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Corresponding Author: Mr. Lu Feng,

Corresponding Author's Institution: Aarhus University

First Author: Lu Feng

Order of Authors: Lu Feng; Yolanda Maria Lemes Perschke; Doline Fontaine; Marcell Nikolausz; Ulisses Nunes da Rocha; Felipe Borim Corrêa; Alastair James Ward; Jørgen Eriksen; Peter Sørensen; Henrik Bjarne Møller

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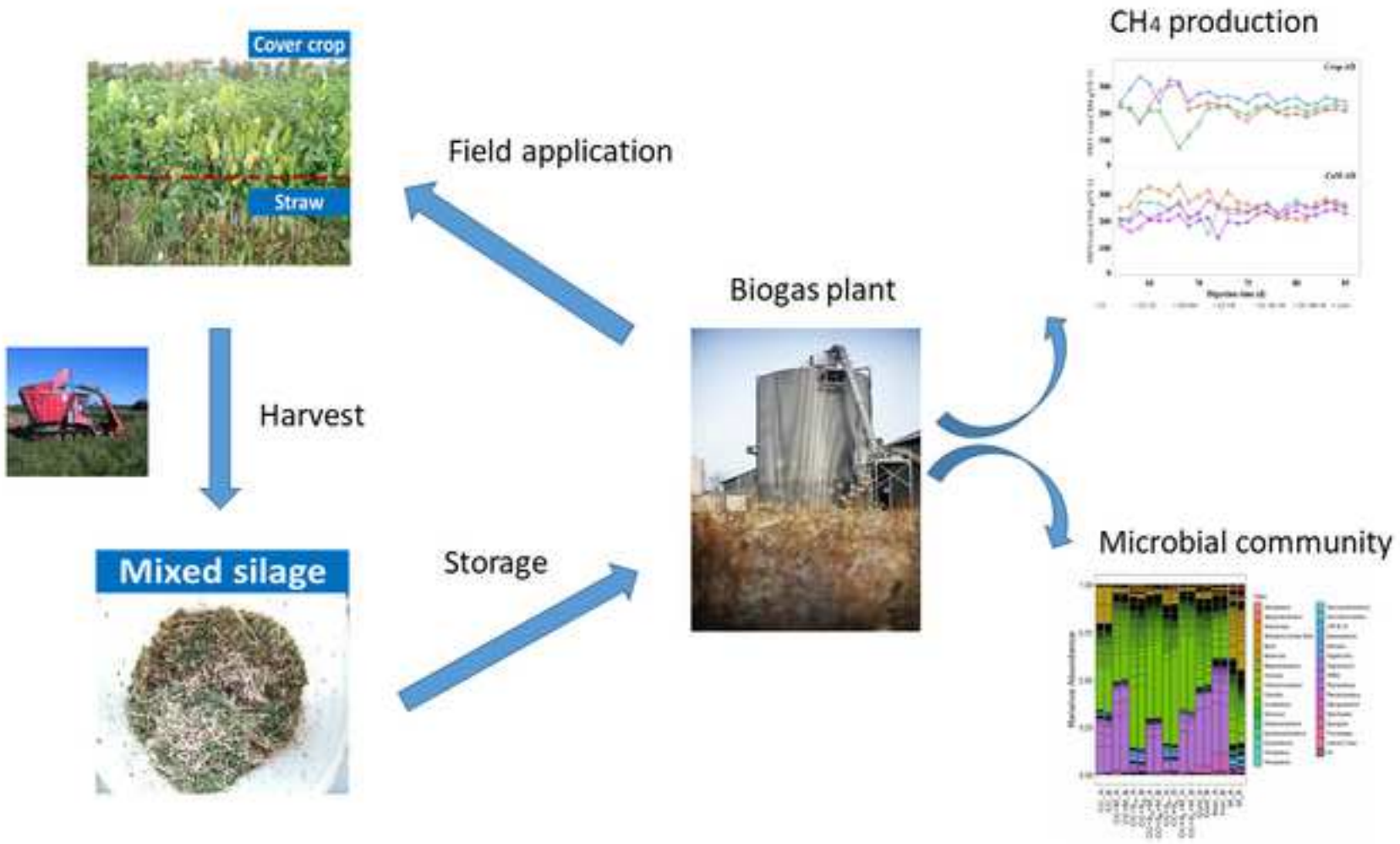
Graphical Abstract



Highlight

- Anaerobic digestion of co-ensiled cover crop and straw with manure were tested.
- Cover crop is feasible for biogas either alone or together with straw, manure or both.
- Co-digestion of cover crops with manure and less straw had superior performance.
- High straw addition led to distinct microbial communities.

Anaerobic digestion of co-ensiled cover crop and barley straw: effect of co-ensiled ratios, manure addition and impact on microbial community structure

Lu Feng[a,*], Yolanda Maria Lemes Perschke[a], Doline Fontaine[b], Marcell Nikolausz[c], Alastair James Ward[a], Ulisses Nunes da Rocha[c], Felipe Borim Corrêa[c], Jørgen Eriksen[b], Peter Sørensen[b], Henrik Bjarne Møller[a].

[a] Department of Engineering, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark.
[b] Department of Agroecology, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark
[c] Department of Environmental Microbiology, Helmholtz Centre for Environmental Research-UFZ, 04318 Leipzig, Germany

*Corresponding author: email: lufeng@eng.au.dk; Telephone: +45 9145427

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Lu Feng[a,*], Yolanda Maria Lemes Perschke[a], Doline Fontaine[b], Marcell Nikolausz[c], Alastair James Ward[a], Ulisses Nunes da Rocha[c], Felipe Borim Corrêa[c], Jørgen Eriksen[b], Peter Sørensen[b], Henrik Bjarne Møller[a].

[a] Department of Engineering, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark.

[b] Department of Agroecology, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark

[c] Department of Environmental Microbiology, Helmholtz Centre for Environmental Research-UFZ, 04318 Leipzig, Germany

*Corresponding author: email: lufeng@eng.au.dk; Telephone: +45 9145427

Abstract:

Cover cropping is important for nutrient management of agricultural systems and has largely unexploited potential for biogas production. Co-harvest of cover crops and straw and storage as silage blends prior to biogas is promising as it could enhance the long-term storability and lead to synergies for anaerobic digestion. However, it is necessary to evaluate the feasibility of using co-ensiled blends and process optimization based on continuous test. In this study, semi-continuous thermophilic anaerobic digestion experiments were carried out with feeding of cover crop silage, co-ensiled cover crop and straw (with mixing ratios in relation to various harvest strategies) with or without addition of cattle manure. The main objective is to determine the feasibility of aforementioned mixtures for biogas production and the influence on microbial community structures in response to various feeding compositions. Results demonstrated that cover crop (silage) is feasible for digestion alone or with addition of barley straw, cattle manure or both, while manure addition led to higher CH_4 yield/buffer capacity, and enhanced the volatile solids reduction. Microbial community compositions were found to have been affected by the feeding, while high straw addition led to a distinct community structure.

Keywords: Anaerobic digestion; Cover crop; Lignocellulosic biomass; Silage; Methane.

Abbreviation

AD	Anaerobic digestion
ADF	Acid detergent fiber
ADL	Acid detergent lignin
BMP	Biochemical methane potential
CC	Cover crop
C:N	Carbon to nitrogen ratio
CSTRs	Continuous stirred tank reactor
FM	Fresh matter
HRT	Hydraulic retention time
IA	Intermediate alkalinity
NDF	Neutral detergent fiber
NMDS	Nonmetric multidimensional scaling
OLR	Organic loading rate
PA	Partial alkalinity
RMP	Residual CH_4 potential
TA	Total alkalinity
TAN	Total ammonia nitrogen
TE	Trace elements
TKN	Total Kjeldahl nitrogen
T-RFLP	Terminal restriction fragment length polymorphism
TS	Total solids

VFA	Volatile fatty acid
VS	Volatile solids
WSC	Water soluble carbohydrates

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1. Introduction

Cover cropping plays an important role in organic farming systems as it can recycle nutrients that could be lost by leaching during the rainy season. Cover crops are subsequently used in the form of green manure fertilizer to increase nutrients availability for the following crops (Vogeler et al, 2019). However, cover cropping may be associated with nitrogen losses following cutting and mulching of the residues due to atmospheric emissions (NH_3, N_2O, NO and N_2) (Frøseth et al., 2014). Therefore, it is promising to harvest the cover crop to produce biogas *via* anaerobic digestion (AD), with potential benefits in the form of reduction of greenhouse gas (GHG) emissions, production of renewable energy, and redistribution of nutrients in space and time via the use of digestate as mobile biofertilizer (Brozyna et al., 2013; Li et al., 2015; Michel et al., 2010., Stinner et al, 2008). Conversion of cover crops to biogas requires storage prior to biogas production as biogas plants are operated continuously (Feng et al., 2018). Ensiling is the most widely used technique for preservation of wet-biomass for the livestock feed industry and is today being considered as a feasible method of feedstock preservation for the biogas sector (Baldini et al., 2017; Kholif et al., 2017; Vervaeren et al., 2010). Maintaining of the quality of ensiled cover crop is a critical issue since the total solids (TS) content of cover crop is usually lower than the recommendation (TS of 25-35%) for limiting the release of leachate and avoiding undesirable microbial activities such as clostridia fermentation under wet conditions or fungal activity under high-solids conditions (Franco et al., 2016; Liu et al., 2016). One possible solution is to mix cover crop and straw together as this could optimize the content of total solids (TS) and simultaneously provide sufficient water soluble carbohydrates (WSC) for rapid formation of organic acids to reduce the pH (Thompson et al., 2005). This can be achieved by two strategies: 1). When harvesting mature grain from a cereal with undersown cover crop, leaving the straw

with high stubble in the field until the cover crop is harvested in late autumn, which would reduce the cost for straw baling since the cover crop and the straw are collected in one operation. At the same time, the grain harvest will be faster and cheaper since only the ears and grain of the cereal crop will go through the combine harvester; or 2). Harvesting straw in summer with the grain harvest and storing it until the cover crop is harvested in the autumn. The mixing ratios of cover crop to straw is generally higher in the first strategy because weight loss of straw is expected to take place until the autumn harvest due to leaching of water-soluble compounds (Collins et al., 1990). Beside that, utilization of co-ensiled cover crop and straw is also good for anaerobic digestion as it will optimize the C:N ratio and lead to synergistic effects (Feng et al., 2019; Hillion et al., 2018). On the other hand, biomass, such as agricultural residues/by-products, are not commonly used as exclusive feedstocks for biogas plants (Tsapekos et al., 2015). One of the major concerns is the risk of system failure due to deficiency of trace elements (TEs) or reduction in buffering capacity over time (Thamsiriroj et al., 2012; Wahid et al., 2018; Xie et al., 2011). To avoid these problems, the most common practice for crops-to-biogas is to co-digest crops with animal manure or other liquid substrates to promote homogenous and stable conditions (Murphy et al., 2011): animal manure is rich in nutrients (both macro and micro), has a good buffering capacity that provides optimal growth conditions for microorganisms (Mulat and Horn, 2018; Thamsiriroj et al., 2012), and is also an excellent inoculum providing microbial resources needed for effective anaerobic digestion (Leite et al., 2016).

In this study, semi-continuous anaerobic digestion experiments were operated at thermophilic temperature by feeding of cover crop and co-ensiled blends of cover crop and straw, with or without manure addition. Digestate was collected at the end to determine the microbial community structure to gain a deeper insight into changes within these communities in response

to various feeding compositions. The work is based on our finding from previous study under batch scale test which carried out under semi-continuous basis to give more comprehensive and reliable results closed to realistic biogas production. To the best of our knowledge, there are very limited studies investigating anaerobic digestion of co-ensiled blends and compare the effect of manure addition under semi-continuous anaerobic digestion tests. The aims of this study were to: 1) investigate the feasibility of using co-ensiled cover crop and barley straw for biogas; 2) determine the influence of manure addition on anaerobic digestion of cover crop, with or without straw and, 3) reveal the impact on microbial community structures in response to the various feeding compositions.

