



## Research paper

# Anaerobic co-digestion of grass and forbs – Influence of cattle manure or grass based inoculum



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## ARTICLE INFO

## Keywords:

Anaerobic co-digestion  
Biogas  
Forbs  
Inoculum source  
Multi-species grassland

## ABSTRACT

Anaerobic co-digestion of agricultural by-products or wastes with complementarity characteristics is commonly used to enhance methane yield. This study firstly explores the possibility of co-digesting grass and forb species (white clover, chicory and plantain) differing in nutrient composition in enhancing methane yield. This was examined with two inocula (a cattle manure-based inoculum and a grass-based inoculum) in a batch assay. Results showed that co-digesting grass and forbs synergistically enhanced methane yield potential on average by 31 L kg<sup>-1</sup> volatile solids (+11%) and reduced lag phase time by 0.8 day in the grass-based inoculum, but not in the cattle manure-based inoculum. Mixtures containing plantain showed more consistent synergistic effect than chicory. Synergistic effects were attributed to more balanced nutrient composition (especially C/N ratio) in grass-forb mixtures. We demonstrate that anaerobic co-digestion of grass and forbs is feasible for enhancing methane yield, which promotes the utilization of multi-species grasslands for bioenergy production.

## 1. Introduction

Biogas produced through anaerobic digestion of agricultural by-products, wastes or energy crops plays an important role in providing renewable bioenergy [1,2]. Biogas production can be enhanced through mixing two or more substrates with complementary characteristics because the anaerobic co-digestion process facilitates a better nutritional balance (e.g. N) and/or decreases the probability of inhibition (e.g. ammonia), leading to improved efficiency of microorganisms involved in anaerobic digestion [3,4].

Animal manure is a commonly used substrate co-digested with agricultural residues and energy crops to enhance biogas production and improve fertiliser values of digestates [5,6]. That is because animal manure contains higher nutrient concentrations (e.g. N) can facilitate the degradation of nutrient-poor agricultural residues. However, the production and pre-storage of animal manure are often associated with high risks of environmental pollution, such as greenhouse gas emissions (e.g. methane) and nutrient losses (e.g. ammonia) [7,8]. Hence, search for alternative and sustainable feedstock to animal manure has been put on the research agenda.

There is an increasing interest in using grassland biomass for bioenergy production in Europe and North America [9,10]. Current studies have been mainly focusing on different grass species or varieties [11,12]. Yet, the reported biogas production from grass species is

generally low and varies widely among various energy crops [1,10], probably because grasses alone contain relatively lower and unbalanced nutrients.

Recent studies show that some deep-rooting forbs species (e.g. chicory) are rich in some macro- and micro-nutrients (potassium, sulphur, zinc and boron) due to their uptake from deeper soil layers [13], while legume species (e.g. white clover) are well known to be rich in nitrogen (N) in plant tissues. These forbs have recently shown notable biogas yields [14,15]. Hence, co-digesting grass and forbs with complementary nutrient composition could have a higher probability of synergistically enhancing methane production (i.e. higher methane production in mixtures than the sum of methane production from individual species digested separately). In addition, this positive effect would be more likely to occur in nutrient-poor inoculums produced from grass species than nutrient-rich inoculums from animal manure. That is because grass-forb mixtures provide nutrient composition that is more balanced than with single species, thereby compensating the possible nutrient limitation for microbial growth in nutrient-poor inoculum.

This study is to examine (1) how co-digesting grass and forbs in different substrate composition influences methane production potential and other kinetic parameters either synergistically, antagonistically, or neutrally; and (2) whether these effects depend on inoculum source. Two sources of inocula were used: one was produced based on

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commonly used cattle manure and agricultural residues (hereafter called as “Manure inoculum”); the other was produced using organically managed grass ley (“Grass inoculum”). To address these questions, the batch test was employed because it enables us to characterise the kinetics of specific substrates under relative stable conditions (e.g. constant temperature) during anaerobic digestion. We hypothesized that synergistic effects occur in the Grass inoculum, but neutral effects occur in the Manure inoculum.

## 2. Materials and methods

### 2.1. Substrates

The substrates were from an existing field experiment established in spring 2014 at the Foulumgaard Experimental Station, Aarhus University, Denmark (56°29'44 N, 9°34'3 E). The experimental site had a mean annual rainfall of 770 mm and mean annual temperature of 7.7 °C. The soil is classified as a Typical Hapludult with 6.4% clay, 8.5% silt, 44% fine sand, 39% coarse sand and 2% organic matter. The experiment followed organically managed practices and hence no fertiliser and pesticide were applied. To address the objectives of this study, pure stands of ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.), chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.) were selected. Each pure stand had two field replicates. Plants were harvested four times (May, July, August and October, respectively) by cutting the herbage at 7 cm stubble height using a Haldrup plot harvester (Haldrup C-85, Denmark). In August 2016, the harvested samples from each of the four pure stands were mixed across replicates. A subsample (ca. 1 kg) was taken from each plant species, with unsown species removed. The subsample was then chopped into 1–2 cm size manually and stored at –20 °C until further analysis.

