# Anaerobic digestion of Crassulacean Acid Metabolism plants: exploring alternative feedstocks for semi-arid lands

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## Abstract

In this work, five Crassulacean Acid Metabolism (CAM) species from the five different genera (*Agave*, *Ananas*, *Euphorbia*, *Kalanchoe*, and *Opuntia*) were selected as alternative feedstocks and their biochemical methane potential (BMP) investigated. Batch assays were performed using sludge and rumen fluid as inocula under uncontrolled pH and at mesophilic temperature (39 °C). Mean methane yields from the CAM plants inoculated with AD sludge ranged from 281 to 382 ml/gVS. These values were not significantly different from the methane yield obtained from maize, a feedstock for biomethane and volatile fatty acid (VFA), suggesting that CAM plants may be viable as bioenergy crops on poor-quality soils in areas with low rainfall that are unsuitable for cultivation of food crops.

*Keywords:*

CAM plants, Rumen fluid, Sludge, Anaerobic digestion, Biomethane

## 1. Introduction

Anaerobic digestion (AD) of lignocellulosic biomass for biogas production has gained considerable research interest over the past decades. Although, maize (corn) stover is one example of a promising energy crop, concerns over competition for water and land resources between fuel and food is increasing. The prediction is that competition between lignocellulosic biomass for energy production and food crops will intensify as global population grows (Ge et al., 2016; Sawatdeenarunat et al., 2015). Harvesting maize stover for bioenergy also affects field operation and production. In other words, more resources such as nutrients and fertilisers need to be supplied to soil (Hess et al., 2009). An average of 2–3 tonnes per acre of corn crop residues should be left unharvested to prevent soil surface erosion and preserve soil water (Yang et al., 2016). Use of maize stover as an energy crop will limit the sustainability of crop residues as sustainable feedstocks. To overcome the disadvantages of using maize stover as an AD substrate, alternative sustainable biomass should be explored.

Crassulacean Acid Metabolism (CAM) plants have recently gained attention as a potential energy crop (Borland et al., 2009; Mason et al., 2015). They possess a specialised mode of photosynthesis systems that allows the stomata on the shoot surfaces to be closed during the day, thereby reducing water loss with $CO_2$ uptake from the atmosphere occurring mainly at night. These features enable CAM plants to thrive in semi-arid and arid habitats with scarce water availability and also to grow relatively well in poor quality soils. An additional

advantage of CAM-plant feedstock over current common sources of lignocellulosic biomass is that they have relatively low lignin content (Mason et al., 2015), a key inhibitor of the cellulolytic activity required for hydrolysis, the first and rate-limiting stage of anaerobic digestion process. Furthermore, lignin and its derivatives can be highly toxic to methanogenic bacteria, so the low lignin content of CAM species offers some potential and significant advantages in terms of microbial conversion of plant biomass to biogas and other high value products (Sierra-Alvarez and Lettinga, 1991).

In the past decade, there have been a few examples of CAM crops exploited for bioenergy production such as *Agave sisalana* Perrine (sisal) and *Opuntia ficus-indica* L. (prickly pear) (Calabrò et al., 2018; Mshandete et al., 2005). The estimated biomass yields for *Agave spp.* previously reported ranged from 7 to 34 dry tonnes per hectare per year (Escamilla-Treviño, 2012). Similarly, the biomass availability of *Opuntia ficus indica* in a recent study was expected to be around 29 dry tonnes/hectare/year (Ramírez-Arpide et al., 2018). According to The Conventional Bale corn stover supply system, the estimated yield of corn stover was 1.6 dry mass tonne/acre/year (3.95 tonne/hectare/year) (Hess et al., 2009). Another report showed the availability of maize stover in the United States could be between 1.8 and 7.9 dry tonne/hectare/year (Gonzalez et al., 2011). Compared to maize stover, the two CAM species have shown that CAM plants could potentially be cultivated in semi-arid areas to compete with typical feedstocks such as corn stover as a viable AD biomass. In addition, there are other CAM species that might be suitable as feedstock for bioenergy production such as *Ananas comosus* (L.) Merr. (pineapple) and *Euphorbia* spp. (Hastilestari

et al., 2013), but these have received less attention in terms of biomass and biogas production.

As well as methane production from plant biomass, there is growing interest in the potential to generate other high value products such as volatile fatty acids (VFAs). These include acetate, propionate and *n*-butyrate, the primary products of the hydrolysis and acidogenesis steps of anaerobic digestion. They are of considerable and growing interest in terms of biorefinery since they provide a sustainable source of platform chemicals providing one of the few alternatives to oil-based feedstocks. However, hydrolysis has been found to be the first and rate-limiting step of anaerobic digestion and is a key issue limiting the appeal of AD (Nopharatana et al., 2007).

The source of microbial inoculum for seeding the AD reactor represents a key factor in the establishment of an AD system. Researchers have found rapid hydrolysis of corn stover and high VFA yields when cow rumen fluid was used as an inoculum (Hu and Yu, 2005). Rumen fluid used for pretreatment of rice straw was also found to help improve substrate degradation efficiency with acetate and propionate being the dominant pretreatment products (Zhang et al., 2016).

Most studies to date have reported on either the production of methane or VFA from different substrates under the same anaerobic conditions employing identical inoculum. The main objective of this study was to evaluate the specific methane potential and VFA production from five different CAM plants employing the biochemical methane potential assay (BMP). The performance of two different inocula sources, anaerobic digester sludge and rumen fluid, on

anaerobic digestion processes of the five CAM plants was assessed. Methane yields, VFA production and pH data were collected for the analysis, and kinetic modelling was performed of methane curves from the BMP experiments.

## 2. Material and methods

### *2.1. Biomass collection*

Biomass from five different Crassulacean Acid Metabolism (CAM) plants was used in this study, pineapple leaves (*Ananas comosus* (L.) Merr.), agave leaves (*Agave angustifolia* Haw.), opuntia cladodes (*Opuntia fragilis* (Nutt.) Haw.), kalanchoe leaves (*Kalanchoe daigremontiana* (Raym.-Hamet & H.Perrier) A.Berger) and euphorbia stems (*Euphorbia virosa* Willd.). Maize shoots (i.e. stalks and leaves combined) (*Zea mays* L.) were also investigated as this is a common energy crop on which there have been many studies. The CAM species were selected to be representative of five major families of plants that encompass a variety of features typical of CAM plants.

All plants were grown in glasshouses at the Department of Plant Sciences and the Oxford Botanic Garden, University of Oxford, where plants were cultivated under natural irradiation and with supplementary heating to maintain minimum temperatures > 10 °C. The harvested materials were coarsely chopped to 5.0 ± 1.0 cm employing a knife and dried in a ventilated oven at 80 °C until the mass was constant. The dried samples were then ground to pieces of approximately 1–2 mm using a commercial blender (Waring Pro® Stainless Steel Blender, 550 W). The main biomass characteristics of each substrate are summarised in **Table 1**.

<<<<<<<<<<<<<<< Insert **Table 1** here >>>>>>>>>>>>>>>

120 *2.2. Inoculum and culture media preparation*

121 *2.2.1. Inoculum*

122 Anaerobic sludge was used as a source of microorganisms that were
123 obtained from Agrivert AD facility in Cassington, Oxford, UK. Rumen fluid was
124 obtained from a fistulated non-lactating cow fed daily with grass or maize silage
125 feed (The Centre for Dairy Research, University of Reading, UK). The fresh fluid
126 was collected in warm vacuum flasks and immediately transferred to the
127 laboratory. The fluid was then filtered through an aluminium mesh strainer (mesh
128 number 10, 1.0 mm). Both inocula were used on the day of collection. The
129 inoculum characteristics are summarised in Table 1.