2. Materials and methods

2.1 Substrate and silage preparation

Cover crop was undersown in spring barley (*Hordeum vulgare L.*) in May 2017 at Research Centre Foulum (56°30’ N and 09°35’ E). After harvest of the spring barley and removal of the straw in summer 2017, the cover crop grew freely and was harvested on October 13, 2017. The botanical composition (dry-matter based) of the cover crop was 88-89% red clover (*Trifolium pretense L.*), 0.1-0.8% mixed weeds and 10-11% chicory (*Cichorium intybus L.*). Barley straw was obtained from a nearby field and stored in plastic bags until October. The cover crop was harvested using a grass harvester (Haldrup F-55 grass harvester, Løgstør, Denmark) with a cutting width of 1.5 m and equipped with a direct weighing system. After harvest, cover crop and barley straw were weighed, chopped together to a particle size of 3-5 cm and fully mixed using a sample chopper (Laborhäcksler, Baumann Saatzuchtbedarf, 74638 Waldenburg, Germany) according to mix ratios of cover crop and barley straw that were determined based on the data

obtained from the field experiments. Ratios of cover crop to barley straw were: 1:0 *w:w* (no straw included, hereafter referred to as CC); 2); 10:1 *w:w* (mixture of high stubble of straw and cover crop harvested together in the autumn, hereafter referred to as $CC+S_L$); 3:1 *w:w* (straw harvested at maturity was later mixed with autumn harvested cover crop, hereafter referred to as $CC+S_H$). These were prepared as silage blends in vacuumed plastic bags (4-5 kg per silage batch) and stored for 4 months. After the 4 months ensiling period, the silage blends were transferred to small plastic bags according to daily a feeding mass (100-300 g per bag) and stored at -18℃. Individual plastic bags were defrosted at 15℃ as required prior to feeding. Cattle manure was collected from the animal facilities at Research Centre Foulum (Aarhus University, Denmark) and kept at -18℃ after sampling. Thermophilic inoculum (51℃) was collected from pilot-scale reactors of 30 m^3 total volume (Aarhus University, Foulum) which had been running with cattle manure as the main feedstock for over six months.

2.2 Laboratory scale continuous stirred tank reactors (CSTR)

The laboratory scale experiment was carried out with seven CSTRs of 15 L working volume. All reactors were manually fed daily and a volume of digestate that was reciprocal to the hydraulic retention time (HRT) of 25 days mutiplied by the digester volume was removed. Description of equipment details regarding the lab-scale CSTRs set-up has been described in Feng et al. (2017). All reactors were operated at thermophilic conditions (51℃) and filled with 15 L of inoculum before start-up. Digestate from each reactor was collected once or twice per week for analysis of pH, total solids (TS) content, volatile solids (VS), volatile fatty acids (VFA), total ammonia nitrogen (TAN). The content of total organic nitrogen (TN), and total/partial/intermediate alkalinity (TA, PA, IA) were measured from samples collected at the end of the experiment. In addition, digestate at the end of the experiment were collected for determination of the residual

CH_4 potential, nutrients concentration (Ca, K, Mg, Na, P, S, Fe, Ni, Co, Cu, Zn), fiber composition (cellulose, hemi-cellulose, lignin), and structure of microbial communities.

The schematic diagram of the experimental reactors is shown in Fig.1. Digesters were set up as Crop-AD (anaerobic digestion of co-ensiled crops), CoM-AD (co-digestion of co-ensiled crops with cattle manure) and control (mono-digestion of cattle manure, Cont). For all digesters, the organic loading rates (OLRs) were adjusted to 3 g VS L^{-1} d^{-1} (at 9% TS) by adding either tap water (Crop-AD) or cattle manure (CoM-AD) (25-80%$_{w:w}$) (Table 1). The entire experiment lasted for over three HRTs (85 days) to ensure relatively stabilized performance towards the end of the experiment.

2.3 Problems of Crop-AD digesters

In this study, co-ensiling of cover crop and barley straw represented two strategies of managing agricultural by-products through either harvest together or separately. During the experimental period, undigested fibers gradually accumulated and further formed a 'dead zone' where the stirring system could not sufficiently mix them into the bulk fluid from Crop-AD digesters without manure addition. Undigested fiber (1 kg, 12-14% TS) were therefore partly removed from the digester CC+S_L at day 44 and from the digesters CC+S_H at day 33 and 42, respectively, to avoid system failure due to mechanical issues.

2.4 Residual methane potential

Digestates were collected at the end of the experiment to determine the residual methane potential (RMP) following the protocol suggested by Moset et al. (2018). The test was set-up using 500 mL infusion bottles. Three infusion bottles were prepared as replicates for each reactor. 200 gram of digestate was added to each bottle which were then tightly sealed with

rubber stoppers and screw caps. All bottles were flushed with N_2 for 2 minutes to replace the headspace air and incubated at 51^{o}C for 50 days. Produced biogas was measured by inserting a needle through the butyl rubber caps. The needle was attached to a tube with inlet to a graduated plastic tube filled with acidified water (pH <2) and the volumes were measured by the water displaced until the relevant two pressures (column and headspace in bottles) were equal. Biogas from infusion bottles travelled through the vial to the column and therefore recorded by reading the scale on the tube. Gas samples were collected using 20 mL flat bottom headspace vials (Agilent technologies, CA 95051, USA) which were connected between the infusion bottle and the acidified water tube. The biogas/CH_4 yields were adjusted to standard conditions (0℃ and 1.013 bar).

2.5 Analyses

TS and VS were measured according to the standard methods (APHA, 2005). Biogas composition from the semi-continuous experiments was analyzed twice per week (biogas composition from the RMP experiment was determined periodically) using gas chromatography (Agilent technologies 7890A, CA 95051, USA). Dissolved VFA was determined using a gas chromatograph (Agilent technologies 7890A, CA 95051, USA). Description regarding the detector, carrier gas, column, temperatures, etc., can be found in Feng et al. (2017). TAN was determined weekly from digestate using photometry (Spectroquant Kit, Merk, NJ, USA). Total Kjeldahl nitrogen (TKN) was determined according to APHA (2005). Crude protein content was calculated by determining total organic nitrogen and multiplying by a factor of 6.25 (Hattingh et al., 1967).

Samples for fibre analysis were dried (48 h at 60°C) and milled to a particle size of 0.8 mm using a CyclotecTM 1093 mill (FOSS, MN, USA). Fibre fractions, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (ADL) were analyzed according to the Van Soest (1991) method. From these fractions, hemicellulose, cellulose and lignin contents were calculated. The hemicellulose content was calculated as the difference between NDF and ADF, the cellulose content as the difference between ADF and ADL, and the lignin content was assumed to be equal to ADL. TA, PA and IA were measured by titration with HCl (0.1 M), which consists of two end points during the titration process: the first to pH 5.75 is due to the existence of bicarbonate and is known as PA; the second to pH 4.3 corresponds to TA (Jantsch and Mattiasson, 2003). The IA, which is related to the VFA concentration, is estimated from the difference between TA and PA. Elemental content (C, H, N, S) was determined using Elementar vario macro cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). Macro-, micro- and trace elements from digestate were determined according to the DIN (1998) method.

2.6 Microbial community structure

Samples taken from CSTRs, the original inoculum, and the manure used as substrate feed were stored at -20°C until microbial analysis. The total genomic DNA was extracted with NucleoSpin Soil kits (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the supplier's protocol using buffer SL2 with enhancer SX. The quality of DNA was checked by 0.8% agarose gel electrophoresis and concentration was measured via a NanoDrop ND-1000 UV/visible spectral photometer (Thermo Fisher Scientific, United States).

The 16S rRNA genes were amplified and sequenced using the MiSeq platform (Illumina V3, 2 x 300 bp). The variable regions V3–V4 of the bacterial 16S rRNA gene were PCR amplified using

160 the primers 341f (5' CCTACGGGNGGCWGCAG 5') and 785r (5'
161 GACTACHVGGGTATCTAAKCC 5') according to Klindworth et al. (2013). Afterwards the
162 PCR products were purified with AMPure XP beads *via* magnetic stand. Index PCR with the
163 purified PCR products was applied with the Nextera XT Index kit to attach dual indices.
164 Subsequently, the PCR products were purified with above methods for Illumina$^{@}$ MiSeq
165 amplicon sequencing. Raw sequencing data from demultiplexed samples was imported and
166 processed with QIIME2 version 2018.11. Denoising of paired-end reads, dereplication, chimera
167 filtering and generation of Amplicon Sequence Variants (ASVs) were made with DADA2 plugin
168 according to developer's instructions (Callahan et al., 2016). Taxonomy was assigned to the
169 ASVs using the MiDAS 2.1.3 reference database built for the respective hypervariable region
170 (McIlroy et al., 2015). The sequences obtained from this study were deposited under the EMBL-
171 EBI accession number PRJEB33585.

172 For a detailed analysis of relative abundances of methanogenic archaea, a similar approach was
173 used but instead of 16S rRNA gene, the methanogen-specific mcrA gene was amplified using the
174 primers mlas (GGTGGTGTMGGDTTCACMCARTA) and mcrA-rev
175 (CGTTCATBGCGTAGTTVGGRTAGT) (Steinberg and Regan, 2008). Taxonomy was assigned
176 using a custom database of mcrA genes (Popp et al., 2017). For the 16S and mcrA amplicons, the
177 ASV frequency table, taxonomy and DNA sequences were exported from QIIME2 objects to text
178 and FASTA files for data analysis.

179 2.7 Calculation and data analysis

180 Residual $CH_{4VS\text{-}C}$ (%) was calculated according to Eq.1:

181 Residual $CH_{4\ VS\text{-}C}$ (%) = $(RMP\times(1\text{-}VS_{\text{-}R}))/(SMY_E + RMP\times(1\text{-}VS_{\text{-}R}))$ (1)

where the RMP (mL CH_4 g^{-1} VS) was directly measured from the RMP batch test and specific methane yield (SMY_E (mL CH_4 g^{-1} VS) was the average CH_4 production calculated based on the last HRT of the continuous experiment. In eq.1, the VS reduction is taken into account (VS_{-C}) since the VS from feeding was partly degraded (VS_{-R}) during the continuous anaerobic digestion process (VS_{-C}, corrected VS with consideration of VS reduced due to anaerobic digestion, $VS_{-R,}$ VS reduced/degraded during anaerobic digestion).

The synergistic effect derived from co-digestion was determined according to Eq.2:

$$\text{Synergistic effect (\%)} = \frac{\text{SMY}_{\text{E-CoAD silage}} - \text{SMY}_{\text{C-CoAD silage}}}{\text{SMY}_{\text{C-CoAD silage}}} \times 100 \quad (2)$$

$$\text{SMY}_{\text{C-CoAD Silage}} = \frac{\text{SMY}_{\text{CO-AD}} \times \text{VS}_{\text{T}} - \text{SMYcont} \times \text{VS}_{\text{M}}}{\text{VS}_{\text{CoAD silage}}} \quad (3)$$

where the SMY_E (mL CH_4 g^{-1} VS) was the average CH_4 yield obtained from the last HRT, $SMY_{C\text{-}CoAD:silage}$ (mL CH_4 g^{-1} VS) (the SMY contributed from silage when co-digested together with manure) was calculated according to Eq.3. VS_T (g VS day^{-1}) represents the daily feeding VS per reactor. $VS_{CoAD\ silage}$ and VS_M represent the VS sourced from either silage or cattle manure fed to corresponded digesters. SMY_{cont} was the SMY (average yield from the last HRT) determined from the control reactor.

For the data analysis of bacteria via Illumina$^{@}$ MiSeq, Simpson index and ASV counts (α-diversity) were determined using the phyloseq R package (McMurdie and Holmes, 2013). Analyses were carried out in two steps: First, differences in bacterial community composition (β-diversity) were calculated using Bray–Curtis dissimilarity indices based on rarefied and square-root-transformed ASV abundances, which are demonstrated via nonmetric multi-dimensional scaling (NMDS) plot. NMDS plots were produced with the phyloseq R package according developer's instructions (McMurdie and Holmes, 2013). JMP 13.0 (SAS Institute Inc, 10740 Cary, USA) was used for graphing.

205 **3. Results and discussions**

206 3.1 Characteristics

207 Fiber composition (cellulose, hemi-cellulose, and lignin), C:N ratio, and VS contribution from
208 each substrate under various feeding compositions are listed in Table 1. Addition of barley straw
209 increased the fiber content, with cellulose content increasing from 24.3 (CC) to 35.1 % (CC+S_H),
210 hemicellulose from 13.1 (CC) to 28.0% (CC+S_H), and lignin from 5.9 (CC) to 9.5% (CC+S_H).
211 C:N ratio is an important parameter to balance the AD process, while the excess nitrogen from
212 the feedstock will lead to high total ammonia nitrogen (TAN) and/or higher VFA accumulation
213 in the digester (Li et al., 2011). In this study, C:N ratios from manure amended systems
214 decreased slightly in CoM-AD digesters, CC+S_L+M and CC+S_H+M, but had almost no change
215 for digester CC+M compared to their corresponding Crop-AD digesters. Co-ensiled mixtures
216 (CC+S_H) had the most favorable C:N ratio (25:1) for anaerobic digestion (Pang et al., 2008).

217 3.2 Semi-continuous anaerobic digestion

218 3.2.1 General performance

219 Regarding Crop-AD digesters, the average SMYs (the last HRT) acquired from digester CC
220 (266.0 mL CH_4 g^{-1} VS in average) was 32% higher compared to digesters CC+S_H (202.0 mL CH_4
221 g^{-1} VS) and 21% higher than digester CC+S_L (219.9 mL CH_4 g^{-1} VS) (Table 2). Similar tendency
222 was observed from CoM-AD digesters, with the highest SMY observed from digester CC+M
223 (268.9 mL CH_4 g^{-1} VS on average) followed by CC+S_L+M (244.1 mL CH_4 g^{-1} VS) and
224 CC+S_H+M (238.4 mL CH_4 g^{-1} VS). Manure addition showed positive influence on anaerobic
225 digestion as the average CH_4 yield from digesters CC+S_L+M and CC+S_H+M was about 11 and
226 18% higher CH_4 yield than their corresponding Crop-AD digesters.