### 2.2. Inoculum preparation

Two sources of inocula were tested in this study, and prepared at the full-scale biogas plant in Research Centre Foulum, Aarhus University, Denmark. One is the Manure inoculum, collected from a full-scale thermophilic 1200 m<sup>3</sup> reactor (53 °C), and fed by 74% cattle manure, 8% deep litter, 8% maize, 8% grass and 2% silage for more than one year. This full-scale thermophilic digester was controlled at organic loading rate (OLR) of approximately 9 kgVS m<sup>-3</sup> d<sup>-1</sup> and HRT was 14 days. The other is the Grass inoculum, collected from a 100 L thermophilic pilot reactor which fed by 100% grass ley (tall fescue) for more than 3 months. The OLR and hydraulic retention time (HRT) for this pilot-scale reactor was 3 kgVS m<sup>-3</sup> d<sup>-1</sup> and 20 days, respectively. The grass ley was established in spring in 2014 and managed with a two-cut strategy without fertiliser and pesticides. In October 2016, the grass ley was harvested and ensiled. The two inocula were filtered using manual sieve and stored for 2 weeks at 53 °C to minimise the residual biogas production and adapted them to batch conditions.

### 2.3. Anaerobic batch experiment

The methane potential of the substrates was analysed by a biogas potential (BMP) test according to the approach described by Møller et al. [16]. Samples from the four species were used to construct 13 substrate compositions, comprising four single species, and nine mixed substrates varying with the forb species and mixture ratios of plant species on a basis of volatile solid (VS) (Table 1). Substrate composition represents biomass proportions of different species commonly observed in agricultural grasslands with different management practices.

The batch experiment was arranged in a factorial design with the 13 substrates and the two inocula as two factors. In addition, two blank controls with only the inoculum (Manure or Grass) were included. All the 28 treatments were examined in triplicate. The batch experiment

**Table 1**  
Species composition of the 13 substrates used for the batch experiment.

Substrates	Substrate composition (% of VS)			
	Ryegrass (G)	White clover (W)	Chicory (C)	Plantain (P)
100G	100			
100W		100		
100C			100	
100P				100
30G-50W-20C	30	50	20	
50G-30W-20C	50	30	20	
30G-30W-40C	30	30	40	
30G-50W-20P	30	50		20
50G-30W-20P	50	30		20
30G-30W-40P	30	30		40
30G-50W-10C-10P	30	50	10	10
50G-30W-10C-10P	50	30	10	10
30G-30W-20C-20P	30	30	20	20

G, ryegrass; W, white clover; C, chicory; P, plantain.

was conducted at thermophilic conditions (53 ± 1 °C) for 96 days. In brief, 5 g VS (about 220–250 g) of inoculum was added in each 500 mL infusion bottle, followed by adding 5 g (VS) substrate, resulting in a ratio of 1:1 (VS<sub>inoculum</sub>: VS<sub>substrate</sub>). The biogas volume was measured nine times at days 2, 5, 7, 12, 16, 26, 37, 61, 96, respectively, by inserting a needle connected to a tube with inlet to a column filled with acidified water (pH < 2) through the butyl rubber. The biogas produced was calculated by recording the volume of water displaced until the pressure between column and bottle headspace was equal. Biogas volume was normalised to standard conditions (0 °C and 1.013 bar). Methane produced from each sample was calculated by multiplying biogas volume with methane fraction, and corrected by subtracting the volume of methane produced from the control.

To assess whether inhibition may occur in the batch experiment, the digestates of four single species in both inocula and two controls at the last measurement (Day 96) were characterised for its VS, pH, volatile fatty acids (VFA) and total ammonia N (TAN).

### 2.4. Analytical methods

Subsamples of four plant species and two inocula were dried at 60 °C for 48 h, and then ground < 0.5 mm (CT 193 Cyclotec™ Sample Mill, Denmark). The total solid (TS) and VS were analysed using the procedure by APHA [17]. Total carbon (C) and N concentrations were analysed using a LECO CNS-1000 analyser (LECO Corp. St. Joseph, MI). The analysis of macro- and micro-nutrient concentrations was performed with ICP-MS (Inductively Couple Plasma-Mass Spectrometry) using a NexION 300 (PerkinElmer, USA). The acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) of plant samples were determined using the Van Soest method [18]. Cellulose was calculated as the difference between ADF and ADL, and hemicellulose as the difference between NDF and ADF. Crude protein (CP) was calculated by multiplying N concentration with 6.25. All samples were analysed in duplicates, and the averaged values were presented.

Biogas composition was analysed by using gas chromatography (7890A, Agilent Technologies, USA) equipped with a thermal conductivity detector (TCD) and helium as the carrier gas. For digestates at the end of the experiment, pH value was measured using a Portamess 911 pH meter (Knick, Berlin, Germany). Dissolved VFA were determined using a gas chromatograph (Agilent technologies, CA 95051, USA) equipped with a flame ionization detector (FID) and helium as the carrier gas. The TAN was determined using photometric kits (Spectroquant® Test Kits, Merck, Germany). All samples were analysed