130 *2.2.2. Culture media*

131 The culture media used contained macronutrients, micronutrients, trace
132 minerals, vitamin mixtures and buffer solution necessary for growth of
133 microorganisms as described by (Angelidaki and Sanders, 2004).

134 *2.3. Biochemical methane potential (BMP) assay*

135 The biochemical methane potential (BMP) experiment was carried out in
136 batch culture based on the modified protocol previously described (Yue et al.,
137 2012). Batch cultures were performed in 250 ml serum vials with 150 ml of
138 working volume. The inoculum to substrate ratio was set as 2:1 (w/w) on a volatile
139 solid (VS) basis. Media solution (120 ml) was filled to the substrate-inoculum
140 mixture to make up the same working volume for all bottles. The experiment was
141 carried out in an anaerobic chamber filled with $N_2$ and $H_2$ gas mixture (95:5). The
142 vials sealed with rubber stoppers and aluminium caps were then placed in a
143 shaking incubator for fermentation at 39 ± 1 °C with constant agitation of 120 rpm.

All experiments were performed using three biological replicates and the average results were presented as means + standard deviations. Blank batches consisting only of inocula and media solution were included to determine endogenous methane production. Microcrystalline cellulose (Avicel®, Sigma-Aldrich, US) was used as a positive control for the inoculum activity test. Biogas production was directly determined by the water displacement method using a eudiometer and the corresponding cumulative methane production is presented. The experiment was terminated when the ratio of daily methane production to the cumulative volume was <1% (Holliger et al., 2016). The gas volume (ml) data were corrected to standard temperature and pressure (STP) at 273.15 K and 101.3 kPa to eliminate any potential errors in biogas quantification according to the procedure described by (Walker et al., 2009).

*2.4. Analytical methods*

Total solid (TS) and volatile solid (VS) of all plant materials and inocula were measured according to the standard procedures (APHA, 1998). Analysis of cellulose, lignin and matrix polysaccharides was performed by the methods as previously described by (Rezende et al., 2018). The gas composition was determined by gas chromatography (Shimadzu GC-2010, Japan) equipped with a thermal conductivity detector (GC-TCD) and a 30 m x 0.53 mm x 0.25 µm HP-PLOT U capillary column. The temperatures of the detector, injector and oven were 200 °C, 150 °C and 75 °C, respectively. Helium was used as the carrier gas at a flow rate of 5.53 ml/min. Liquid samples were centrifuged at 30,000×g for 15 minutes and supernatant was collected into 1.5 ml GC vial and acidified with 3% (v/v) formic acid. VFA analysis was conducted by using a gas chromatography

168 equipped with a flame ionisation detector (GC-FID) and a 30 m x 0.25 mm x 0.25
169 µm fused-silica capillary column (ZB-FFAP). The temperatures of the detector
170 and injector were 350 °C and 250 °C, respectively. The oven temperatures were
171 set at 100 °C for 2 minutes and then increased at a rate of 8 °C/min. The total
172 volatile fatty acid (TVFA) was shown as the sum of acetic acid, propionic acid,
173 isobutyric acid, *n*-butyric acid, isovaleric acid and *n*-valeric acid.

174 *2.5. Kinetic modelling*

175 Modified Gompertz equation (Eq. (1)) was used to model the methane
176 production profile to determine the methane production potential, maximum
177 methane production rate and lag phase for each substrate (Zhao et al., 2018):

178 $$Y = A\exp\{-\exp[(\mu_m e/A)(\lambda - t)+1]\} \quad (1)$$

179 where $Y$ is cumulative methane production at time $t$ (ml/gVS), $A$ is methane
180 production potential (ml/gVS), $\mu_m$ represents maximum methane production rate
181 (ml/gVS/d), $\lambda$ refers to lag phase time (d), $e$ is equal to exp(1) and $t$ is the digestion
182 period (d). The parameters in both equations were estimated by Solve system of
183 nonlinear equations and best-fitting with the experimental data using MATLAB
184 R2018a.

185 *2.6. Statistical analysis*

186 All data were presented as means ± standard deviation of triplicate. Analysis
187 of variance (ANOVA) and Tukey HSD test was performed using R programming
188 software (R i386 3.5.0) to test the statistical significance of the data.

## 3. Results and Discussion

### *3.1. Characteristics of feedstocks and inocula*

#### *3.1.1. Biomass characterisation*

**Table 1** summarises the total solid (TS), volatile solid /total solid (VS/TS), and percentages of crystalline cellulose and lignin in dry mass of substrates from five CAM species comparing with maize. VS/TS ratio is the proportion of biodegradable content in dry solid. The TS contents and the VS/TS ratios in CAM plants were in the range of 8.51 ± 0.04–17.55 ± 0.48% and 67.19 ± 0.54–88.32 ± 0.22%, respectively, the highest of which was for *Euphorbia*. High percentage of VS/TS was considered one of the desirable characteristics of substrates for anaerobic digestion; this means greater quantity of organic materials was available for conversion to biomethane or VFAs (Li et al., 2013). The lignin content of *Agave* (*A. angustifolia*) (14.42 ± 3.85%TS) was similar to literature value of 15.6 ± 0.8% (Pérez-Pimienta et al., 2018). The low lignin percentage of *O.fragilis* (8.26 ± 2.50%TS) was also in accordance to the value of *O. ficus indica* (7.95% dry mass) previously reported (Kuloyo, 2012). Compared to CAM substrates, maize had the highest VS/TS at 95.05 ± 0.60%. In addition, the highest percentages of crystalline cellulose and lignin were also obtained from maize (39.92 ± 4.32% and 18.81 ± 2.19%, respectively). All CAM feedstocks were found to have relatively low lignin compared to maize shoots, although the lignin percentage for pineapple was almost as high as that for maize (17.35 ± 2.28%TS and 18.81 ± 2.19%TS, respectively). The lignin percentages of *Opuntia* and *Kalanchoe* were lower than 10%, with cellulose contents of 16.51 ± 1.34 (%TS) and 20.28 ± 2.18 (%TS), respectively, which were also the lowest among all the

substrates examined. Despite low cellulose content compared to maize, matrix polysaccharides (µg/mg) of some CAM feedstocks such as *Agave*, *Euphorbia* and pineapple were relatively higher (11.37, 12.10 and 10.09, respectively) than that of maize (9.46).

### *3.1.2. Inoculum characterisation*

The characteristics of the inocula are shown in **Table 1**~~**Table *1***~~. AD sludge had higher TS content (4.50 ± 0.02%) compared to rumen fluid (2.99 ± 0.02%), but the VS/TS ratios of both inocula types were relatively similar (60.10 ± 0.22% and 67.22 ± 0.01%). The VS/TS values of the AD sludge from this study were consistent with other sludge data from the literature, ranging from 55.6–76.5% (Gonzalez-Fernandez et al., 2018; Li et al., 2013; Wall et al., 2013). The initial pH of the rumen inoculum (5.94) was much lower than that of sludge (8.02), since the fluid was obtained from a cow rumen where the digestion processes lead to acid generation. Both inocula were tested for their methanogenic activity using microcrystalline cellulose (Avicel®, Sigma-Aldrich, US) to validate the BMP and gas measurement procedure. The activity of the AD sludge measured as methane production was 355.3 ± 20.5 ml/gVS, which is in good agreement with the criteria previously established (Holliger et al., 2016) that methane yield generated from cellulose should be between 352 and 414 ml/gVS. Compared to sludge, the activity of the rumen fluid was considerably lower (189.4 ± 16.1 ml/gVS) and may not have been suitable for methane potential determination.