227 Buffer capacity is often expressed as total alkalinity (TA), which is the equilibrium of carbon
228 dioxide and bicarbonate ions that provides resistance to significant and rapid changes in pH, and
229 the buffering capacity is therefore proportional to the concentration of bicarbonate (Ward et al.,
230 2008). In this study, there is almost no difference in TAs from digesters CC (10448.8 mg $CaCO_3$.
231 L^{-1}) and CC+M (10721.4 mg $CaCO_3$ L^{-1}), indicating that the degradation of cover crop generates
232 extra TA and thus increases the buffer system as a result of the higher protein content. Total
233 alkalinity from the digesters CC+S_L and CC+S_H were lower than those digesters without straw
234 addition. Total alkalinity measured from digesters CC+S_L+M and CC+S_H+M was 9514.4 and
235 7835.0 mg $CaCO_3$ L^{-1}, respectively, while that from CC+S_L and CC+S_H was only 4896.6 and
236 4211.8 mg $CaCO_3$ L^{-1} (Table 2), respectively. pH from digester CC was higher (pH=7.8 on
237 average) compared to CC+S_L (pH=7.6) and CC+S_H (pH=7.5), while all of the co-AD digesters
238 held relative similar pH values (7.8-7.9).
239 3.2.2 Volatile solids (VS) reduction/Residual CH_4 potential (RMP)/Synergistic (or antagonistic)
240 effect
241 VS reduction achieved from digester CC was determined to be 54%, including 48% reduction of
242 cellulose and hemi-cellulose (Table 3). With addition of barley straw, the VS reduction from
243 digesters CC+S_L and CC+S_H were 37.9% and 36.1%, including 25.9% and 40.5% of cellulose
244 reduction, 23.1 and 49.0% of hemicellulose reduction, respectively. Regarding the CoM-AD
245 digesters, the VS reduction from digesters CC+M, CC+S_L+M, CC+S_H+M was 49.7, 44.7, and
246 29.4%, respectively. VS reduction from digester CC+M was slightly lower than that from CC
247 (54% *vs* 50%) (Table 3), while digesters CC+S_L+M, CC+S_H+M had 7-20% increment compare
248 to the corresponding Crop-AD digesters. Hemicellulose degradation achieved from all CoM-AD
249 digesters was quite similar (39.2, 41.5, and 39.0%, respectively). However, the reduction of

cellulose from CC+S_H+M was very poor (only 16% reduction of cellulose). In addition, as the most recalcitrant component during AD (Mulat and Horn, 2018), lignin content determined from the digestates ranged between 16.49 to 20.89% of TS (not including the control digester), corresponding to negative degradation/decreases from -19.4 to -85.3%. It should be noted that the negative degradation values are because the values are as a percentage of the TS, therefore negative degradation of lignin simply means that lignin is a greater proportion of the TS following digestion.

Residual CH_4 potential (RMP) reflects the efficiency of anaerobic digestion and the emission potential after land application (Ruile et al., 2015). RMP determined from Crop-AD digesters CC, CC+S_L, CC+S_H were 115.7, 145.3 and 165.5 mL CH_4 g^{-1} VS, respectively (Table 2). Anaerobic digestion of CC silage (either alone or with manure addition) had lower RMP (16.8% of total CH_4 yield was recoverable during RMP test), while that derived from digesters CC+S_L and CC+S_H accounted for 29.1% and 34.4% of the total CH_4 potential, respectively. For CoM-AD digesters, RMP determined from digesters CC+S_L+M, CC+S_H+M was 23% and 32%, respectively. Synergistic effects, in terms of methane yields, with manure addition were obtained from CoM-AD digesters (according to eq.2) as: CC+M (3.6%), CC+S_L+M (12.0%) and CC+S_H+M (16.2%).

3.2.3 Digestate characteristic

Effluents from digesters CC had the highest TKN and NH_4-N^+ contents (3.1/1.3 g L^{-1}) as CC silage is a nitrogen-rich feedstock than straw, followed by digester CC+S_L (1.8/0.6 g L^{-1}) and CC+S_H (1.4/0.35 g L^{-1}). Digestate from Crop-AD digesters contained 0.4-0.9 g m^{-3} of iron, 6-12 mg m^{-3} of nickel, and 1.7-2.5 mg m^{-3} of cobalt, which was found to be higher from the CoM-AD

digesters (1.8-2.6 g m^{-3} of iron, 11-14 mg m^{-3} of nickel, and 2-2.5 mg m^{-3} of cobalt) as a result of manure addition (Table 4).

3.3 Microbial communities

3.3.1 Diversity and evenness

Bacterial and archaeal communities of digesters, inoculum, and cattle manure were assessed using alpha diversity by the number of observed amplicon sequence variants (ASVs) and Simpson's diversity index with consideration of the evenness of the community (Fig.2). The numbers of bacterial ribotypes per digester ranged from 111-181 (ASVs) with the average of 152 ASVs. Among all the digesters investigated, the highest bacterial richness was observed from digester CC+S_L (177 ASVs observed) and the lowest diversity (excluding digester Cont) was measured from digester CC+M with 111 ASVs reads. The numbers of methanogen types per digester ranged from 20-58 (ASVs), with lower value for the digester CC+M and higher value from digester CC+S_H.

3.3.2 Bacterial community composition

The bacterial diversity in the anaerobic digesters, inoculum, and cattle manure was investigated by amplicon sequencing of 16S rRNA genes. Fig.3 shows the relative abundances of the taxa comprising at least 1% in the digestate samples. Bacterial communities of all digesters were dominated with limited numbers of microbial taxa, while large variations were observed between digesters (Fig.3). The class Clostridia (belonging to Firmicutes) was the most dominant bacteria in all digesters (except inoculum), at a relative abundance from 50-80%. This highly versatile class represents organotrophs, including hydrolytic strains capable of degrading proteins, lipids, and polymeric carbohydrates (Lynd et al., 2002), therefore their dominance is not surprising (Karlsson et al., 2013). Other classes are also represented but at a lower relative abundance, such

as OPB54 (uncultured taxonomic groups exist in the phylum Firmicutes), Bacteroidia, Actinobacteria, Erysipelotrichia, Synergistia, Anaerolineae, Fibrobacteria, and Spirochaetes. It is clear that digesters operated under various feeding compositions led to distinct bacterial communities. For instance, OPB54, which is known to ferment carbohydrates, was found as one of the most abundant bacteria in most of the digesters except CC+S_H and CC+S_L, with increases in their relative abundances for the CoM-AD reactors (*i.e.* with manure added) compared to Crop-AD reactors (*eg.* CC+M > CC; CC+S_L+M > CC+S_L) and their relative abundances decreased in related to straw addition (*eg.* CC+M > CC+S_L+M > CC+S_H+M). Moreover, high straw addition enriched Clostridia (digesters CC+S_H > CC+S_L, CC+S_H > CC+S_H+M, CC+S_L > CC+S_L+M), which corresponds to an increased requirement for cellulolytic activity. This was also observed in case of many other cellulolytic members, such as classes Bacilli, Fibrobacteria and Anaerolinea (König, 2006; Ransom et al., 2012; Xia et al., 2016). Fibrobacteria and Anaerolinea were in general rare in the reactor samples and only appeared in higher abundances from digesters CC+S_H and CC+S_L. When manure was also added together with straw, their selective advantage disappeared and remained rare members of the community.

3.3.3 Archaeal community composition

Methanogenic communities from digesters were mainly composed of the genera: *Methanobacterium, Methanosarcina, Methanocelleus, Methanothermobacter, Methanoregula, Methanobrevibacter, Methanosaeta* and *Candidatus Methanoplasma* (Fig.4). *Methanobacterium* was found to be the most predominant genus among Crop-AD digesters, which are mainly hydrogenotrophic methanogens to utilise H_2/CO_2 and sometimes formate and alcohols as substrates for growth and methane production (Whitman et al., 2006). *Methanosarcina*, which are generalist that can utilize methanol, methylamines, acetate, and many species also utilize H_2

318 (Liu and Whitman, 2008), was found to be the most abundant methanogen in all CoM-AD
319 digester correlated with the manure in feedstock (Ziganshin et al., 2016). *Methanosarcina* also
320 correlated well with increased pH and TAN: appearance of *Methanosarcina* is often associated
321 with stressed digesters due to their low affinity for acetate and ammonia, and thus their ability to
322 withstand relatively high concentrations of these intermediates (Calli et al., 2005). Although
323 none of the digesters in this study appeared particularly stressed in this study, it is postulated that
324 the increased abundance could make the respective digesters better equipped to deal with shock
325 loading in the future, although their lower affinity for acetate could reduce performance at lower
326 acetate concentrations. *Methanoculleus* and *Methanebrevibacter*, which were the most abundant
327 genus originally detected from the inoculum, were obviously lower in all experimental reactors
328 apart from the control.

329 3.4 Influence of feeding compositions on anaerobic digestion and structures of microbial
330 communities

331 3.4.1 Comparison of Crop-AD and CoM-AD

332 In general, regarding to methane production, CoM-AD configurations had superior performance
333 over Crop-AD, with higher SMY and synergistic effects, when both cover crop and straw were
334 fed but had almost no difference between digesters CC and CC+M. Similarly, residual CH_4
335 values, which are used as another indicator of efficiency during anaerobic digestion, were found
336 to be quite similar (16%) (with VS reduction taken into account) which were slightly higher than
337 from the digesters CC+S+M.

338 As described in 2.4, there were fibers accumulated in digesters CC+S_H and CC+S_L which further
339 led to risk on interlock of the digesters or even the stirring system over time. Thamsiriroj et al.
340 (2012) reported failure of the mechanical agitator after operation up to one year during anaerobic

digestion of grass silage, which probably led to the inhibition of acetogenesis, and further to the accumulation of lactic acid, drop in pH, reduced CH_4 yield and biodegradability. According to a survey to several full-scale biogas plants in Europe, biogas plant fed with crops alone might lead to depletion of micronutrients over a longer time span (Schattauer et al., 2011). Lebuhn et al. (2008) stated that long-term anaerobic digestion using crops alone would lead to a reduction of the methanogenic population, since trace elements (TEs) in the feed are insufficient. TEs are important in metabolic pathways and enzymatic reactions (Bougrier et al., 2018; Wintsche et al., 2016). Table 4 summarizes the concentrations of iron (Fe), nickel (Ni) and cobalt (Co) from various feeding compositions in this study and the optimal or stimulatory concentration for batch cultures of methanogens suggested by Takashima et al. (1990).

The results indicated that the concentration of iron (Fe) is more than sufficient for all digesters while Ni content for Crop-AD digesters were below the recommended value for anaerobic digestion of energy crops, crop residues and animal excreta, especially for digesters $CC+S_L$ and $CC+S_H$. Anaerobic digestion of cover crop alone performed quite normal during the entire experimental period. This might be explained by micronutrients from cover crop being much higher than with barley straw, which probably slows down the depletion of nutrients over time. However, long-term tests on cover crop are necessary to justify this observation.

