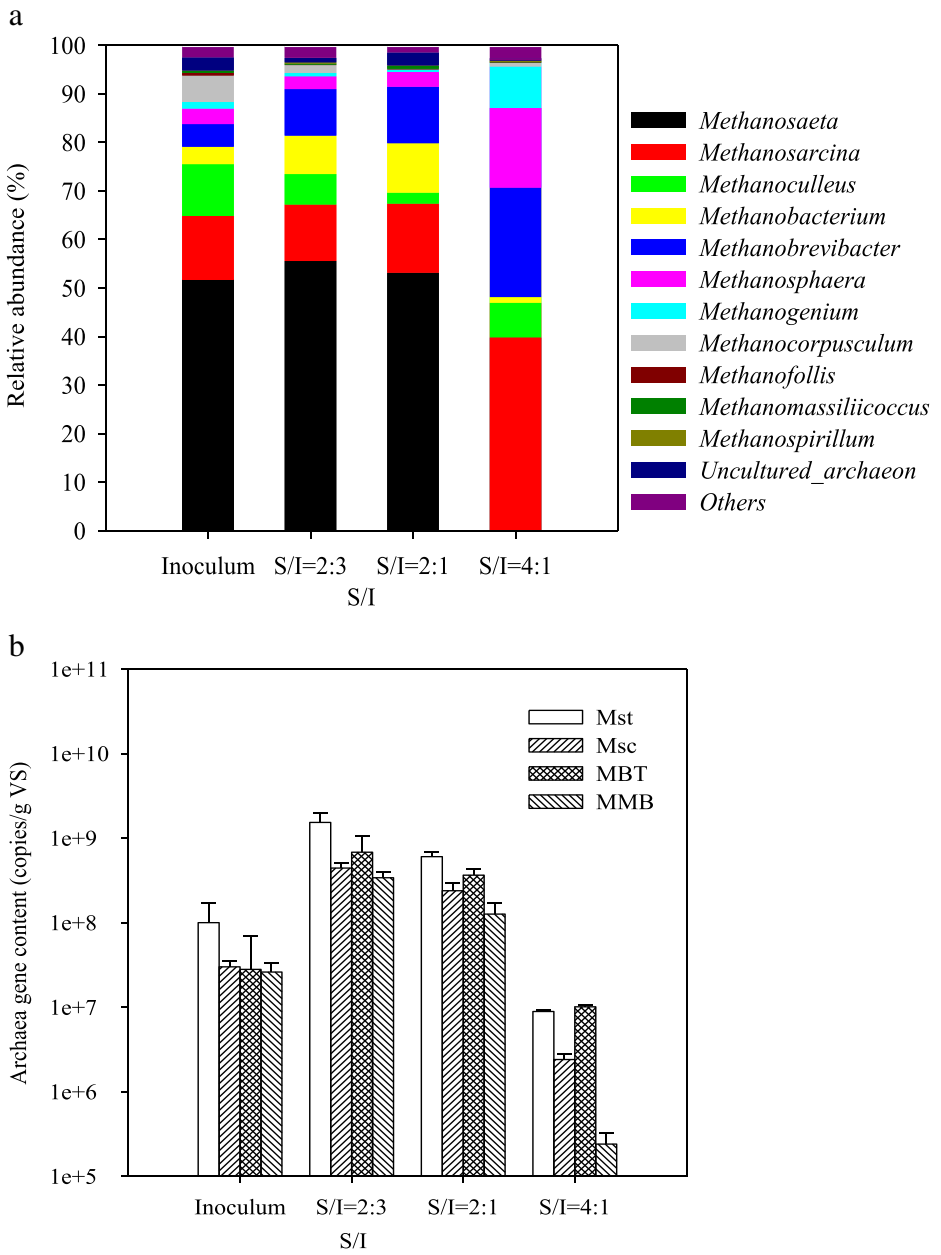

Fig. 5 (A) Relative abundance of different archaeal genera and (B) total archea population numbers of inoculum and samples collected on day 12 of digestion substrates at different substrate/inoculum (S/I) ratios. Mst: *Methanosaetaceae*; Msc: *Methanosarcinaceae*; MBT: *Methanobacteriales*; MMB: *Methanomicrobiales*

hydrogenotrophic *Methanobacterium* in the sour digesters (22.5%) increased sharply compared to that of S/I ratios of 2:3 (7.9%) and 2:1 (10.2%). The other two hydrogenotrophic methanogens, *Methanobrevibacter* and *Methanosphaera*, showed similar tendencies for change as *Methanobacterium*. Our results were consistent with those of Blume et al. [45], who reported