### *3.2. Anaerobic digestion of plant materials by sludge*

#### *3.2.1. Experimental methane yields from CAM substrates*

The distinctive BMP production curves of anaerobic digestion of the different substrates inoculated with AD sludge are depicted in **Fig. 1**. The shape of BMP curves is largely governed by the substrate degradability, operating conditions and any inhibitory effects occurring during digestion process. Methane production started immediately as the substrate was introduced into the vials, but similar patterns of cumulative methane curves were observed for all six plant substrates where methane production stopped after around 40 days of incubation. The cumulative methane yields of the five CAM plants, in ascending order, were 280.5 ± 14.1, 297.9 ± 28.0, 300.8 ± 19.2, 337.6 ± 21.9, and 381.7 ± 19.0 ml/gVS for *Euphorbia*, pineapple, *Opuntia*, *Kalanchoe* and *Agave*, respectively, which compared with 307.8 ± 21.9 for maize. Only *Kalanchoe* and *Agave* gave biomethane yields exceeding 200 ml/gVS after the first 10 days (**Figs. 1a and 1c**). These initial steeper slopes for *Agave* and *Kalanchoe* suggest that the hydrolysis and methane production rates (ml/gVS/d) had the potential to be greater than those of other plants. This was subsequently confirmed by the modified Gompertz model parameter, $\mu_m$, which represents the maximum methane production rate (**Table 2**). *Agave* and *Kalanchoe* generated the highest maximum methane production rates, $\mu_m$, at 26.5 and 21.2 ml/gVS/d, respectively, whereas maize had the lowest rate at 15.7 ml/gVS/d, similar to the literature values which ranged between 8.5–26.7 ml/gVS/d (Li et al., 2015). By day 38, the ratio of daily methane yield to cumulative methane yield was <1%, at which point the experiment was terminated.

<<<<<<<<<<<<<<< Insert **Fig.1** here >>>>>>>>>>>>>>>

<<<<<<<<<<<<<<< Insert **Table 2** here >>>>>>>>>>>>>>>

Total volatile fatty acid (TVFA) concentration and pH were also analysed alongside biomethane production and are presented in **Fig.1**. TVFA was taken to be a mixture of acetic, propionic, *n*-butyric, isobutyric, *n*-valeric and isovaleric acids. The first three were found to be the major VFA products in all batches, whereas low concentrations of the latter three acids could still be detected.

The peak of TVFA concentrations was evident after 24 hours of digestion in all substrates, especially *Agave* and *Kalanchoe*, inoculated with the AD sludge (**Fig. 1**). The change was the highest in *Kalanchoe* (**Fig.1c**) where the TVFA increased from 98.7 ± 24.5 mg/l (day0) to 334.3 ± 34.3 mg/l (day1). The TVFA levels after 24 hours were 134.0 ± 20.4, 177.0 ± 31.6, 201.6 ± 34.1, 239.7 ± 13.9 and 271.6 ± 19.3 mg/l for *Euphorbia*, maize, pineapple, *Opuntia* and *Agave*, respectively. After 72 hours, the TVFA levels for all substrate significantly dropped and reached around 10 mg/l by the end of day 5. This implies that the fast hydrolysis of easily biodegradable soluble substrates occurred within the first 24–72 hours of the digestion period. For all substrates, there was little accumulation of TVFA after day 5 until the end of the study, whereas methane volume continued to rise until the digestion finished. The inoculum to substrate ratio of 2:1 (w/w VS) used for this experiment was therefore deemed suitable, as there was no sign of VFA accumulation that could cause inhibition of anaerobic digestion processes (Holliger et al., 2016).

pH and TVFA are closely linked and influence each other in anaerobic digestion. As also shown in **Fig. 1**, all substrate vials had the starting pH of 8.5 which then decreased to about 8.0 after the first 24-48 hours of digestion, when TVFA level also increased accordingly. Increasing production of TVFA leads to a decline in pH and vice versa (Li et al., 2017). For all substrates, the pH gradually dropped over the course of digestion and reached final values between 7.58 ± 0.10 and 7.70 ± 0.05. Nonetheless, a small but gradual pH drop after day 5 of digestion when little VFA accumulation was detected suggested that there may have been other organic acids or long-chain fatty acids (LCFAs) accumulating, which were not detected by the analytical methods used in this study, and could contributed to the decrease in pH.

The methane yields from individual CAM substrates in this study were compared with CAM species previously investigated. Comparison of data obtained from the same reactor mode, batch digestion, methane yields obtained from *Agave angustifolia* Haw. (381.7 ml/gVS) revealed it to be higher than the value for *Agave sisalana*, 320 ml/gVS previously reported (Mshandete et al., 2004). As well as *A. tequilana*, which has long been used for ethanol production, *A. angustifolia* is known for the production of Spanish alcoholic beverages. Other potential applications of *A. angustifolia* include medicine, prebiotic and additive (Stewart, 2015). There have been several reports of using *Euphorbia tirucalli* as substrate for biogas production in the past without determining methane content (Hastilestari et al., 2013; Rajasekaran et al., 1989). Hence, it is not straightforward to compare the methane yield from *Euphorbia virosa* with those previously reported. This study is the first to report the biomethane yield from

304 *Kalanchoe daigremontiana* (336.9 ml/gVS). Despite *K. daigremontiana* not being
305 a commercial crop, it is cultivated horticulturally and often known as the 'mother
306 of thousands'. It possesses some key properties which are attributed for potential
307 bioenergy feedstock: most notably it is easy to propagate and harvest with low
308 lignin content. The methane yield from *Opuntia fragilis* used in this study (300.8
309 ml/gVS) was relatively higher than those of previously reports from *O. ficus indica*
310 *and O. maxima* (289 L/kgVS, 233.6 ml/gVS, and 236.7-260.1 $m^3$/tVS),
311 respectively (Calabrò et al., 2018; Ramos-Suárez et al., 2014; Valenti et al.,
312 2018). In recent years, there has been a growing interest in *Opuntia* species as
313 a bioenergy crop due to its desirable characteristics such as ease of propagation,
314 suitability for coppicing, and low lignin content (Mason et al., 2015). High methane
315 yields obtained from some CAM crops, such as *Agave* and *Kalanchoe* in the
316 absence of further optimisation have already given promising economic benefit
317 in terms of substrate conversion. Nonetheless, methane yield is largely
318 dependent upon the type of substrate and its characteristics, the operating
319 conditions and experimental scale. In addition, growth stage and harvest time of
320 bioenergy crops are also important as they affect total biomass yield, moisture,
321 and lignin content, key factors that determine the biomethane yield of each crop.