3.4.2 Comparison of high straw and low straw addition

Feeding of mixed silages and the impact of straw addition on anaerobic digestion were compared under the same configuration (with or without manure addition). In general, the average SMYs from digesters $CC+S_L$ (+M) and $CC+S_H$ (+M) (based on the last HRT) were slightly lower with increased straw usage (Table 2), accompanied with higher RMP and less VS reduction, as barley straw is fibrous and resistant to anaerobic degradation. In terms of CoM-AD digesters, SMYs

364 measured from digesters CC+S_L+M and CC+S_H+M were very similar (244.1 and 238.4 mL CH_4
365 g^{-1} VS), while the RMPs were determined to be 23.0 % and 32.4%, respectively, corresponding
366 to VS reduction of 44.7 and 29.4%. The anaerobic degradation rate (VS reduction) was lower
367 from digester CC+S_H+M (even though there was no blockage risk observed) than other CoM-AD
368 digesters. Thus, feeding of co-ensiling mixtures, *i.e.* CC+S_L, under CoM-AD systems is more
369 feasible to CSTR reactors.
370 3.4.3 Influence on microbial community
371 In general, co-digestion of crops and animal manure appeared to cause an overall decrease in
372 diversity and evenness of reactor communities, which was more pronounced in the case of the
373 methanogenic communities but a similar trend was also observed in bacterial communities.
374 Addition of straw might result in a more diverse community as the digester CC+S_H led to the
375 highest diversity among all digesters (Fig.2). This effect was also clear in the detailed
376 community structure (Fig. 3), considering the increase of the relative abundance of typical
377 cellulolytic taxa, such as *Clostridia, Bacilli, Anaerolineae* and *Fibrobacteria,* in straw digesting
378 reactors, which was not apparent when manure was supplemented. Manure addition in fact
379 reduced the relative contribution of lignocellulosic straw biomass, influenced the C:N of the
380 complex feedstock, TEs concentrations and buffer capacity. On the other hand, feeding of cover
381 crop has low impact on the microbial communities, as the compositions of cover crop and cover
382 crop plus manure are relatively similar (Fig.5).
383 Additionally, microbial community profiles from cattle manure were also different compared to
384 the digesters or inoculum, indicating that the manure may have been the origin of microbes
385 which cannot be well established in the digesters even under regularly feeding. Beside that, we
386 also observed lower microbial community diversity from CoM-AD digesters than that of Crop-

AD digesters. This is not in agreement with the results of Zealand et al. (2018) who found the addition of manure contributed to an increased diversity providing additional and varied microbes to the system (El-Mashad and Zhang, 2010). To further investigate this phenomenon, the numbers of unique, shared and core ASVs (representing unique bacterial taxa) in the digesters CC, CC+S_H, and CC+S_H+M were plotted against the control digester (Cont) fed with manure, with the original inoculum (Inoc), and with untreated cattle manure (M) used as a substrate, and between each other (Fig.6). Digesters CC and CC+S_H shared only 1 and 2 ASVs with manure, respectively. The pilot-scale biogas digester that were used as a source of inoculum for all investigated reactors were also partially fed with cattle manure, therefore indirect influence of the manure microbiota was expected even in Crop-AD reactors. The number of shared ASVs between CC+S_H+M and M were relatively high (23 ASVs) because of the continuous supplement of cattle manure, and even higher in case of the control reactor (Cont) fed solely with manure (63 ASVs). Comparison between the digesters CC, CC+S_H, CC+S_H+M were completed as well (Fig.6d), with the observation of 46 ASVs shared between all three digesters and 65-75 ASVs shared between each two digesters.

To summarize, it seems that the communities from Crop-AD digesters adapted to the fibrous feedings (especially the digesters fed with high share of barley straw) and, therefore, formed the most distinct and diverse microbial communities. Meanwhile, digesters with addition of cattle manure have lower diversity than that from the Crop-AD digesters, which was unexpected. This is likely due to that most of the manure-originated microorganisms are mesophilic, which do not belong to the core biogas microbiota at thermophilic temperature, and, as a result, are mainly washed out instead of becoming established. Another assumption is that feed containing a high share of straw could act as bio-carrier the anaerobic digester to enrich the microbes in a positive

410 way. Tsapekos et al. (2017) reported clear differences in microbial community compositions
411 between the microbes firmly attached to solid fraction of digested grass and planktonic microbes
412 floating freely in the liquid medium within the same reactor.
413 3.5 Economic perspective related to harvest strategies.
414 The cover crop used in this study was collected from a field in which an experiment was
415 established in 2017 in order to assess the effect the main crop harvest time and cutting height on
416 variation of cover crop yields. The experiment was repeated in two years and therefore energy
417 yields were calculated based on the cover crop yields obtained over the two years (Table 5).
418 The total energy output for the only harvest of cover crop ranged between 319-585 Nm^3 CH_4 ha^{-1}
419 for both Crop-AD and CoM-AD systems. Addition of straw at a level of 3:1 or 10:$1_{w:w}$ ($CC+S_H$
420 and $CC+S_L$ in response to the semi-continuous digestion test) increases the amount of harvested
421 total VS by up to 3.4-6.1 tons ha^{-1}, depending on the cover crop yields. In consequence, the total
422 energy output reached 440-800 Nm^3 CH_4 ha^{-1} in the mixture $CC+S_L$ (10:$1_{w:w}$) and 686-1232 Nm^3
423 CH_4 ha^{-1} in the mixture $CC+S_H$ (3:$1_{w:w}$). Those results are within the range of 486-702 m^3 CH_4
424 ha^{-1} found by Molinuevo-Salces et al (2013) for cover crop and straw blends. Compared to
425 harvest of cover crop and straw separately, the available straw yields were reduced by 41% (%
426 VS) under simultaneous harvest, which was probably because of leaching of soluble compounds
427 (Collins et al, 1990) during the three months that separated a normal harvest of straw (summer)
428 and the late harvest of straw/cover crop (autumn). Harvest of straw during summer could
429 increase the VS conservation and therefore enhance the total methane production per hectare.
430 However, the higher residual CH_4 yield (32% of the total CH_4 potential) suggests that a longer
431 retention time (more than 25 days) would be necessary for treating co-ensiled mixtures with a
432 high share of straw. It should be noted that all comparisons in this part are made only according

to CH_4 yield obtained in the semi-continuous experiment and total biomass yield based on a two-year field experiment. More information regarding energy input/requirement in response to two harvest strategies, including the energy consumptions of straw harvest, baling, transportation, ensiling, mixing, are still required to finalize the evaluation between the two methods in practice.

4. Conclusion

The present study demonstrated that harvested cover crop (conservation as silage) is feasible for anaerobic digestion alone or together with either straw, manure or both. Co-digestion with manure with less straw addition was recommended as it has relatively higher CH_4 yield, VS removal and buffer capacities within the reactor. Microbial community compositions were affected by the feeding compositions, while a high share of straw altered the bacterial community structure most. Further investigations into optimization of anaerobic digestion adapted to high straw addition and monitoring over a long time span will be necessary.

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Figures and Tables

Figures

Figure 1. Schematic diagram of the experiment. (Crop-AD, anaerobic digestion of co-ensiled crops; CoM-AD, co-digestion of crops with manure; Cont, mono-digestion of cattle manure)

Figure 2. Alpha diversity of the bacterial and methanogenic communities based on the number of observed unique amplicon sequence variants (ASVs) of the (a) 16S rRNA (in duplicates) and (b) mcrA gene amplicon sequence libraries and by the Simpson index. Values calculated from all randomly subsampled (without replacement) libraries down to the lowest number of sequences per sample. (Crop-AD, anaerobic digestion of co-ensiled crops; CoM-AD, co-digestion of crops with manure; Cont, mono-digestion of cattle manure; CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with addition of cattle manure)

Figure 3. The relative abundances of selected bacterial classes dominant in the investigated reactor systems. Taxonomic affiliation was based on 16S rRNA gene amplicon sequences. (CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; Cont, mono-digestion of cattle manure; M, cattle manure; +M, co-digestion with cattle manure; _A/B, technical replicates from the same reactor).

Figure 4. Relative abundances of the methanogenic genera detected in the microbial communities based on the sequences of the mcrA gene amplicons. (CC, Cover crop; CC+SH, Cover crop with high straw addition; CC+SL, Cover crop with low straw addition; Cont, mono-digestion of cattle manure; M, cattle manure; +M, co-digestion with addition of cattle manure) .

Figure 5. Nonmetric multidimensional scaling analysis plot (NMDS) of the bacterial communities from various reactors, the inoculum, and manure (Samples). The results were

based on the amplicon sequencing data of the 16SrRNA genes using Bray-Curtis dissimilarity index. The taxa correlating with the community differences (at phylum level) are also shown in the right plot (Taxa). (Crop-AD, anaerobic digestion of co-ensiled crops; CoM-AD, co-digestion of crops with manure; Cont, mono-digestion of cattle manure ; CC, Cover crop; CC+SH, Cover crop with high straw addition; CC+SL, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with addition of cattle manure; The data refer to the same digester was the technical replicates sourced from the same reactor).

Figure 6. Venn diagram of unique, shared, and core ASVs of the bacterial communities. The inoculum (Inoc), cattle manure (M), and control reactor fed with cattle manure only (Cont) is compared to samples from reactor fed only with cover crop (CC), with cover crop and straw (CC+SH), or with cover crop and straw supplemented with cattle manure (CC+SH+M).

725

726 Table 1. Characteristics of cover crop silage, co-ensiled mixtures, cattle manure and inoculum

Digesters[a]	Cellulose (% of TS)	Hemicellulose (% of TS)	Lignin (% of TS)	C:N	Proportion (% of FM)			Proportion (% of VS)		
					CC	S	M	CC	S	M
CC	24.3	13.1	5.9	10.4	100	0	0	100	0	0
CC+S_L	31.4	20.5	7.4	16.4	90.9	9.1	0	62.8	37.2	0
CC+S_H	35.1	28.0	9.5	24.9	75	25	0	33.6	66.4	0
M	21.6	22.5	7.8	13.9	0	0	100	0	0	100
CC	23.9	14.5	6.2	10.8	62.6	0	37.4	85.6	14.4	0
CC+S_L+M	28.3	21.1	7.5	15.5	27.7	2.8	69.6	43.7	25.9	30.4
CC+S_H+M	30.1	26.0	8.9	19.4	15	5	80	21.7	42.8	35.5

727 a. CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M, cattle manure; +M, co-
728 digestion with addition of cattle manure.

729 Table 2. CH_4 yield, residual CH_4 potential, and characteristics after anaerobic digestion.
730

Parameters	Crop-AD[b]			CoM-AD[b]			Cont[b]
	CC	CC+S_L	CC+S_H	CC+M	CC+S_L+M	CC+S_H+M	
SMY[a] (mL $CH_4.g^{-1}$ VS)	266.0	219.9	202.0	268.9	244.1	238.4	212.2
RMP (mL $CH_4.g^{-1}$ VS)	115.7	145.3	165.5	105.8	131.6	161.6	110.5
Residual CH_4 (%)[c]	16.8	29.1	34.4	16.5	23.0	32.4	24.3
Effluent TS (%)	5.39	5.12	5.67	5.85	5.47	6.54	6.20
Effluent VS (%)	4.30	4.38	4.97	4.57	4.43	5.56	4.30
VFA (mg L^{-1})	919.8	2310.0	1417.8	1604.3	1461.0	1720.4	1632.5
TKN (g L^{-1})	3.1	1.8	1.4	3.4	2.7	2.5	4.2
NH_4-N (g L^{-1})	1.34	0.60	0.35	1.64	1.44	1.17	2.66
pH	7.80	7.58	7.49	7.95	7.95	7.81	8.13
TA (mg $CaCO_3$ L^{-1})	10448.8	4896.6	4211.8	10721.4	9514.4	7835.0	14547.3
PA (mg $CaCO_3$ L^{-1})	8014.6	3098.5	2724.1	7611.9	6932.7	5600.0	10676.6
IA (mg $CaCO_3$ L^{-1})	2434.1	1798.0	1487.7	3109.5	2581.7	2235.0	3870.6
Cellulose (% TS)	24.57	34.64	30.62	27.20	27.37	33.49	16.77
Hemi-cellulose (% TS)	13.12	23.45	20.99	15.23	19.86	20.92	16.07
Lignin (% TS)	20.89	15.66	16.70	18.85	17.12	16.49	11.79

731 a. Calculated based on the data acquired at the last HRT
732 b. Digesters: Crop-AD, anaerobic digestion of co-ensiled crops; CoM-AD, co-digestion of crops with manure; Cont, mono-digestion of cattle manure ; CC, Cover crop;
733 CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with addition of cattle manure.
734
735

736 Table 3. Volatile solids and fiber (cellulose, hemi-cellulose, lignin) reduction.
737

Parameters[a]	Crop-AD[b]			CoM-AD[b]			Cont[b]
	CC	CC+S_L[c]	CC+S_H[c]	CC+M	CC+S_L+M	CC+S_H+M	
VS reduction (%)	53.5	37.9	36.1	49.7	44.7	29.4	38.4
Cellulose Reduction (%)	47.0	25.9	40.5	34.2	39.9	15.7	43.2
Hemi-cellulose reduction (%)	47.5	23.1	49.0	39.2	41.5	39.0	47.6
Lignin reduction (%)	-85.3	-42.4	-19.4	-76.2	-41.7	-40.8	-11.2

738 a. Based on the digestate collected at the end of continuous test.
739 b. Crop-AD, anaerobic digestion of co-ensiled crops; CoM-AD, co-digestion of crops with manure; Cont, mono-digestion of
740 cattle manure ; CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M,
741 cattle manure; +M, co-digestion with addition of cattle manure.
742 c. Undigested fibers were partly removed from digesters CC+S_L and CC+S_H.
743

744

745 Table 4. Fe, Ni and Co concentrations from the feeding of Crop-/CoM-AD and the value

746 recommended by Takashima et al. (1990).