**Table 2**  
Chemical characteristics of four plant species.

Parameters	Units	Ryegrass	White clover	Chicory	Plantain
Total solid (TS)	% <sub>FM</sub>	20.0 ± 0.1	15.7 ± 0.2	14.8 ± 0.3	18.3 ± 0.1
Volatile solid (VS)	% <sub>FM</sub>	18.0 ± 0.1	14.0 ± 0.1	13.0 ± 0.3	16.5 ± 0.1
NDF	% <sub>VS</sub>	53.2 ± 0.6	30.9 ± 0.3	39.6 ± 0.5	50.8 ± 0.6
ADF	% <sub>VS</sub>	30.5 ± 0.8	23.0 ± 0.4	31.6 ± 0.5	39.1 ± 0.6
ADL	% <sub>VS</sub>	2.42 ± 0.09	3.06 ± 0.04	2.06 ± 0.14	6.04 ± 0.18
Hemicellulose	% <sub>VS</sub>	22.7 ± 0.22	7.88 ± 0.69	7.92 ± 0.00	11.7 ± 0.05
Cellulose	% <sub>VS</sub>	28.1 ± 0.7	19.9 ± 0.3	29.6 ± 0.4	33.1 ± 0.4
Crude protein	% <sub>VS</sub>	13.3 ± 0.2	26.7 ± 0.6	12.2 ± 0.7	10.9 ± 0.2
Carbon (C)	% <sub>FM</sub>	8.02 ± 0.04	6.23 ± 0.02	5.52 ± 0.01	7.29 ± 0.02
Nitrogen (N)	% <sub>FM</sub>	0.38 ± 0.00	0.60 ± 0.02	0.25 ± 0.02	0.29 ± 0.01
C/N	/	21.4 ± 0.19	10.5 ± 0.24	22.3 ± 0.82	25.4 ± 0.72
Phosphorus (P)	g kg <sub>FM</sub> <sup>-1</sup>	0.71 ± 0.01	0.42 ± 0.01	0.54 ± 0.01	0.57 ± 0.00
Potassium (K)	g kg <sub>FM</sub> <sup>-1</sup>	5.39 ± 0.33	3.79 ± 0.23	4.97 ± 0.38	4.32 ± 0.29
Calcium (Ca)	g kg <sub>FM</sub> <sup>-1</sup>	0.79 ± 0.04	1.89 ± 0.02	2.06 ± 0.13	1.78 ± 0.15
Magnesium (Mg)	g kg <sub>FM</sub> <sup>-1</sup>	0.28 ± 0.01	0.33 ± 0.01	0.27 ± 0.04	0.31 ± 0.02
Sulphur (S)	g kg <sub>FM</sub> <sup>-1</sup>	0.52 ± 0.01	0.34 ± 0.02	0.57 ± 0.01	0.59 ± 0.04
Sodium (Na)	g kg <sub>FM</sub> <sup>-1</sup>	0.17 ± 0.04	0.15 ± 0.05	0.28 ± 0.14	0.08 ± 0.01
Iron (Fe)	mg kg <sub>FM</sub> <sup>-1</sup>	35.86 ± 2.70	40.10 ± 6.30	30.30 ± 2.60	22.26 ± 0.83
Manganese (Mn)	mg kg <sub>FM</sub> <sup>-1</sup>	14.07 ± 1.71	8.22 ± 0.49	8.55 ± 0.39	4.14 ± 0.17
Boron (B)	mg kg <sub>FM</sub> <sup>-1</sup>	0.55 ± 0.00	2.46 ± 0.07	2.37 ± 0.00	1.81 ± 0.00
Zinc (Zn)	mg kg <sub>FM</sub> <sup>-1</sup>	4.13 ± 0.10	2.99 ± 0.09	4.60 ± 0.33	4.20 ± 0.45
Copper (Cu)	mg kg <sub>FM</sub> <sup>-1</sup>	1.08 ± 0.06	1.05 ± 0.01	1.31 ± 0.17	1.12 ± 0.03
Molybdenum (Mo)	mg kg <sub>FM</sub> <sup>-1</sup>	0.26 ± 0.04	0.13 ± 0.02	0.11 ± 0.01	0.07 ± 0.01
Cobalt (Co)	mg kg <sub>FM</sub> <sup>-1</sup>	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Nickel (Ni)	mg kg <sub>FM</sub> <sup>-1</sup>	0.87 ± 0.23	0.42 ± 0.13	0.31 ± 0.06	0.16 ± 0.02
Selenium (Se)	mg kg <sub>FM</sub> <sup>-1</sup>	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.05 ± 0.00

'±' Represents standard error of duplicates measurements.

based on one replicate.

## 2.5. Calculation methods and statistical analysis

### 2.5.1. Modified Gompertz model

The methane yield production during 96 days was modelled by fitting the experimental data to the modified Gompertz equation (Eq. (1)):

$$y = B_0 \exp \{-\exp [\mu_m e/B_0 (\lambda - t) + 1]\} \quad (1)$$

where  $y$  represents the cumulative methane yield (L kg<sup>-1</sup> VS<sup>-1</sup>) at time ( $t$ ) expressed in days;  $B_0$  is the cumulative methane yield (L kg<sup>-1</sup> VS<sup>-1</sup>) at the end of the batch (Day 96);  $\mu_m$  is the maximal methane production rate (L kg<sup>-1</sup> VS<sup>-1</sup> d<sup>-1</sup>), which is given by the slope of the line when methane is produced exponentially;  $e$  is the Euler's number, 2.718;  $\lambda$  is the lag phase time (d) [19].

The first-order kinetic model,  $y = B_0 [1 - \exp(-k t)]$ , was also used to fit the methane yield production. But given that the modified Gompertz equation generally provided a better goodness of fit than the first-order kinetic model, only the results derived from the modified Gompertz equation were reported.

The parameters  $\mu_m$  and  $\lambda$  were optimised by minimising the root-mean-squared error (RMSE). The coefficient of determination ( $R^2$ ) between predicted and observed methane yields was used to evaluate the precision of the model fit.

Two-way analysis of variance (ANOVA) was employed to analyse the effects of substrate composition and inoculum source on the parameters  $B_0$ ,  $\mu_m$  and  $\lambda$ . In case of significant main factor effect, differences between treatments were tested using Tukey's *post hoc* test. Data were log transformed where necessary for statistical analysis to meet the assumptions of normality and heterogeneity.