322 ### *3.3. Anaerobic digestion of plant materials by rumen fluid*

323 #### *3.3.1. Experimental methane yields*

324 The cumulative methane yields of different substrates employing rumen fluid
325 is shown in **Fig.2**, which depicts a long digestion period of all substrates which
326 terminated around 90 days after inoculation. The cumulative methane yields of
327 CAM crops attained were 98.3 ± 22.9, 168.2 ± 21.5, 177.6 ± 11.8, 184.4 ± 12.9

328 and 210.1 ± 11.3 ml/gVS for *Opuntia*, pineapple, *Euphorbia*, *Agave* and
329 *Kalanchoe*, respectively, which compared with 143.1 ± 14.3 ml/gVS for maize
330 (**Table 3**). Methane production from all substrates began from the first day of the
331 experiment, however a noticeable lag period of around 18 days was observed.
332 The minimal methane volume produced in the beginning of digestion were
333 potentially from soluble biodegradable substrates which were readily available for
334 more immediate utilisation by the inoculated microorganisms. With their
335 depletion, the microbial community became deprived of readily available
336 substrate and so entered a period of activity lag (Budiyono et al., 2009). The
337 absence of biogas production with the first 10 days of inoculation has previously
338 been correlated with the microbial growth exhibited lag (Elhassan et al., 2015).
339 Very low, and in some cases, no gas production was detected from substrates
340 during the lag phase. Since all substrate experienced similar lag phase patterns,
341 it is unlikely that biomass recalcitrance had a major effect on substrate
342 degradation despite a notable difference in lignin composition, ranging from 8.06
343 to 18.81 (%TS).

344 The starting TVFA concentration for all substrates at day 0 was 2.25 ± 0.09
345 g/l. As represented in **Fig. 2**, high initial TVFA concentration of rumen fluid may
346 have affected the anaerobic digestion. Not least, the hydrolysis of cellulose is
347 known to be inhibited at TVFA concentrations above 2,000 mg/l (Siegert and
348 Banks, 2005). A small fluctuation in TVFA concentrations was observed but VFA
349 accumulation persisted as the TVFA levels remained high (~2,000 mg/l) over the
350 course of 15 days, whilst the corresponding pH stayed between 6.0 and 7.0. The
351 highest TVFA concentrations detected, 2.73 ± 0.39 g/l was determined for

352 *Kalanchoe* with 7 days of incubation with rumen fluid. As a result of this prolonged
353 acid accumulation and the pH drop (from 7.5 to 6.3 for *Kalanchoe*, as an example)
354 as a result of substrate feedback inhibition, methane production ceased during
355 this period.

356 The pH of the batches inoculated with rumen fluid started lower than those
357 with sludge. A pH drop from 7.5 to around 6.0 was detected after 24 hours of
358 anaerobic digestion for all the plant species examined. It remained relatively low
359 (~6.5) for the first 10 days of digestion and then recovered to around 7.5 by day
360 20. The slight increase in pH may have been due to microbial consumption of
361 VFA, which correlated with a rise in methane yield, and/or the production of
362 ammonia from protein degradation. The final pH values for all substrates were in
363 the range of 7.1–7.7. For most substrates, an increase in pH after day 20
364 corresponded to a decrease in TVFA concentration and accordingly an increase
365 in methane production. At larger scale, the buffering system should be carefully
366 monitored to prevent this initial sharp decline of pH, although such monitoring
367 was not feasible for this BMP study. By adjusting the starting pH before
368 inoculation, the pH drop effect could be reduced. Methanogenic bacteria are
369 sensitive to pH changes, whilst microbial growth and activity in the AD system
370 can be inhibited by acid accumulation. The narrow pH range of between 6.8 and
371 7.2 was assumed to be optimal for the AD process for biogas production (Ward
372 et al., 2008). Different optimal pH values, however, can be expected, depending
373 on the types of substrates used.

374 The lag period during the first 18 days could also be explained by substrate
375 inhibition by volatile fatty acid (VFA) accumulation, the result of substrate

hydrolysis. Between day 18 and day 40, a decline in cumulative methane yields of all substrates was due to a negative methane yield on the day the gas was collected. The negative value is a consequence of the rumen fluid blank producing greater endogenous methane volume than the vials with substrates. For most substrates except *Opuntia*, the pHs and TVFAs during this period were in a reasonable range for AD processes (around 7.5 and below 1.0 g/l, respectively), this slight decline in methane yield may have been due to the effect of individual VFA accumulation e.g. propionic acid or butyric acid. For *Opuntia*, the TVFA concentration above 1 g/l between day 18 and day 58, which caused a decrease in pH to 6.92 by day 43, could have resulted in an inhibition of methane production, which caused a negative cumulative methane yields shown as 0 ml/gVS in **Fig. 2d**.

The cumulative methane production for individual substrates reached its maximum at different times, but for most substrates, their cumulative methane production curves began to level off between day 50 and day 60. Only the cumulative methane production curves of *Agave* and *Opuntia*, reached a plateau after day 70 and day 80, respectively (**Figs. 2a and 2d**). The longer digestion period for *Agave* and *Opuntia* could have been due to a greater time it took for the methanogens to acclimatise (Carucci et al., 2005). It has been suggested that one of the factors that affects the acclimatisation period of methanogens is a high proportion of fat in the substrate (Palatsi et al., 2010). Fat can be converted to long chain fatty acids (LCFA), which can greatly inhibit methanogens, although the LCFA inhibition eventually subsides once methanogens adapted to the

399 conditions (Carucci et al., 2005). In this case, further analysis of fat content of
400 these substrates would be beneficial in order to confirm the hypothesis.

401 <<<<<<<<<<<<<<< Insert **Fig. 2** here >>>>>>>>>>>>>>>

402 *3.4. Statistical analysis of methane yields*

403 The specific methane yields (ml/gVS) from the anaerobic digestion of
404 different substrates using AD sludge and rumen fluid as inocula are shown as
405 boxplot diagram in **Fig 3**. Maize was used in this BMP experiment as a
406 representative of typical energy crops. There was no significant difference ($P$ =
407 0.35 and 0.071, for sludge and rumen fluid, respectively) in methane yields
408 between the different CAM plants tested and maize ($P < 0.05$) as shown in **Figs.**
409 **3a. and 3b**. This suggests that CAM plants from different families could compete
410 with maize in terms of biomethane production and utilised as potential feedstocks
411 for anaerobic digestion, especially in arid and semi-arid regions where maize
412 grows poorly.

413 It is apparent that methane yields of all substrates inoculated with rumen fluid
414 were lower than the batch inoculated with AD sludge. *Opuntia*, which
415 demonstrated a considerable lag period of around 60 days (**Fig. 2d**), produced
416 significantly less methane ($P < 0.05$) than *Kalanchoe* (**Fig. 3b**). Non-paired t-tests
417 of each substrate also confirmed that methane yields obtained from most of the
418 substrates inoculated with AD sludge were significantly greater than the yields of
419 the same substrate employing rumen fluid as the inoculum (**Fig. 3c**). Only
420 *Euphorbia* and pineapple showed no statistical difference in terms of methane
421 yields between the two inocula ($P$ = 0.059 and $P$ = 0.1485, respectively). The
422 large standard deviations in methane yield of replicate samples of pineapple

clearly demonstrated a considerable variance in methane production. This suggests that despite replicates being treated in the same way, the bacterial community in each vial could behaved quite differently.

### *3.5. Kinetic analysis and prediction of biomethane yield*

Modified Gompertz model parameters were determined from the experiment methane yield data by using non-linear regression. All kinetic constants obtained are presented in **Table 2**. There was a direct influence on the lag phase by the inoculum activity. The values of lag phase, $\lambda$, for trials with AD sludge calculated (0.67–1.45 days) were considerably lower than those with rumen fluid (5.50–59.9 days) regardless of feedstocks. The modified Gompertz model could render negative values of lag phase, which are mathematically possible but have no biological meaning and therefore are omitted from **Table 2** (Morales et al., 2017; Nevot et al., 2008). The results suggested anaerobic digestion using AD sludge inoculum experienced a rapid initial methane production with negligible lag phase observed. This shorter lag phase duration for sludge may have been due to a greater methanogenic activity of sludge (**Table 1**) and acid accumulation in rumen fluid case. The values for methane production potential ($A$), were relatively lower in substrates inoculated with rumen fluid. Likewise, lower values for the maximum methane production rate ($\mu$) indicated slower production rates from the batch using rumen inoculum, which corresponded to the lag phase behaviour described earlier. The modified Gompertz model developed to predict methane yield ($Y_{pre}$) showed very good regression ($R^2$ = 0.93–1.00) for all cases except *Opuntia* in a rumen fluid batch ($R^2$ = 0.62). As shown in **Fig. 2d**, *Opuntia* experienced accumulated TVFA above 1g/l between day 25 and day 58, which resulted in a

drop in pH and inhibition of methane production during this period. Hence, the modified Gompertz model, which produced an extensive lag period of 59.9 days, did not fit well with the experimental methane profile of *Opuntia* and may not be applied to *Opuntia*.