Elements	Unit	Crop-AD[a]			CoM-AD[a]			Cont[a]	Optimum concentration (Takashima et al., 1990)
		CC	CC+S_L	CC+S_H	CC+M	CC+S_L+M	CC+S_H+M		
Iron (Fe)	($g.m^{-3}$)	0.87	0.51	0.39	1.8	1.9	2.6	6.3	0.28-50.4
Nickle (Ni)	($mg.m^{-3}$)	11.9	7.5	6	14.2	10.7	13.3	22.5	12-5000
Cobolt (Co)	($mg.m^{-3}$)	2.5	1.7	2	2.5	2	2.5	3.3	5.9-120

747 a. Crop-AD, anaerobic digestion of co-ensiled crops; CoM-AD, co-digestion of crops with manure; Cont, mono-digestion of
748 cattle manure ; CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M,
749 cattle manure; +M, co-digestion with addition of cattle manure.
750

751 Table 5. Energy output generated by anaerobic digestion of cover crop and straw harvested separately or simultaneously.

Types of digestion	Strategy	Barley straw addition	SMY_{silage}[b] (N mL $CH_4.g^{-1}VS$)	Biomass yield (ton VS. ha^{-1})	Reduction biomass yield between 2 harvest strategies (%)	Total Energy output (Nm^3 CH_4 ha^{-1})	Reduction energy output between 2 harvest strategies (%)
Crop-AD[a]	Cover crop	-	266	1.2-2.2		319-585	
	Harvest separately	High	202	3.4-6.1	41	686-1232	44
	Harvest together	Low	220	2.0-3.6		440-792	
CoM-AD[a]	Cover crop	-	269	1.2-2.2		322-592	
	Harvest separately	High	238	3.4-6.1	41	809-1452	44
	Harvest together	Low	244	2.0-3.6		488-878	

752 a. Crop-AD, anaerobic digestion of co-ensiled crops; CoM-AD, co-digestion of crops with manure.
753 b, SMY observed from either Crop-AD or CoM-AD digesters.

Figure 1

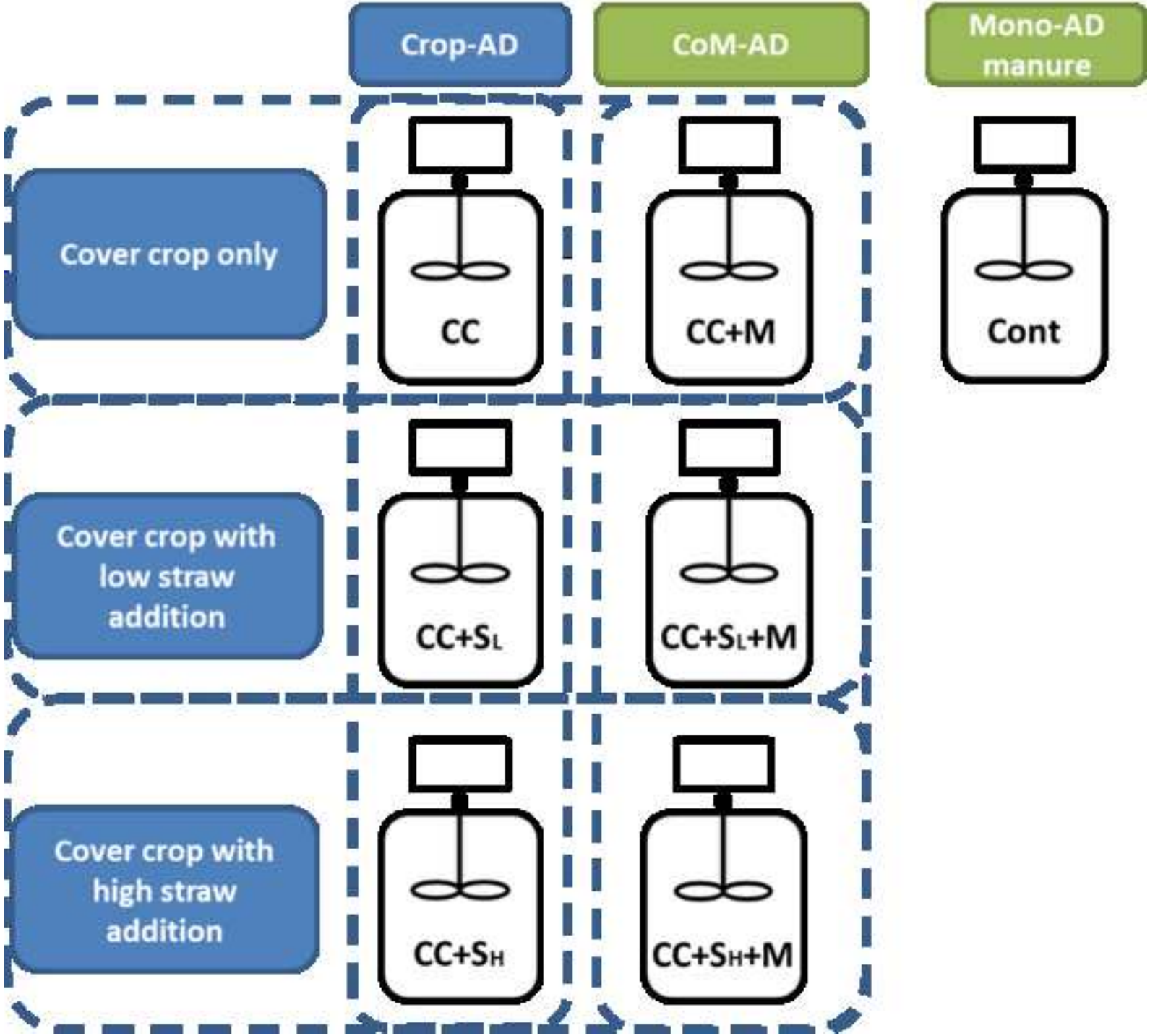

Crop-AD
CoM-AD
Mono-AD manure
Cover crop only
Cover crop with low straw addition
Cover crop with high straw addition
CC
CC+M
Cont
CC+SL
CC+SL+M
CC+SH
CC+SH+M

Figure 2A

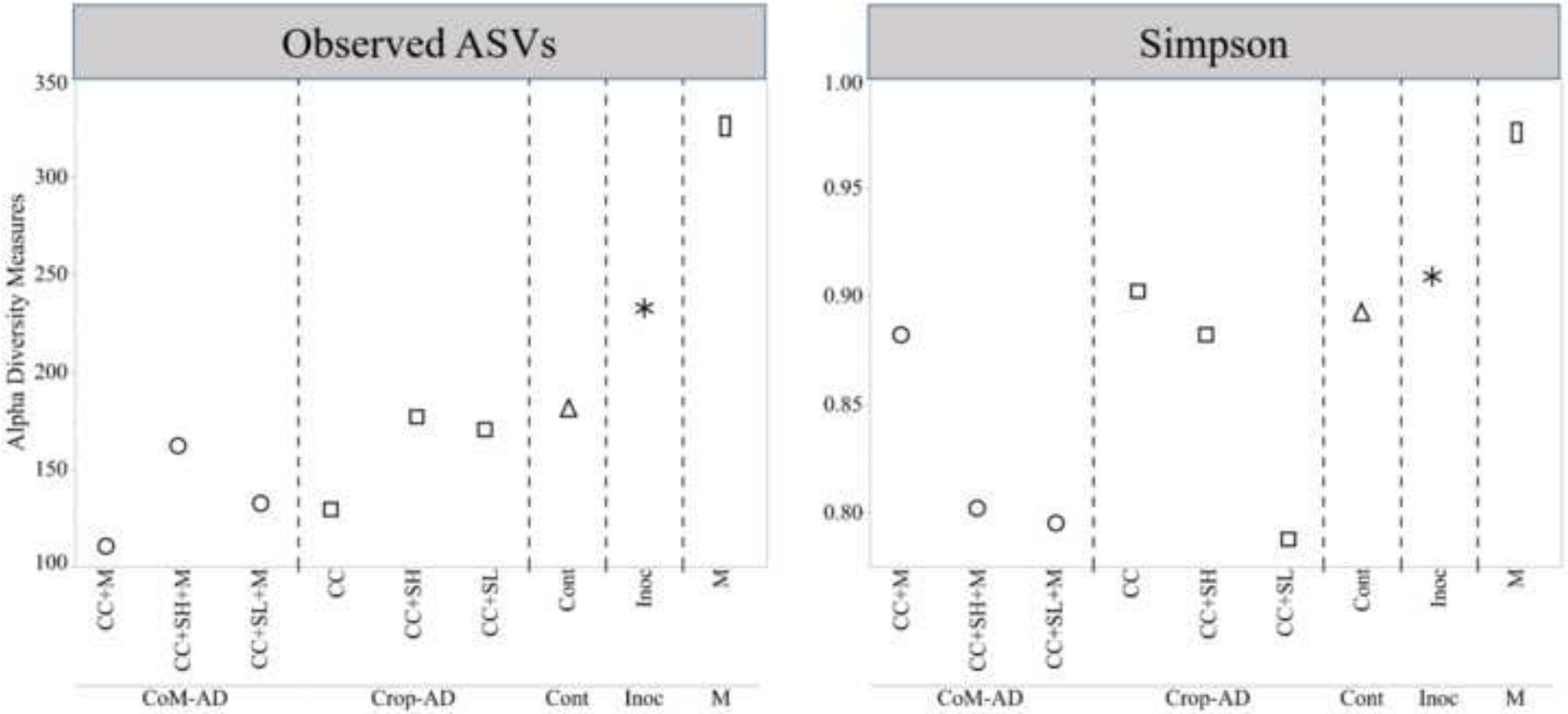

Observed ASVs
Simpson
Alpha Diversity Measures
350
300
250
200
150
100
1.00
0.95
0.90
0.85
0.80
CC+M
CC+SH+M
CC+SL+M
CC
CC+SH
CC+SL
Cont
Inoc
M
CoM-AD
Crop-AD
Cont
Inoc
M

Figure 2B

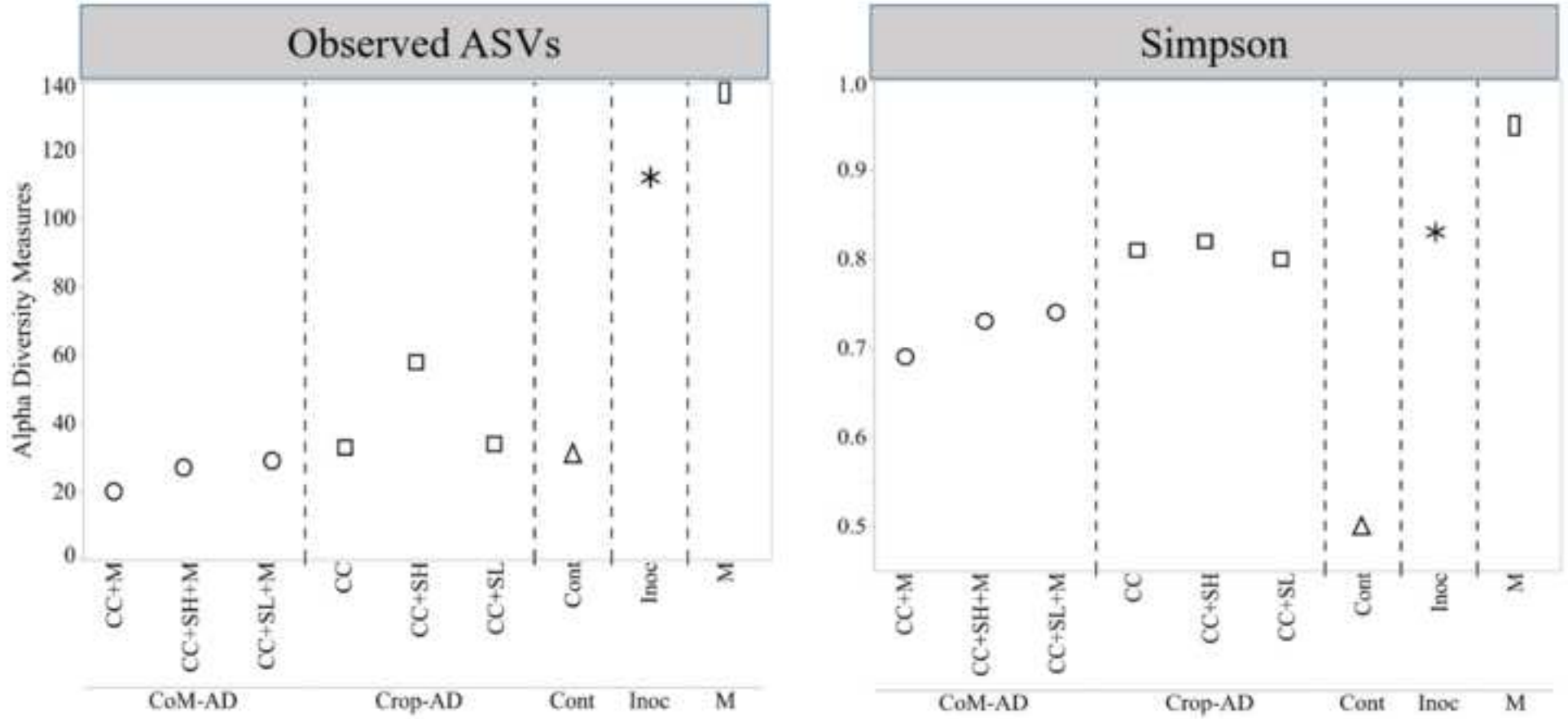

Observed ASVs
Alpha Diversity Measures
140
120
100
80
60
40
20
0
CC+M
CC+SH+M
CC+SL+M
CC
CC+SH
CC+SL
Cont
Inoc
M
CoM-AD
Crop-AD
Cont
Inoc
M
Simpson
1.0
0.9
0.8
0.7
0.6
0.5
CC+M
CC+SH+M
CC+SL+M
CC
CC+SH
CC+SL
Cont
Inoc
M
CoM-AD
Crop-AD
Cont
Inoc
M