### 2.5.2. Estimation of co-digestion (synergistic or antagonistic) effect

Two linear models (i.e. the full model and the additive model) were established to examine the co-digestion effect on parameters  $B_0$ ,  $\mu_m$  and  $\lambda$ . The full model was constructed with all 13 substrate compositions, two inocula and their interactions as explanatory variables (Eq. (2)). The additive model consisted of only four plant species as explanatory

variables, assuming no interactive effects of mixing species on these parameters in nine mixed substrates (Eq. (3)).

$$Y_{\text{full model}} = (\beta_G P_G + \beta_W P_W + \beta_C P_C + \beta_P P_P + \beta_{j P_G P_W P_C} + \delta_k P_G P_W P_P + \delta_l P_G P_W P_C P_P) \gamma_m + \varepsilon \quad (2)$$

$$Y_{\text{additive model}} = (\beta_G P_G + \beta_W P_W + \beta_C P_C + \beta_P P_P) \gamma_m + \varepsilon \quad (3)$$

where coefficients  $\beta_i$  estimate the performance of species  $i$  (G: ryegrass, W: white clover, C: chicory, P: plantain).  $P_i$  is the proportion of species  $i$  in a mixture and if  $P_i = 1$ ,  $\beta_i$  measures the species' performance in a pure stand. Coefficients  $\delta_j$ ,  $\delta_k$  and  $\delta_l$  measure the interactions between ryegrass, white clover with one forb (chicory or plantain), or with both forbs, respectively. Coefficients  $\gamma_m$  estimate the effects of two inoculums (Manure and Grass).

The co-digestion effect was calculated as the difference between the outputs from the full model and the additive model. First, the  $F$  test was conducted to test whether the co-digestion effect significantly occurred across the mixed substrates. Then, the matrix contrasts were defined for each of the nine mixed substrates to examine in which mixed substrate the co-digestion effect was significant. Taking the mixture 30G-50W-20C for example, we subtracted the methane yield of this mixture estimated from Eq. (3) from that estimated from Eq. (2), and then examined whether the difference was significantly from zero. If the outcome is significantly larger than zero, the co-digestion effect is synergistic, otherwise the effect is antagonistic. Using the similar approach, we also examined whether the co-digestion effect was significantly different between the two inocula for a given mixed substrate. All analyses were performed using the  $R$  software version 3.4.3.

## 3. Results and discussion

### 3.1. Substrates and inoculum characteristics

The main chemical characteristics of four plant species are summarised in Table 2. Ryegrass had the highest hemicellulose content, while plantain had the highest cellulose and lignin content, which was in line with our recent studies [20,21]. In contrast, white clover

**Table 3**  
Chemical characteristics of the Manure and Grass inocula.

Parameters	Units	Manure	Grass
Total solid (TS)	% <sub>FM</sub>	5.9 ± 0.6	3.1 ± 0.01
Volatile solid (VS)	% <sub>FM</sub>	4.8 ± 0.5	2.4 ± 0.01
Phosphorus (P)	g kg <sub>FM</sub> <sup>-1</sup>	0.31	0.34
Potassium (K)	g kg <sub>FM</sub> <sup>-1</sup>	1.43	2.18
Calcium (Ca)	g kg <sub>FM</sub> <sup>-1</sup>	0.96	0.86
Magnesium (Mg)	g kg <sub>FM</sub> <sup>-1</sup>	0.21	0.29
Sulphur (S)	g kg <sub>FM</sub> <sup>-1</sup>	0.22	0.17
Sodium (Na)	g kg <sub>FM</sub> <sup>-1</sup>	0.16	0.54
Iron (Fe)	mg kg <sub>FM</sub> <sup>-1</sup>	76.03	67.90
Manganese (Mn)	mg kg <sub>FM</sub> <sup>-1</sup>	15.84	10.94
Boron (B)	mg kg <sub>FM</sub> <sup>-1</sup>	0.82	0.77
Zinc (Zn)	mg kg <sub>FM</sub> <sup>-1</sup>	10.70	6.41
Copper (Cu)	mg kg <sub>FM</sub> <sup>-1</sup>	2.46	1.79
Molybdenum (Mo)	mg kg <sub>FM</sub> <sup>-1</sup>	0.10	0.20
Cobalt (Co)	mg kg <sub>FM</sub> <sup>-1</sup>	0.04	0.02
Nickel (Ni)	mg kg <sub>FM</sub> <sup>-1</sup>	0.29	0.15
Volatile fatty acids	mg l <sup>-1</sup>	148 ± 49	159 ± 86
Total ammonia N	mg l <sup>-1</sup>	1147 ± 111	950 ± 200
pH	/	7.6 ± 0.1	7.6 ± 0.1
Methane in biogas	%	55.2 ± 1.5	57.5 ± 1.0
Specific methane yield	l CH <sub>4</sub> kg <sub>VS</sub> <sup>-1</sup>	234 <sup>a</sup>	126
Volumetric biogas yield	m <sup>3</sup> biogas t <sub>FM</sub> <sup>-1</sup>	20.3 <sup>a</sup>	5.3

<sup>a</sup> ± ' Represents standard error of duplicates measurements.

<sup>a</sup> Methane and biogas yields in the Manure inoculum were calculated from the data of the cumulative biogas production of the whole period (62 days).

contained the lowest fibre composition. As expected, white clover contained larger N content and thus crude protein than grass and forb species, leading to a low C/N ratio (10.5). Consistent with previous studies [13,22], we observed that chicory generally held the highest concentrations of macro- and micro-nutrients among species because its deep roots facilitate larger uptake from deeper soil layers.