**Table 2** also summarises the experimental methane yields ($Y_{exp}$) and predicted methane yields ($Y_{pre}$) for the different substrates and inoculum types, as well as the percentage differences of the two values for each substrate and inoculum pair. The range of differences in predicted and experimental yields were 0.55%–1.97% and 0.58%–7.64%, for sludge and rumen fluid, respectively. Marginal differences between experimental and predicted data were less than 10%, suggesting that the modified Gompertz model could be used to predict the BMP profile accurately (Raposo et al., 2009).

### *3.6. Comparison of overall performance between sludge and rumen fluid as inocula for anaerobic digestion of CAM plants*

#### *3.6.1. Biogas composition*

The above results confirmed that the two inocula sources resulted in differences in gas yield from the biomass feedstocks tested. In general, higher and more sustained biogas production was achieved in batch inoculated with sludge. After 7 days of digestion, the batch methane compositions were in the range 49.7%–59.2% in sludge compared to 17.5%–29.0% using rumen fluid. Methane percentages of the rumen fluid batch increased over time and reached 59.5 ± 3.3% on average by day 25. There was virtually no difference in methane compositions between sludge and rumen fluid by day 38, when methane production terminated in the case of batches inoculated with sludge. The average

biomethane percentages were 67.1 ± 0.8% and 66.0 ± 5.8%, in sludge and rumen fluid, respectively.

### 3.6.2. Volatile fatty acids (VFAs) production from CAM plants

Volatile fatty acids are the major products of the hydrolysis and acidogenesis steps and AD metabolic pathway could be studied by observing the VFA production profile. The total volatile fatty acid concentrations for individual substrates plotted in **Fig. 4** were subtracted from the total volatile fatty acid concentration of the uninoculated control; from henceforth, TVFA is replaced by sTVFA (subtracted total volatile fatty acid). The sTVFA concentrations and VFA percentages for individual substrates and inocula are shown in **Fig. 4**. sTVFA yields from substrates inoculated with rumen fluid were considerably higher compared to those of the sludge. As shown in **Fig. 4a**, using *Agave* as an example, the sTVFA concentrations in sludge and rumen fluid batches were 0.27 ± 0.02 mg/l and 0.22 ± 0.04 mg/l, respectively, after 24 hours of inoculation, however, there was substantial decline in sTVFA to almost zero in the sludge batch by day 7 whereas the sTVFA concentration in the batch using rumen fluid continued to rise and peaked at 1.14 ± 0.09 g/l on day 15. This difference in VFA production between the sludge and rumen fluid may have been a consequence of a greater proportion of hydrolytic and acidogenic microorganisms in rumen inoculum.

<<<<<<<<<<<<<<< Insert **Fig. 4** here >>>>>>>>>>>>>>>

Comparing the sTVFA levels of the plant species tested using rumen fluid, maize produced the lowest sTVFA throughout the digestion period. The prominent sTVFA difference between maize and *Agave* was detected on day 15

495 when the concentrations were 0.69 g/l and 1.14 g/l, respectively (**Fig. 4ii**). Maize
496 was the most fibrous of the substrates tested with the highest lignin percentage
497 (18.8 ± 2.2 %TS) (**Table 1**). It has been widely reported that hydrolysis rates are
498 slower for tough fibrous materials with higher lignin content, as they are more
499 difficult to degrade (e.g. Dechrugsa et al., 2013). Therefore, the greater
500 accumulation of VFA products from the CAM-plant substrates may have been a
501 function of the lower lignin content of this succulent biomass feedstock (**Table 1**).

502 Of the volatile fatty acids investigated, acetate was the predominant
503 compound produced in the first phase of digestion (**Fig. 4**). Acetic acid is an
504 important AD intermediate and a precursor of biomethane production. After 24
505 hours of inoculation, the substrates inoculated with AD sludge had a peak
506 production of acetate of 0.07–0.27 g/l (accounting for 86.3%–98.8% of sTVFA
507 production). A similar scenario was also observed with the CAM substrates
508 inoculated with rumen fluid, for which acetate concentrations were in the range
509 0.06–0.32 g/l (accounting for 86.3%–98.8% of sTVFA produced). There was no
510 VFA produced from maize on the first day (**Fig. 4b**) and the propionate and
511 butyrate present were derived solely from other organic matter occurring in the
512 rumen fluid. Since the sTVFA levels were very low or negligible from day 7
513 onwards, the following section focuses on the individual VFAs produced from
514 substrates inoculated with rumen fluid.

515 The acetic acid concentrations produced by all substrates by day 15 were
516 between 0.64 and 1.04 g/l. It has been reported that propionic acid degradation
517 can be inhibited by acetic acid concentrations exceeding 0.50 g/l (Gorris et al.,
518 1989; Mawson et al., 1991), which may account for the small changes in propionic

519 acid concentrations from day 1 until day 25 for most of the substrates examined,
520 with exception of *Euphorbia* and *Opuntia*.

521 The sequence of accumulated VFAs was similar for all substrates and could
522 be described as (1) acetic acid accumulation from inoculation day until day 15 (2)
523 butyric acid accumulation by day 25, and (3) propionic acid accumulation by day
524 38. Conversion of VFAs to other products was achieved by a series of
525 biochemical reactions performed by acidogens and acetogens. Propionate,
526 butyrate and valerate were degraded through proton-reducing acetogenic
527 pathways or the homoacetogenic pathway to acetate, which was further
528 consumed by acetoclastic methanogens to produce methane. Propionic acid
529 degradation was reported to be the most thermodynamically unfavourable in the
530 AD system (Shi et al., 2017). The accumulation of acetate may have been a
531 consequence of the production rate exceeding its degradation by acetoclastic
532 methanogens, possibly because the high acetic acid concentration inhibited
533 methanogenic growth (Amani et al., 2011). This acetic acid accumulation caused
534 a decrease in pH (**Fig. 2**) during the first 18 days of incubation. This pH drop also
535 inhibited degradation of propionate and butyrate. As the retention time increased,
536 microorganisms were able to adapt to low pH and the removal efficiency of
537 acetate consequently increased. This was followed by conversion of butyrate, a
538 process which is not as thermodynamically unfavourable as propionate oxidation.
539 Propionate was therefore the last remaining VFA species before it was reduced
540 to acetate and methane.