Figure 3

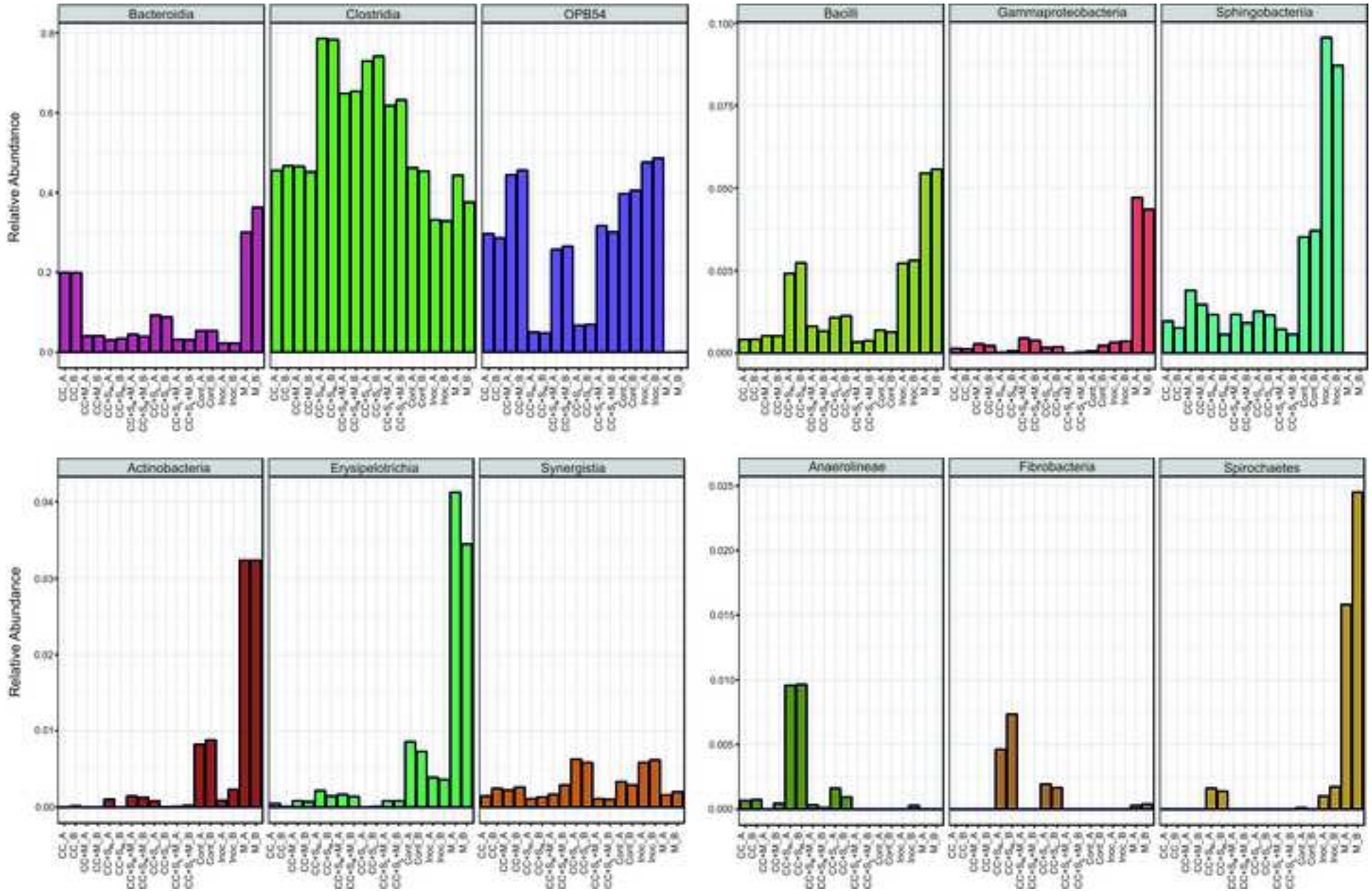

Bacteroidia
Clostridia
OPB54
Bacilli
Gammaproteobacteria
Sphingobacteria
Actinobacteria
Erysipelotrichia
Synergistia
Anaerolineae
Fibrobacteria
Spirochaetes
Relative Abundance
Relative Abundance

Figure 4

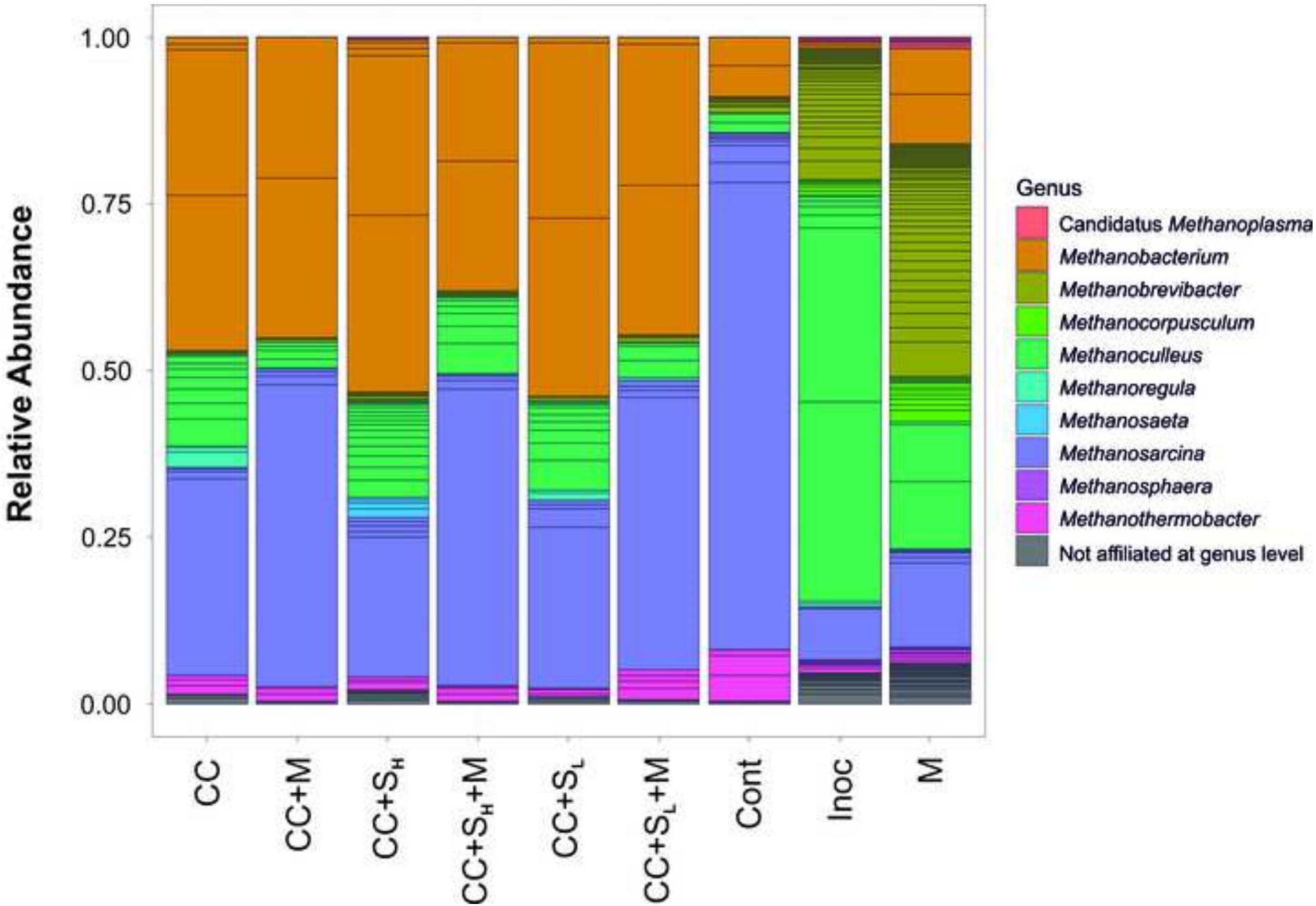
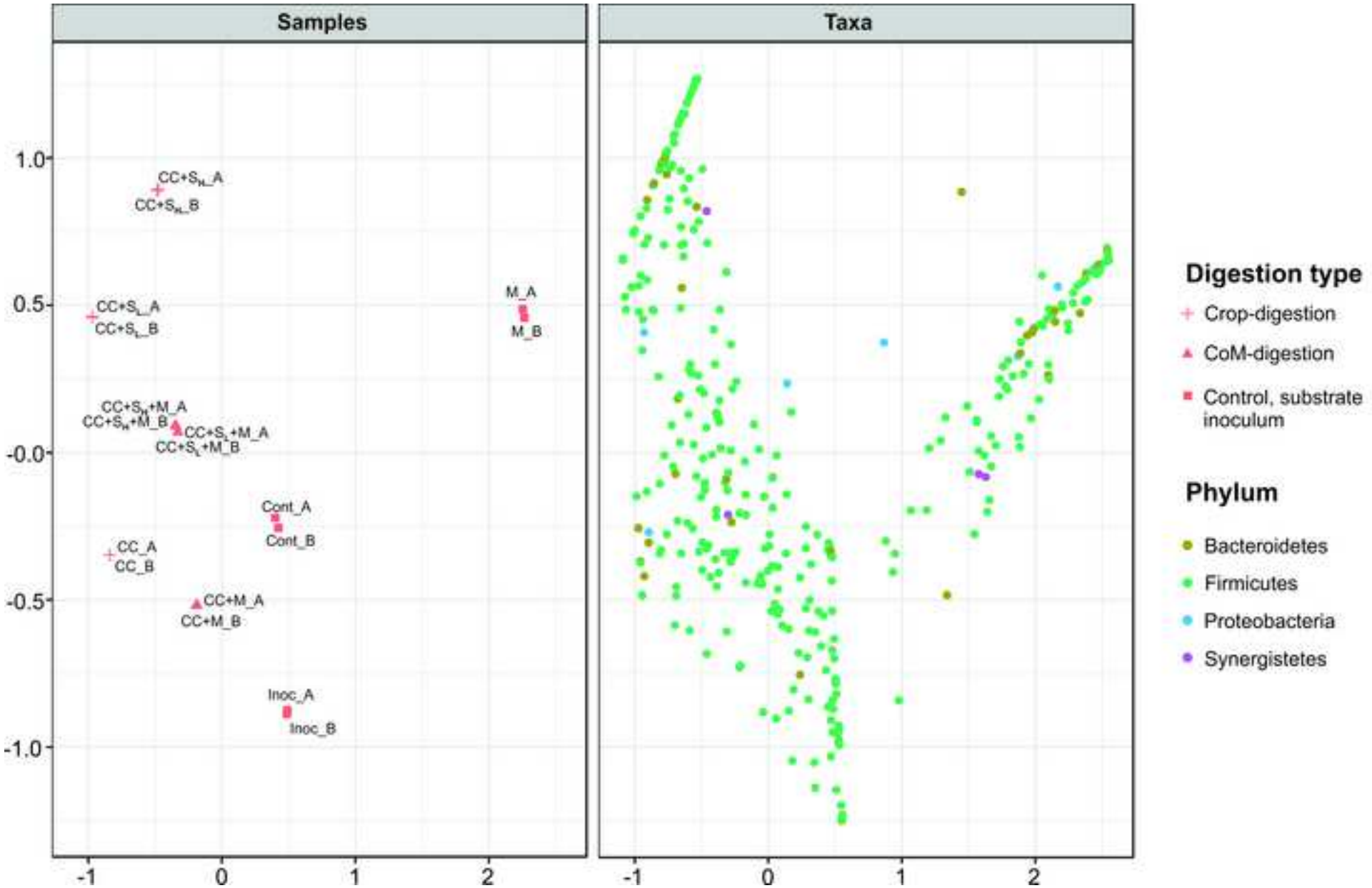

Relative Abundance
1.00
0.75
0.50
0.25
0.00
CC
CC+M
CC+S$_H$
CC+S$_H$+M
CC+S$_L$
CC+S$_L$+M
Cont
Inoc
M
Genus
Candidatus *Methanoplasma*
Methanobacterium
Methanobrevibacter
Methanocorpusculum
Methanoculleus
Methanoregula
Methanosaeta
Methanosarcina
Methanosphaera
Methanothermobacter
Not affiliated at genus level