that *Methanobacteriales* were more abundant than members of the families *Methanosarcinaceae*, *Methanomicrobiaceae*, and *Methanosaetaceae* in environments with high total acid concentrations and low pH. We observed similar results in that *Methanosaeta* and *Methanosarcina* were strongly inhibited, while *Methanobacteriales* was significantly increased at acetate concentrations of up to 8 g/L [46] and was even able to grow at pH < 5.0 [47]. In the present study, the hydrogenotrophic order *Methanobacteriales*, including the genera *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera*, was the most abundant group of methanogens and, despite the resistance of these archaea to acidic and low pH environments, methanogenesis was inhibited. This inhibition resulted from the greater accumulation of VFAs and acetic acid at concentrations > 9.0 g/L. Therefore, methanogens were highly influenced by the S/I ratio through VFA accumulation and pH lowering during HSS-AD.

To better understand archaeal population changes in digesters with different S/I ratios during HSS-AD processes, four groups of archaea, including Mst, Msc, MBT, and MMB, which are generally considered important for biogas production, were quantified from digester samples by qPCR (Fig. 5B). In general, the abundances agreed well with those determined from the Illumina sequencing data for all genera quantified. The highest amount of each methanogen was detected in samples from the digester at an S/I ratio of 2:3 and gradually decreased as the S/I ratio increased. Mst was the most abundant family in all samples, ranging from 1.5×10^9 copies/g VS at an S/I ratio of 2:3 to only 8.8×10^6 copies/g VS at an S/I ratio of 4:1. However, the degree of population decrease among the methanogens was significantly different, with the amount of Mst, Msc, MBT, and MMB approximately 170-, 180-, 70-, and 1400-fold lower, respectively, in the 4:1 digesters compared to that in the 2:3 digesters. This suggests that MBT had the greatest acid tolerance while MMB showed the lowest tolerance. Moreover, consistent with the sequencing data, acetoclastic Mst and hydrogenotrophic MBT were the most predominant in rancid samples from the 4:1 digesters. Leclerc et al. [48] showed that the most frequent archaeal sequences were associated with *Methanosaeta* and *Methanobacterium* in 84% and 73%, respectively, of digesters located in eight different countries and fed with seven different types of waste. Considering the cessation of methane production in the 4:1 digesters in the present study after 12 days of fermentation, the role and importance of the two different pathways for methanogenesis in methanogens are likely related to the acid resistance and subsequent recovery of methane production.

HSS-AD of lignocellulosic feedstocks is a complex process carried out by synergistic microbes that perform different functions during the production of methane. A proper balance among VFA levels and acetotrophic and hydrogenotrophic methanogens is crucial for improving methane production, which is dependent on the optimum S/I ratios. In most previous studies, the S/I ratio was generally expressed in terms of VS ratio. However, this is not an accurate criterion because both substrate characteristics and microbial structure synergistically impact the production of VFAs and methane during the initial phase of HSS-AD. Therefore, a better indicator should be considered, such as the content of easily digestible matter in the feedstocks and activity of bacteria and methanogens in the inoculum (e.g., ratio of bacteria to methanogens).

Conclusion

The S/I ratio (based on VS) affected the process stability and methane production during co-digestion of RS and DM in an HSS-AD system. Different S/I ratios resulted in differences in

methane production and structures of microbial communities. Increasing the S/I ratio shortened the start-up period of HSS-AD. The highest methane yield of 209.1 mL/g VS_{added} and highest volumetric methane production of 0.4 L/(L·d) was achieved at S/I ratios of 2:3 and 2:1, respectively. In digesters with an S/I ratio of 4:1, the amounts of bacteria and archaea on day 12 were significantly reduced because of excess accumulation of VFAs and low pH. At the same time point, the predominant genus of bacteria gradually shifted from *Firmicutes* in digesters with an S/I ratio of 2:3 to *Bacteroidetes* in digesters with an S/I ratio of 4:1. The predominant archaea in all digesters was related to acetoclastic *Methanosaeta*, but the relative abundance of hydrogenotrophic *Methanobacterium* increased as the S/I ratio was increased. Our results provide useful information for enhancing the efficiency of methane production and improve the understanding of the microbiome in HSS-AD of RS and DM at different S/I ratios. To improve RS digestion, further studies are needed to optimize the physical, chemical, and biological growth conditions of bacteria and archaea and to identify the individual functions of microbes during the HSS-AD process using metagenomics.

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