541 Overall, sludge outperformed rumen fluid in terms of methane production,
542 regardless of substrate type. The main reasons of poor AD processing using

543 rumen fluid as inoculum could be the high initial VFA concentrations of the fluid,
544 which led to acid accumulation and consequently inhibited methane production
545 (2.25 ± 0.09 g/l, as shown in **Table 1**). Rapid substrate hydrolysis after
546 inoculation, which further amplified the effect of VFA accumulation, may also halt
547 methanogenic functions of other bacteria in the community. Pre-incubation of
548 inoculum is sometimes performed to minimised the endogenous methane
549 contributed by the inoculum to avoid biasing the BMP result (Elbeshbishy et al.,
550 2012). In this case, pre-incubation of rumen fluid may be recommended to reduce
551 the initial VFA level in the fluid and lessen the impact of acid accumulation which
552 would shorten the lag phase duration and methane production during anaerobic
553 digestion. However, non-incubated fresh rumen fluid might be employed for
554 substrate pre-treatment or VFA production to exploit the synergies between
555 bacterial communities specialised in substrate hydrolysis.

556 **4. Conclusions**

557 The methane yields achieved from the CAM substrates are at least as high as
558 those obtained from maize, which is one of the widely used feedstocks for AD.
559 Sludge was shown to have higher methanogenic activity than rumen fluid,
560 however, the higher hydrolytic activity of rumen fluid is favourable for VFA
561 production. Until now, the ability to convert CAM crops to both biomethane and
562 volatile fatty acids has received very little research attention. With over 16,000
563 species to further explore, CAM plants could become highly attractive as new
564 potential feedstocks for bioenergy and biorefinery industries.

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## 6. Appendices A., B., C., D. and E. Supplementary data

E-supplementary data associated with this article can be found in the online version of the paper

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773 **Table 1**

774 Biomass characteristics of plant samples and inocula. Values are means of three samples ± standard deviations

| Characteristics[1] | Agave | Euphorbia | Kalanchoe | Opuntia | Pineapple | Maize | AD sludge | Rumen fluid |
|---|---|---|---|---|---|---|---|---|
| **%TS (%FM)** | 8.51 ± 0.04 | 17.55 ± 0.48 | 9.96 ± 0.06 | 9.46 ± 1.73 | 17.52 ± 0.41 | 15.63 ± 0.57 | 4.50 ± 0.02 | 2.99 ± 0.02 |
| **%VS/TS** | 67.19 ± 0.54 | 88.32 ± 0.22 | 80.23 ± 0.80 | 85.75 ± 2.97 | 85.68 ± 0.65 | 95.05 ± 0.60 | 67.22 ± 0.01 | 60.10 ± 0.22 |
| **Crystalline cellulose (%TS)** | 27.39 ± 6.04 | 27.63 ± 1.04 | 20.28 ± 2.18 | 16.51 ± 1.34 | 32.56 ± 3.71 | 39.92 ± 4.32 | - | - |
| **Lignin (%TS)** | 14.42 ± 3.85 | 13.89 ± 3.64 | 8.09 ± 2.83 | 8.26 ± 2.50 | 17.35 ± 2.28 | 18.81 ± 2.19 | - | - |
| **Matrix polysaccharide (mg/g)** | 11.37 | 12.10 | 2.41 | 8.70 | 10.09 | 9.46 | - | - |
| **Initial pH** | - | - | - | - | - | - | 8.02 | 5.94 |
| **Inoculum activity (ml/gVS)** | - | - | - | - | - | - | 355.3 ± 20.5 | 189.4 ± 16.1 |
| **Acetic acid (g/l)** | - | - | - | - | - | - | 0.42 ± 0.12 | 4.78 ± 0.23 |
| **Propionic acid (g/l)** | - | - | - | - | - | - | 0.08 ± 0.01 | 2.72 ± 0.11 |
| **isobutyric acid (g/l)** | - | - | - | - | - | - | - | 0.16 ± 0.02 |
| ***n*-butyric acid (g/l)** | - | - | - | - | - | - | - | 2.51 ± 0.10 |
| **isovaleric acid (g/l)** | - | - | - | - | - | - | - | 0.59 ± 0.08 |
| ***n*-valeric acid (g/l)** | - | - | - | - | - | - | - | 0.49 ± 0.03 |

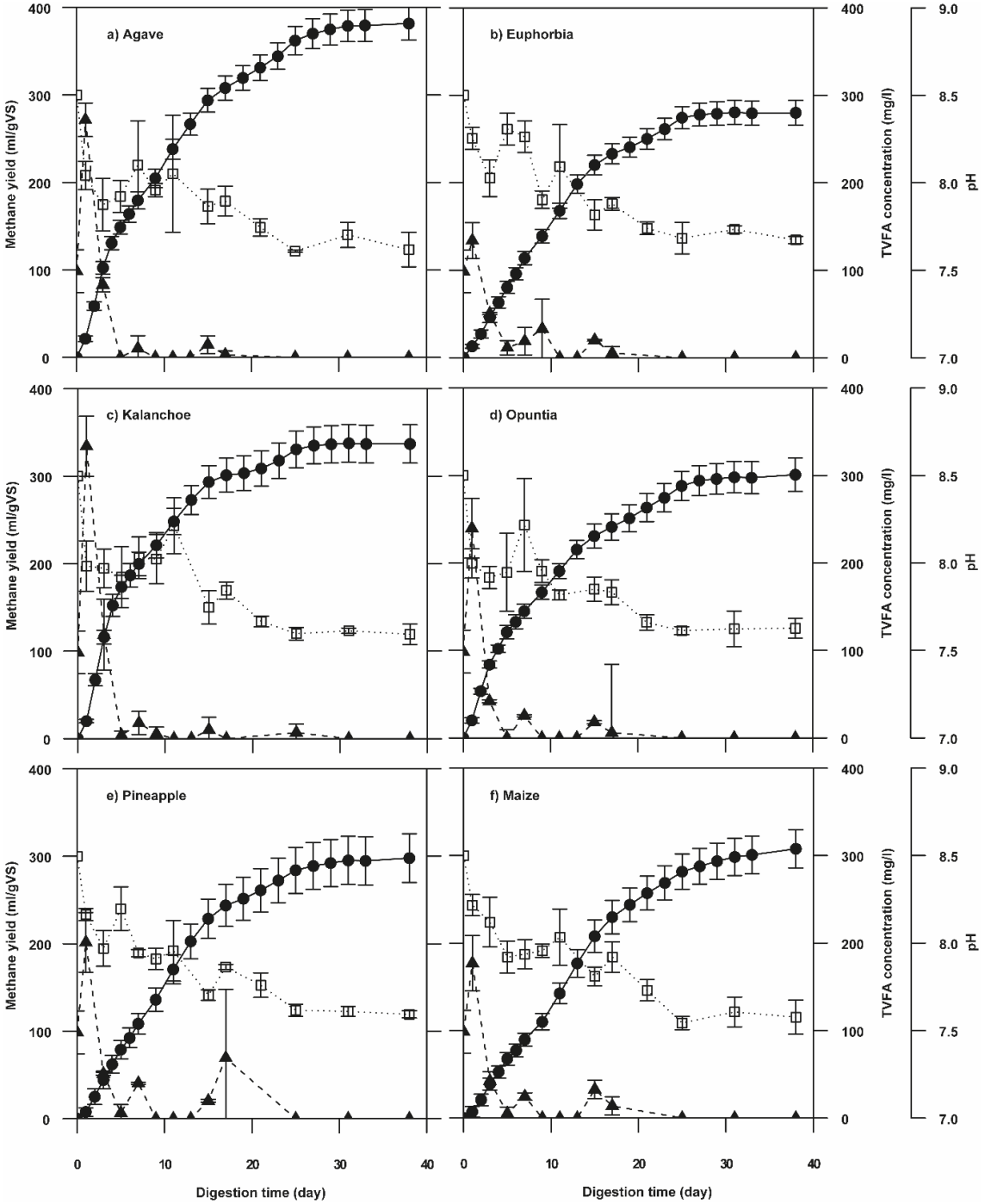
775 [1]FM – Fresh mass; TS – Total solid; VS – Volatile solid; Inoculum activity refers to ml $CH_4$ produced per gVS