As expected, the Manure inoculum generally contained higher TS, VS and macro- and micro-nutrients than the Grass inoculum, except for potassium (K), magnesium (Mg) and sodium (Na) (Table 3). The Manure inoculum had slightly higher TAN, but similar VFA and pH as the Grass inoculum. Given higher concentrations of nutrients and VS, the Manure inoculum had higher biogas yield potential than the Grass inoculum.

### 3.2. Methane yield potential, maximal production rate and lag phase

Table 4 shows that the measured ultimate methane yield (B<sub>0</sub>) after

**Table 4**

The ultimate methane yield (B<sub>0</sub>), maximal methane production rate (μ<sub>m</sub>) and lag phase (λ) estimated from the modified Gompertz model under the Manure and Grass inocula.

Substrates	B <sub>0</sub> (L kg <sup>-1</sup> VS)		μ <sub>m</sub> (L kg <sup>-1</sup> VS d <sup>-1</sup> )		λ (d)		R <sup>2</sup>	
	Manure	Grass	Manure	Grass	Manure	Grass	Manure	Grass
100G	311 <sup>ab</sup>	351 <sup>a</sup>	24.3 <sup>ab</sup>	26.8 <sup>a</sup>	0.12 <sup>b</sup>	0.00 <sup>c</sup>	0.987	0.980
100W	340 <sup>a,*</sup>	226 <sup>c</sup>	28.1 <sup>a,*</sup>	8.0 <sup>c</sup>	1.30 <sup>a,*</sup>	9.08 <sup>a</sup>	0.986	0.984
100C	291 <sup>ab</sup>	320 <sup>ab</sup>	17.6 <sup>b</sup>	16.1 <sup>b</sup>	0.09 <sup>b</sup>	0.00 <sup>c</sup>	0.984	0.960
100P	343 <sup>a</sup>	326 <sup>ab</sup>	23.9 <sup>ab,*</sup>	16.5 <sup>b</sup>	0.00 <sup>b</sup>	0.00 <sup>c</sup>	0.984	0.981
30G-50W-20C	328 <sup>ab</sup>	318 <sup>ab</sup>	26.7 <sup>a,*</sup>	16.2 <sup>b</sup>	0.44 <sup>b,*</sup>	4.49 <sup>b</sup>	0.983	0.991
50G-30W-20C	304 <sup>ab</sup>	301 <sup>b</sup>	21.6 <sup>ab,*</sup>	14.7 <sup>b</sup>	0.04 <sup>b,*</sup>	1.78 <sup>d</sup>	0.985	0.990
30G-30W-40C	291 <sup>ab</sup>	287 <sup>b</sup>	20.3 <sup>ab,*</sup>	14.1 <sup>b</sup>	0.25 <sup>b,*</sup>	2.34 <sup>c</sup>	0.983	0.988
30G-50W-20P	312 <sup>ab</sup>	304 <sup>ab</sup>	23.5 <sup>ab,*</sup>	16.0 <sup>b</sup>	0.07 <sup>b,*</sup>	3.75 <sup>bc</sup>	0.983	0.993
50G-30W-20P	326 <sup>ab</sup>	333 <sup>ab</sup>	24.6 <sup>ab</sup>	19.5 <sup>b</sup>	0.00 <sup>b,*</sup>	1.98 <sup>d</sup>	0.983	0.984
30G-30W-40P	324 <sup>ab</sup>	332 <sup>ab</sup>	24.4 <sup>ab</sup>	18.4 <sup>b</sup>	0.00 <sup>b,*</sup>	1.29 <sup>d</sup>	0.983	0.982
30G-50W-10C-10P	303 <sup>ab</sup>	310 <sup>ab</sup>	23.1 <sup>ab,*</sup>	15.7 <sup>b</sup>	0.43 <sup>b,*</sup>	4.54 <sup>b</sup>	0.983	0.992
50G-30W-10C-10P	272 <sup>b,*</sup>	307 <sup>ab</sup>	18.6 <sup>b</sup>	16.5 <sup>b</sup>	0.22 <sup>b,*</sup>	1.81 <sup>d</sup>	0.985	0.988
30G-30W-20C-20P	323 <sup>ab</sup>	335 <sup>ab</sup>	23.2 <sup>ab</sup>	19.2 <sup>b</sup>	0.03 <sup>b,*</sup>	2.16 <sup>c</sup>	0.984	0.985

Means with different lowercase letters in the same column represent significant differences ( $P < 0.05$ ) between substrates using Tukey's *post hoc* test. Means with an asterisk refer to significant differences ( $P < 0.05$ ) between the two inocula for a given substrate. VS, volatile solid. The coefficient of determination ( $R^2$ ) was used to measure the goodness of a fit of the model. Abbreviations can be referred to Table 1.

96 days significantly differed between substrates ( $P < 0.001$ ), but these differences strongly interacted with inoculum source ( $P < 0.001$ ). In Manure inoculum, the highest B<sub>0</sub> was found in single species of plantain (343 L CH<sub>4</sub> kg<sup>-1</sup> VS) and white clover (340 L CH<sub>4</sub> kg<sup>-1</sup> VS), being significantly higher than that in the mixed substrate with 50% ryegrass, 30% white clover, 10% chicory and 10% plantain (50G-30W-10C-10P). We observed higher B<sub>0</sub> for plantain than the reported from Wahid et al. [15] (273 L CH<sub>4</sub> kg<sup>-1</sup> VS). This could be due to approx. 10% higher cellulose and hemicellulose contents of plantain in this study. As for Grass inoculum, ryegrass produced the highest B<sub>0</sub>, with significantly higher yield than the two mixtures containing high amount of chicory (50G-30W-20C and 30G-30W-40C). Yet, the lowest B<sub>0</sub> (226 L CH<sub>4</sub> kg<sup>-1</sup> VS) was observed in single species of white clover.