Figure 5


Samples
Taxa
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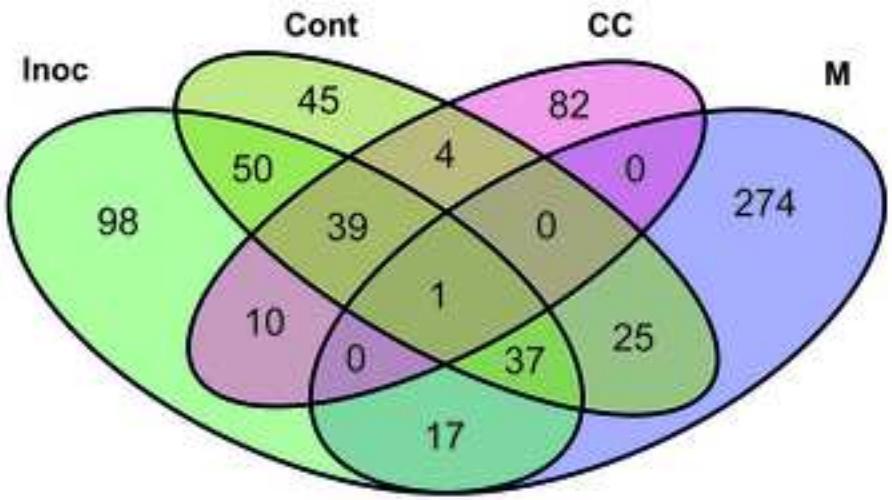
-0.5
-1.0
-1
0
1
2
CC+S_N_A
CC+S_N_B
CC+S_L_A
CC+S_L_B
M_A
M_B
CC+S_N+M_A
CC+S_N+M_B
CC+S_L+M_A
CC+S_L+M_B
Cont_A
Cont_B
CC_A
CC_B
CC+M_A
CC+M_B
Inoc_A
Inoc_B
Digestion type
Crop-digestion
CoM-digestion
Control, substrate inoculum
Phylum
Bacteroidetes
Firmicutes
Proteobacteria
Synergistetes

Figure 6


Cont
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Inoc
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98
39
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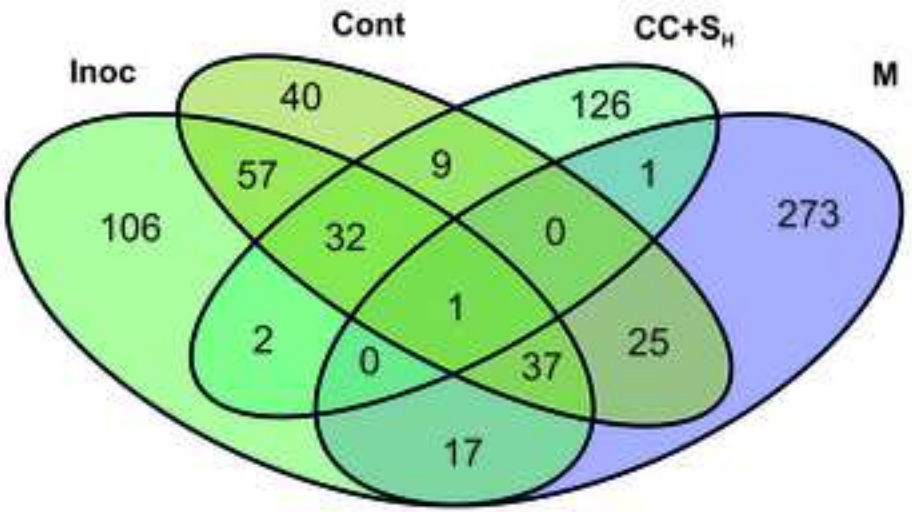
10
0
37
25
17


Cont
CC+S$_H$
Inoc
M
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9
1
106
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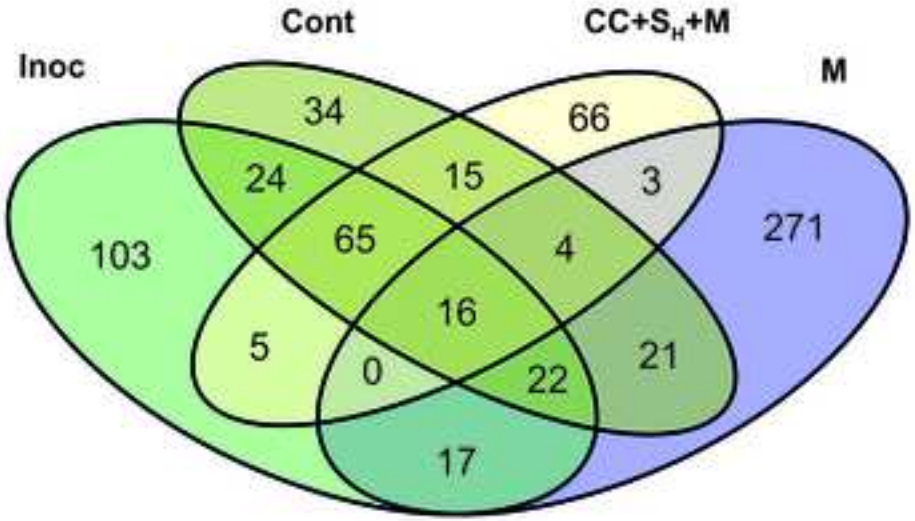
17


Cont
CC+S$_H$+M
Inoc
M
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66
24
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3
103
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4
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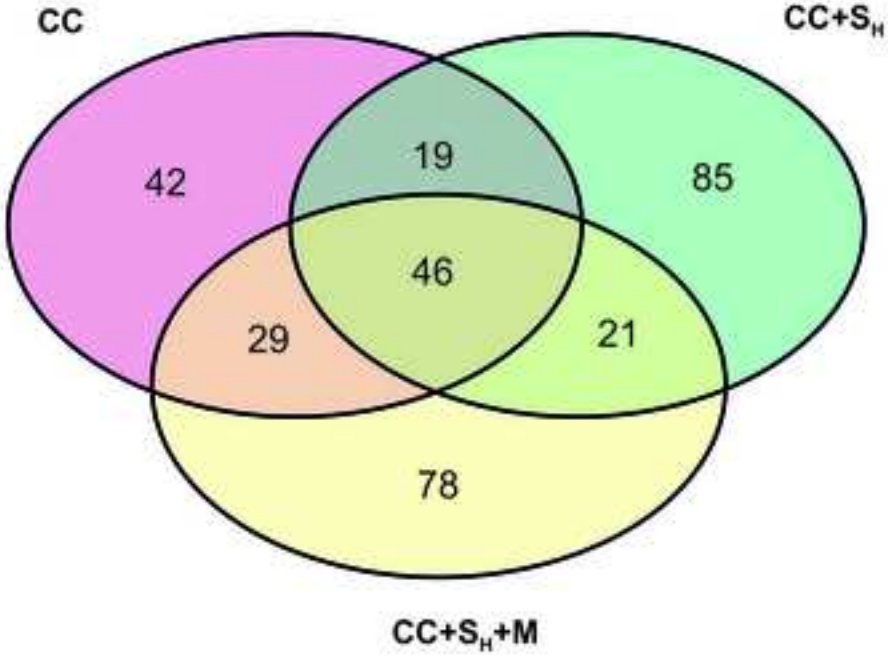
21
17


CC
CC+S$_H$
42
19
85
46
29
21
78
CC+S$_H$+M

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author contribution statement

Title

Anaerobic digestion of co-ensiled cover crop and barley straw: effect of co-ensiled ratios, manure addition and impact on microbial community structure

L.Feng, Y.M. Perschke, and D.Fontaine carried out the experiment (biogas part). L.Feng, M.Nicolausz carried out the microbial community analyses. L.Feng, Y.M.Perschke, D.Fontaine, J.Eriksen, P.Sørensen, H.B.Møller conceived and planned the experiment. L.Feng wrote the manuscript with support from Y.M. Perschke, D.Fontaine, M.Nicolausz, and A.J.Ward. M.Nicolausz, U.N. Rocha, F.B.Corrêa aided in analyzing the experimental data, drafting the figures, and worked on the manuscript (microbiology part). All authors discussed the results, commented, and revised the manuscript.

Supplemental information

Anaerobic digestion of co-ensiled cover crop and barley straw: effect of co-ensiled ratios, manure addition and impact on microbial community structure

Lu Feng[a,*], Yolanda Maria Lemes Perschke[a], Doline Fontaine[b], Marcell Nikolausz[c], Ulisses Nunes da Rocha[c], Felipe Borim Corrêa[c], Alastair James Ward[a], Jørgen Eriksen[b], Peter Sørensen[b], Henrik Bjarne Møller[a].

[a] Department of Engineering, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark.

[b] Department of Agroecology, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark

[c] Department of Environmental Microbiology, Helmholtz Centre for Environmental Research-UFZ, 04318 Leipzig, Germany

*Corresponding author: email: lufeng@eng.au.dk; Telephone: +45 91454277

Tables

Table S1. Characteristics of ensiled cover crop (CC), co-ensiled CC and barley straw, cattle manure, and inoculum.

Parameters	TS[a]	VS[a]	C	N	H	S	C:N	Crude Protein	Cellulose	Hemi-cellulose	Lignin
Unit	(%)	(%)	($\%_{TS}$)	($\%_{TS}$)	($\%_{TS}$)	($\%_{TS}$)		(%)	($\%_{TS}$)	($\%_{TS}$)	($\%_{TS}$)
Barley straw[b]	81.6	78.7	46.1	0.7	6.7	0.06	65.9	4.4	45.2	36.2	6.7
CC silage	13.8	12.4	43.5	4.2	6.4	0.20	10.4	26.0	24.3	13.1	5.9
$CC+S_L$	19.8	18.3	44.2	2.7	6.8	0.11	16.4	16.8	31.4	20.5	7.4
$CC+S_H$	27.2	25.4	44.9	1.8	6.7	0.08	25.1	11.3	35.1	28.0	9.5
M	8.47	6.99	43.1	3.1	7.3	0.6	13.9	19.4	21.6	22.5	7.8
Inoc	6.46	4.37	nd	nd	nd	nd	nd	nd	nd	nd	nd

a. The TS/VS was the original data from raw silages.
b. The data from barley straw was listed as reference data. There is no raw straw used in this study.
ND. Not detected
CC, Cover crop; $CC+S_H$, Cover crop with high straw addition; $CC+S_L$, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with cattle manure.

Table S2. Macro-, micro- or trace elements from AD effluent (end of the experiment) and inoculum

Element	Units	Crop-AD						CoM-AD						Cont		Ino
		CC		$CC+S_L$		$CC+S_H$		CC+M		$CC+S_L+M$		$CC+S_H+M$				
		In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	
Calcium (Ca)	($g.kg_{FM}^{-1}$)	0.84	1.17	0.45	0.74	0.39	0.35	0.92	1.31	0.76	0.91	0.71	0.82	1.25	1.24	1.16
Potassium (K)		2.08	2.73	1.12	1.28	0.96	0.82	2.30	2.75	1.91	2.48	1.80	1.44	3.19	3.20	3.21
Magnesium (Mg)		0.15	0.17	0.08	0.10	0.06	0.08	0.20	0.23	0.19	0.21	0.19	0.20	0.41	0.59	0.58
Sodium (Na)		0.04	0.07	0.02	0.05	0.02	0.06	0.12	0.12	0.17	0.17	0.19	0.16	0.48	0.48	0.52
Phosphorus (P)		0.22	0.24	0.12	0.16	0.11	0.11	0.26	0.28	0.23	0.25	0.23	0.24	0.43	0.57	0.60
Sulfur (S)		0.12	0.12	0.07	0.09	0.06	0.06	0.17	0.19	0.18	0.14	0.19	0.14	0.41	0.32	0.27
Iron (Fe)	($mg.kg_{FM}^{-1}$)	10.38	70.7	6.11	23.9	5.91	19.5	21.59	69.2	28.85	33.7	31.52	57.1	75.5	76.9	98.6
Nickel (Ni)		0.14	2.42	0.09	1.38	0.09	1.38	0.17	1.37	0.16	0.55	0.16	4.32	0.27	0.58	0.24
Cobalt (Co)		0.03	0.04	0.02	0.02	0.03	0.02	0.03	0.05	0.03	0.03	0.03	0.11	0.04	0.05	0.05
Copper (Cu)		0.83	1.12	0.44	0.84	0.36	1.14	1.88	2.55	2.56	2.96	2.78	3.29	7.01	7.28	7.50
Zinc (Zn)		2.11	2.96	1.11	1.89	0.92	1.90	3.91	4.30	4.87	4.52	5.19	4.75	12.6	13.0	14.3
Total solid[a]	(%)		5.24		5.5		4.3		5.6		5.2		5.9		5.9	4.4

CC, Cover crop; $CC+S_H$, Cover crop with high straw addition; $CC+S_L$, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with cattle manure.