**Table 2**

Kinetic parameters of a modified Gompertz model obtained from anaerobic digestion with different inoculum types and comparison between experimental and predicted methane yields from the model

| Inoculum type | Plant materials | Parameters from Modified Gompertz model | | | $R^2$ | RMSE | $Y_{pre}$ (ml/gVS) | $Y_{exp}$ (ml/gVS) | %Δ |
|---|---|---|---|---|---|---|---|---|---|
| | | $A$ (ml/gVS) | $\mu_m$ (ml/gVS/d) | $\lambda$ (d) | | | | | |
| **Sludge** | **Agave** | 376.9 | 21.18 | - | 0.98 | 16.30 | 374.3 | 381.7 | 1.97% |
| | **Euphorbia** | 283.4 | 17.08 | 0.67 | 1.00 | 5.74 | 281.7 | 280.5 | 0.44% |
| | **Kalanchoe** | 326.1 | 26.54 | - | 0.97 | 17.23 | 325.9 | 337.6 | 3.45% |
| | **Pineapple** | 298.3 | 17.75 | 1.02 | 1.00 | 5.79 | 296.3 | 297.9 | 0.55% |
| | **Opuntia** | 296.9 | 16.81 | - | 0.98 | 12.77 | 294.9 | 300.8 | 1.95% |
| | **Maize** | 312.5 | 15.71 | 1.45 | 1.00 | 5.17 | 306.8 | 307.8 | 0.34% |
| **Rumen** | **Agave** | 231.7 | 2.77 | 5.50 | 0.95 | 13.61 | 192.4 | 184.4 | 4.22% |
| | **Euphorbia** | 174.3 | 4.72 | 7.69 | 0.95 | 14.81 | 173.0 | 177.6 | 2.63% |
| | **Kalanchoe** | 261.1 | 3.41 | 4.60 | 0.93 | 20.32 | 226.8 | 210.2 | 7.64% |
| | **Pineapple** | 183.4 | 3.36 | 5.65 | 0.95 | 14.38 | 175.3 | 168.2 | 4.14% |
| | **Opuntia** | 97.0 | 8.81 | 59.96 | 0.62 | 20.75 | 96.8 | 97.3 | 0.58% |
| | **Maize** | 152.6 | 4.17 | 14.11 | 0.95 | 13.56 | 150.9 | 143.1 | 5.27% |

[1] $A$ – Methane production potential; $\mu_m$ – Maximum methane production rate; $\lambda$ – Lag phase time; RMSE – Root mean square error; $Y_{pre}$ – Predicted methane yield after 40 days; $Y_{exp}$ – Experimental methane yield after 40 days; %Δ – Percentage difference between predicted and experimental methane yields




781
782 **Fig. 1.** BMP assay curves showing cumulative methane yield (ml/gVS) from different substrates
783 using AD sludge as inoculum. (●), (□), and (▲) denote the experimental methane yield, pH, and
784 total volatile fatty acid (TVFA) concentration, respectively. Error bars denote standard deviations
785 (*n* = 3). The methane yield of the substrate (ml/gVS) is determined by subtracting the methane
786 production of the blank from the methane production of the substrate.

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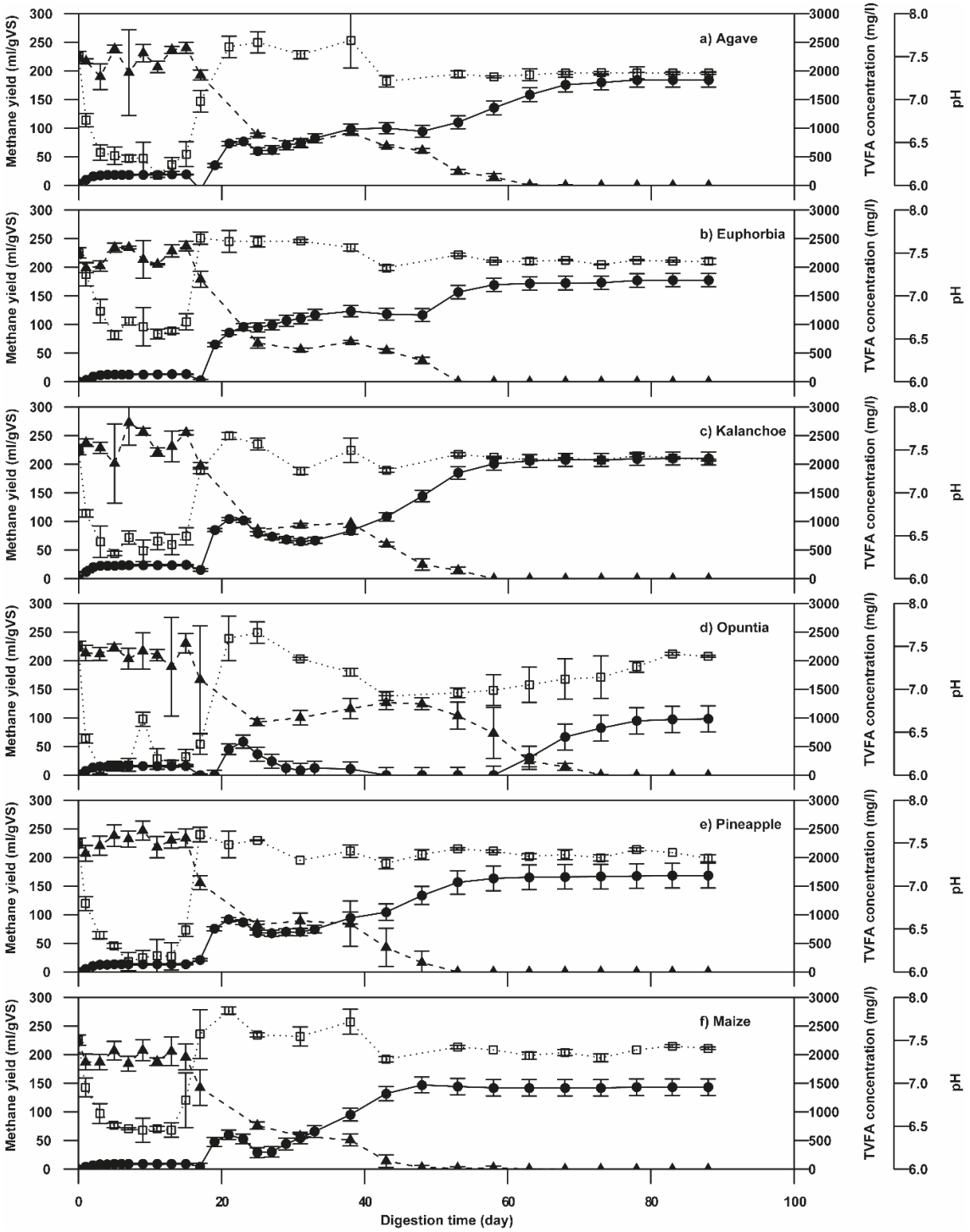


791

792 **Fig. 2.** BMP assay curves showing cumulative methane yield (ml/gVS) from different substrates
793 using rumen fluid as inoculum. (●), (□), and (▲) denote the experimental methane yield, pH, and
794 total volatile fatty acid (TVFA) concentration, respectively. Error bars denote standard deviations
795 (*n* = 3). The methane yield of the substrate (ml/gVS) is determined by subtracting the methane
796 production of the blank from the methane production of the substrate.