The modified Gompertz model was used to estimate the maximal methane production rate μ<sub>m</sub> and lag phase time λ, two important parameters measuring the performance of anaerobic digestion. The model had an R<sup>2</sup> of 0.987 ± 0.006 (SD) across all cases, indicating a high precision of the model fit. Consistent with B<sub>0</sub>, white clover held the highest μ<sub>m</sub> among all substrates in Manure inoculum, but the lowest one in Grass inoculum (Table 4). White clover had the highest λ in both inocula, but with an especially long lag phase in Grass inoculum. In addition, the substrates containing white clover generally had lower μ<sub>m</sub> and higher λ in Grass inoculum than in Manure inoculum.

### 3.3. Mechanisms related to low production performance of white clover in grass inoculum

The low production performance of white clover in Grass inoculum seemed not related to micronutrient levels of white clover since it contained either the largest or the intermediate concentrations of Fe, Mo, Co and Ni (Table 2) among four species, which are key micro-nutrients for methanogenesis [23]. However, white clover had high protein content and low C/N ratio (10.5), indicating that ammonia inhibition may occur during anaerobic co-digestion [3,24].

To test this, we measured pH and the concentrations of VFA and TAN of four single substrates in both inocula at the end of batch experiment. The results showed that pH ranged between 7.3 and 7.8, and VFA concentrations maintained at very low levels (< 80 mg L<sup>-1</sup>) across substrates and two inocula (Table 5). The TAN concentrations of four substrates ranged from 1.01 to 1.47 g L<sup>-1</sup> in Grass inoculum, which was lower than the reported inhibitory values from 1.7 to 14 g L<sup>-1</sup> [24]. Moreover, the highest TAN concentration (2.21 g L<sup>-1</sup>) was observed in white clover digested in Manure inoculum. These results indicated that low production performance of white clover in Grass inoculum could

**Table 5**  
Chemical characterization of digestates of four single species under the Manure and Grass inocula at the end of the batch experiment (Day 96).

Inocula	Substrates	Volatile solid (VS, % <sub>FM</sub> )	pH	Volatile fatty acids (VFA, mg L <sup>-1</sup> )	Total ammonia N (TAN, g L <sup>-1</sup> )
Manure	100G	2.04	7.61	66	1.83
	100W	2.20	7.75	74	2.21
	100C	2.03	7.55	55	1.59
	100P	2.16	7.55	59	1.89
	CK <sup>a</sup>	1.92	7.81	63	1.39
Grass	100G	1.85	7.45	56	1.26
	100W	2.70	7.56	43	1.47
	100C	1.86	7.32	35	1.01
	100P	1.70	7.31	33	1.06
	CK	1.64	7.51	29	0.93

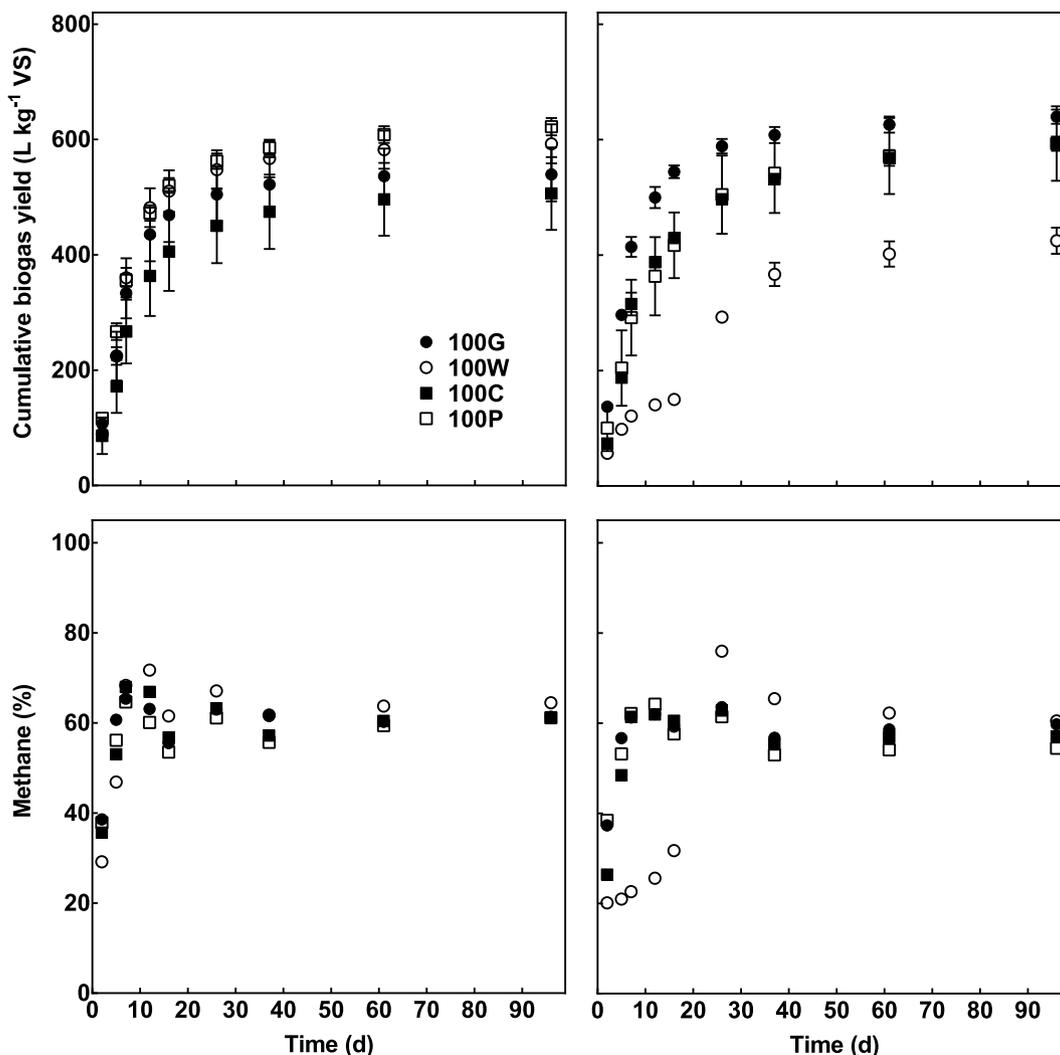
<sup>a</sup> CK refers to the corresponding inoculum without added substrate. Abbreviations can be referred to Table 1.