Table S3. Alpha diversity indexes calculated based on the ASVs obtained by amplicon sequencing the 16S rRNA and mcrA genes.

Digester	Observed	Chao1	ACE	Shannon	Simpson	InvSimpson	Fisher
Bacterial communities							
CC	130	129.75	129.70	3.10	0.90	10.23	18.90
CC+S_L	171	171.71	171.42	3.03	0.79	4.72	26.10
CC+S_H	177	177.50	177.58	3.47	0.88	8.49	27.31
CC+M	111	110.50	110.59	3.06	0.88	8.47	15.72
CC+S_L+M	133	132.66	132.91	2.62	0.80	4.88	19.42
CC+S_H+M	162	162.06	162.23	2.78	0.80	5.06	24.59
Cont	181	181.43	181.49	3.46	0.89	9.28	28.00
M	326	329.33	328.86	4.55	0.98	40.70	56.81
Inoc	233	233.95	234.20	3.49	0.91	10.97	37.70
Archaeal communities							
CC	33	33.0	33.56	2.10	0.81	5.13	3.97
CC+S_L	34	34.0	34.00	2.01	0.80	4.88	4.11
CC+S_H	58	58.0	58.47	2.38	0.82	5.68	7.57
CC+M	20	20.0	20.00	1.49	0.69	3.24	2.25
CC+S_L+M	29	29.0	29.00	1.74	0.74	3.79	3.43
CC+S_H+M	27	27.0	27.00	1.77	0.73	3.70	3.16
Cont	31	31.0	31.00	1.39	0.50	1.99	3.70
M	137	137.0	137.00	3.70	0.95	19.58	20.55
Inoc	112	114.5	114.03	2.73	0.83	5.98	16.23

CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with cattle manure.

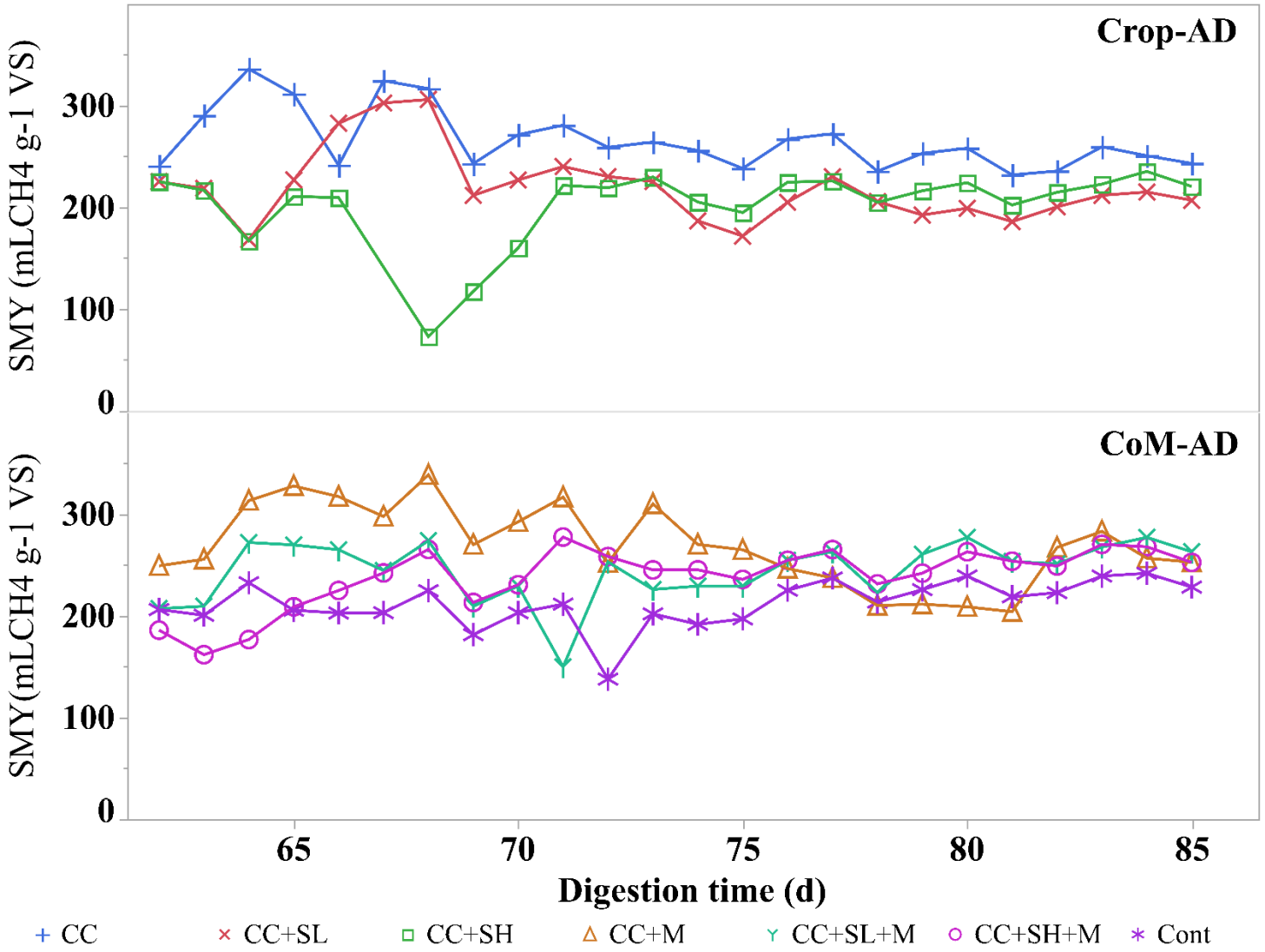
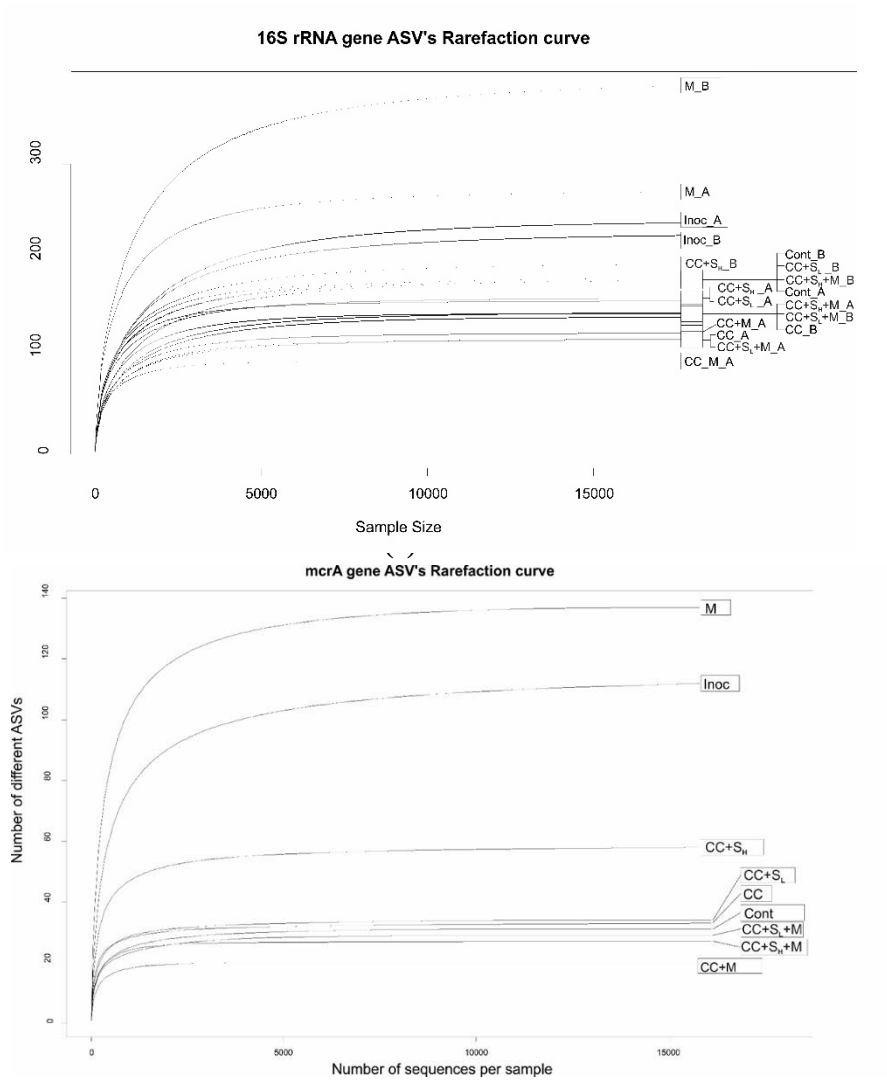


Figure S1. Specific CH_4 yield obtained from the last HRT. (CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with cattle manure)


16S rRNA gene ASV's Rarefaction curve
M_B
M_A
Inoc_A
Inoc_B
CC+S_H_B
Cont_B
CC+S_L_B
CC+S_L+M_B
CC+S_H_A
Cont_A
CC+S_L_A
CC+S_L+M_A
CC+S_L+M_B
CC+M_A
CC_B
CC_A
CC+S_L+M_A
CC_M_A
0
100
200
300
0
5000
10000
15000
Sample Size
mcrA gene ASV's Rarefaction curve
M
Inoc
CC+S_H
CC+S_L
CC
Cont
CC+S_L+M
CC+S_H+M
CC+M
Number of different ASVs
Number of sequences per sample

(b)

Figure S2 Rarefaction curves of the amplicon sequencing data of the (a) 16S rRNA and (b) mcrA genes from various reactors, inoculum, and cattle manure. (CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with cattle manure; _A/B, technical replicates from the same reactor)

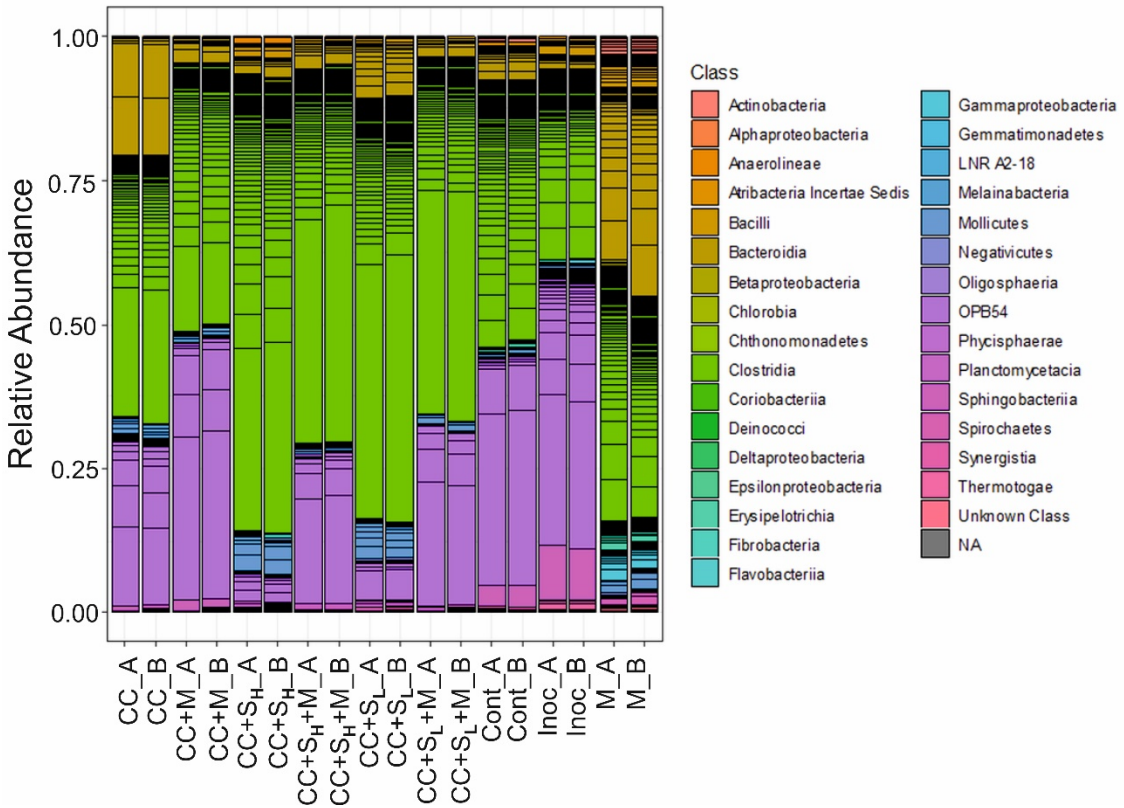


Figure S3. Relative abundances of the bacterial classes of the microbial communities based on the sequences of the 16S rRNA gene amplicons. . (CC, Cover crop; CC+S_H, Cover crop with high straw addition; CC+S_L, Cover crop with low straw addition; M, cattle manure; +M, co-digestion with cattle manure; _A/B, technical replicates from the same reactor)