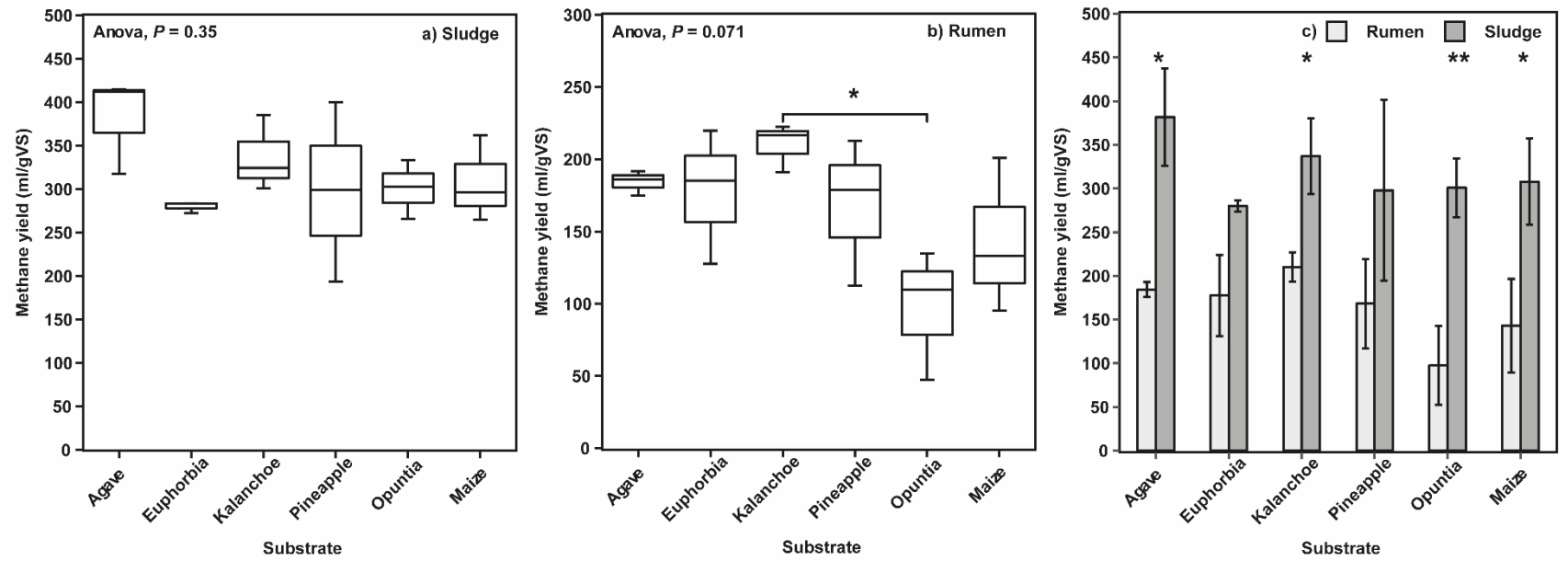


**Fig. 3.** a) and b) Box plots summarising the specific methane yields of different substrates between two different inoculum types. The bold horizontal line crossing each box represents median; the top and bottom of the box refer to the upper and lower quartiles. The maximum and minimum values are represented by the whiskers. c) T-test comparison of mean methane yields between rumen fluid and sludge. * and ** denote significant differences ($P < 0.05$ and $P < 0.01$, respectively). Error bars denote standard deviations ($n = 3$).

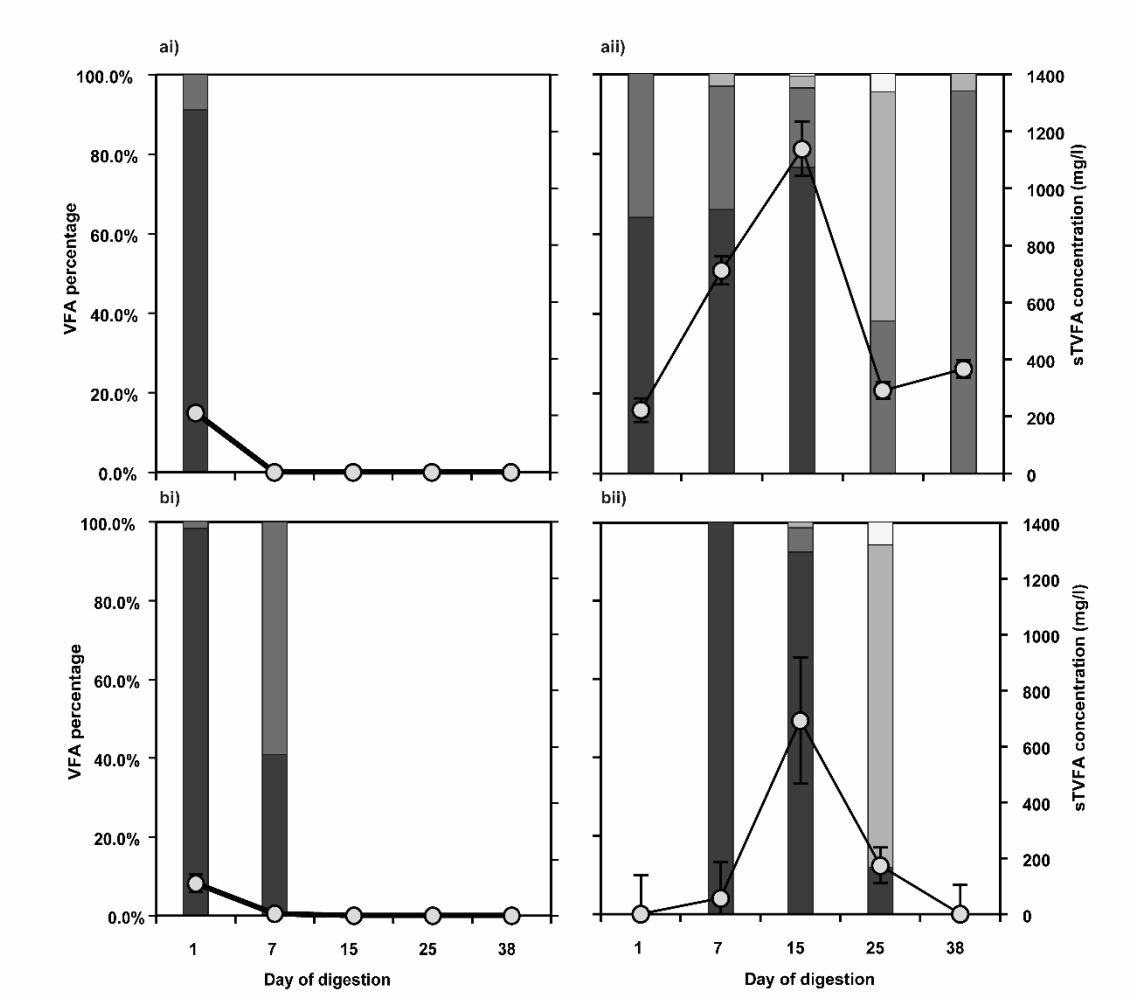


**Fig. 4.** sTVFA concentration profiles on selected days obtained from anaerobic digestion of (a) *Agave* and (b) maize using (i) AD sludge and (ii) rumen fluid as inoculum are presented as percentages of acetate (■), propionate (■), *n*-butyrate (■), *n*-valerate (□), and concentration in mg/l of sTVFA (—○—). Error bars denote standard deviations (*n* = 3)