not be attributed to inhibition due to reduced pH, or accumulation of ammonia or fatty acids.

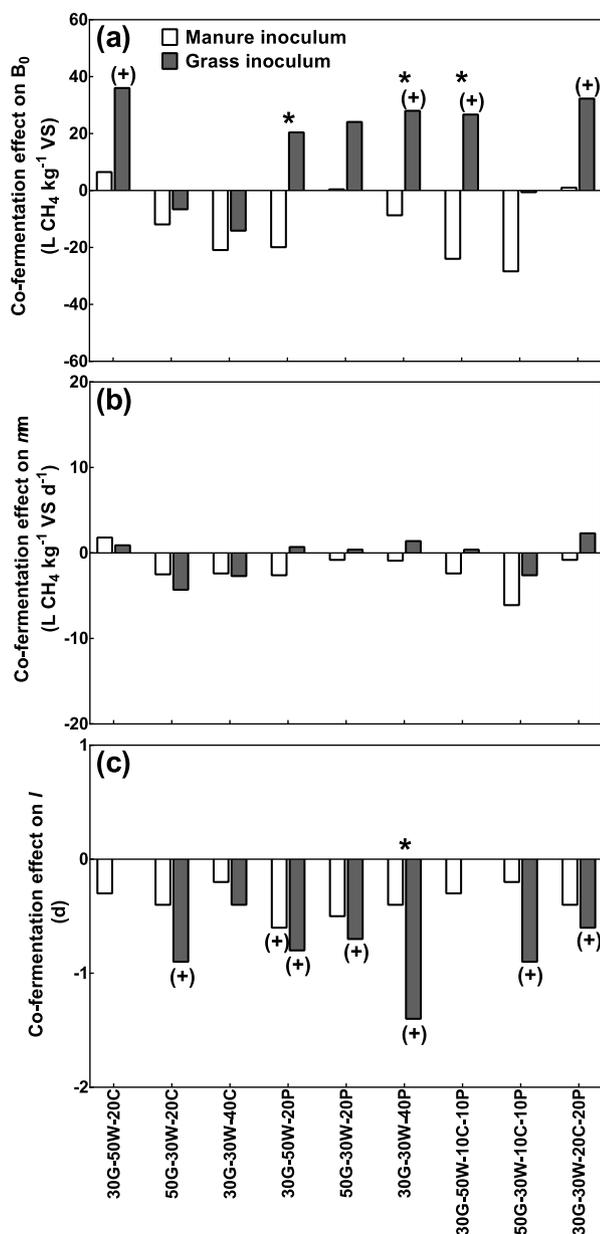
We further found that low methane yield of white clover in Grass inoculum were from both its low biogas yield and methane fraction, especially in the first 16 days (Fig. 1b, d). This suggests that microorganisms in the grass-based inoculum did not adapt to white clover

with low C/N ratio in the beginning probably because the Grass inoculum was previously solely fed on grass hay with higher C/N ratio. Such explanation was further supported by consistently high biogas yield and stable methane fraction of ryegrass digested in Grass inoculum throughout the digestion period (Fig. 1b, d). Interestingly, both biogas yield and methane fraction substantially increased in the following 10 days, suggesting an improved microbial adaptation over time. In addition, we found that methane fraction of white clover went up to 80% at day 26, but stabilised at 60% at day 37. This suggests that both acetotrophic and hydrogenotrophic methanogens have jointly contributed to methane production during this period [25].

Nevertheless, we did not observe improved ultimate methane yield of white clover in Grass inoculum (Table 4), which was confirmed by the highest VS remained after 96 days (Table 5). We further estimated N mineralisation rate of white clover by calculating the relative proportion of increased TAN concentrations during digestion in white clover N and assuming 1% loss of ammonia during gas sampling. We found only 55% N from white clover was mineralised in Grass inoculum, but up to 95% in Manure inoculum. These results indicate a limited ability of the microbial community in adapting to substrates with different characteristics in batch conditions. This is further supported by recent studies showing that initial microbial community structure is crucial for determining ultimate methane production [26,27]. A number of studies have shown that microbial community structure sourced from different



**Fig. 1.** Cumulative biogas yield (a, b) and methane percentage (c, d) of four single species (100G, ryegrass; 100W, white clover; 100C, chicory; 100P, plantain) as a function of time from the two inocula (Manure: a, c; Grass: b, d).



**Fig. 2.** The synergistic and antagonistic effect on ultimate methane yield  $B_0$  (a), maximal methane production rate  $\mu_m$  (b) and the lag phase  $\lambda$  (c) in nine mixed substrates under two inocula (Manure, open column; Grass, filled column). The significant synergistic effects ( $P < 0.05$ ) were indicated by (+). Asterisk (\*) referred to significant difference in the co-digestion effect between the two inocula in a given mixed substrate. Abbreviations can be referred to Table 1.

inoculums initially differed but became similar in a continuous digester over time possibly due to a gradual acclimation to a given substrate [26]. Nevertheless, it is still unclear whether the degree of microbial acclimation varies with substrates or depends on initial microbial community. Further work would be relevant to explore whether and to what degree methane yield of white clover digested in the Grass inoculum can be improved via co-digestion with ryegrass in continuous reactors. Collectively, these results suggest low methane yield of white clover is unlikely due to inhibition, but probably because the initial microbial community in the Grass inoculum had a limited ability in digesting white clover.

#### 3.4. Synergistic and antagonistic interactions in grass-forb mixtures

In line with our hypothesis, we found that co-digesting grass and

forbs significantly enhanced methane yield potential  $B_0$  in Grass inoculum in four of nine substrates (Fig. 2a). This enhancement was on average by  $31 \text{ L CH}_4 \text{ kg}^{-1} \text{ VS}$  (+11%). In addition, co-digesting grass and forbs did not significantly influence maximal methane production rate  $\mu_m$  (Fig. 2b), but significantly shortened the lag phase time  $\lambda$  by 0.8 days in six of nine substrates (Fig. 2c). As expected, synergistic effects did not occur in Manure inoculum across all substrates. Certain mixed substrates tended to show an antagonistic effect on  $B_0$ , but not statistically significant.

Improved production potential of grass-forb mixtures in Grass inoculum was probably because fermentative microorganisms had a better adaptation to grass-forb mixtures containing more balanced composition (especially C/N ratio) than single species of white clover. Such mechanism has often been proposed to explain the synergistic effects occurring in co-digesting slaughterhouse wastes or sewage sludge with agricultural wastes such as animal manure and crop residues [3,4]. These findings imply that co-digesting multiple grassland species with complementary characteristics is an effective way to avoid low biogas production occurring in mono-digestion of single species.

Our results showed that the occurrence of synergistic effects appeared to depend on substrate composition, with higher frequency happening in mixtures containing plantain than chicory. This could be because plantain contained higher C/N ratio and fibre composition than chicory (Table 2). Consequently, mixing plantain with white clover characterised by low C/N ratio and fibre content provided a more balanced nutrient composition to facilitate methane production. Yet, we also observed a significant synergistic effect in the chicory-containing mixture (30G-50W-20C), suggesting that complex interactions among grass, white clover and chicory may function. Recent work has employed the simplex-centroid mixture design (SCMD) to examine the role of substrate combinations in contributing to methane production [28–30]. However, our experiment design did not rigidly follow up the SCMD approach (e.g. lack of two-species combination), which refrained us from performing further analysis for possible mechanisms. Further work may carefully follow this approach or more sophisticated approach such as artificial neural network to unravel which species combinations could lead to the synergistic effects, and to design the optimal mixture ratios for maximal methane production.

Furthermore, these results were based on one cutting strategy (four-cut strategy) in the batch mode. Our recent work showed that cutting frequency influenced fibre and nutrient composition of grass-forb mixtures and thus affected biogas yields in continuous stirred tank reactors [14]. It is worthwhile to explore whether such synergistic effect is subjected to cutting strategy and continuous mode.

#### 3.5. Grass-forb mixtures as alternative bioenergy feedstock

We found that ryegrass, as a commonly used feedstock for bioenergy production, had the highest performance in terms of ultimate methane yield, maximum methane production rate and lag phase when inoculated with Grass inoculum (Table 4). However, the ryegrass-dominated grasslands are often associated with large input of mineral fertilisers, thus at the expense of high energy consumption and negative environmental impacts (Taube et al., 2014). Adding legumes (e.g. white clover) and non-leguminous forbs (e.g. plantain) to the ryegrass-dominated grasslands has been recently found to deliver multiple ecosystem services, such as providing high and stable biomass feedstock for sustainable vehicle fuels [31], increasing nutritive value for livestock [20] and enhancing pollinations for arable crops [32]. Our results showed that co-digesting these forbs (white clover, chicory and plantain) with ryegrass maintained high methane yield potential as ryegrass digested alone over a wide range of substrate ratios in Manure inoculum and largely in Grass inoculum (Table 4). Hence, this finding could furthermore contribute to promoting the use of forb-based multi-species grasslands in enhancing ecosystem multifunctionality of agricultural grasslands.

#### 4. Conclusions

This study demonstrates that co-digesting grass and forbs synergistically enhanced methane yield potential and reduced lag phase time in a grass-based inoculum, but not in a cattle manure-based inoculum. These synergistic effects occurred more often in mixtures containing plantain than chicory. Synergistic effects were probably because grass-forb mixtures provided a more balanced C/N ratio and fibre composition that facilitated the growth of initial microbial community in the grass-based inoculum. Furthermore, mixing forbs with grass in a wide range of substrate ratios maintained as high specific methane yield as commonly used grass from pure stands, providing alternative grassland feedstock for bioenergy production.

#### 5. Declarations of interest

None.

#### Acknowledgements

This work was supported by the Green Development and Demonstration Program ‘MultiPlant’ project [grant number 34009-13-0678] and coordinated by the International Centre for Research in Organic Food Systems. The authors gratefully acknowledge the technical assistance of the staff at Foulumgaard Experimental Station, and thank Radziah Wahid for earlier discussions and Rodrigo Labouriau for statistical advice.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biombioe.2018.09.009>.

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