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Anaerobic co-digestion of *Euphorbia tirucalli* with pig blood for volatile fatty acid production

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ABSTRACT

Acidogenic fermentation of biomass to produce volatile fatty acids provides a renewable pathway to industrial chemicals ordinarily derived from petrochemicals. Crassulacean acid metabolism plants such as *Euphorbia tirucalli* are cultivable on marginal land and offer promising feedstocks for this purpose. This study investigated how the refining of *E. tirucalli* biomass to fatty acids could be augmented with a high-protein co-substrate, pig blood. Blood mono-digestions provided the highest titres of total fatty acid (up to 38 ± 2 g/L), while at high substrate concentrations, acetic acid was maximal in co-digestions. 75 % blood with 25 % *E. tirucalli* produced acetic acid titres 40.8 % ($p < 0.001$) and 30.8 % ($p = 0.001$) higher than those in mono-digestions of *E. tirucalli* and blood, respectively. Where acetate is the desired product, inclusion of blood as a co-substrate offers significant benefit for *Euphorbia* biorefining.

1. Introduction

Against a backdrop of accelerating climate change, renewable alternatives to petrochemicals are increasingly being sought. Anaerobic digestion (AD) is commonly employed to produce biomethane but may instead be configured to refine biomass into volatile fatty acid (VFA) from which industrial chemicals can be produced. VFAs generated via acidogenic fermentation (AF), and derivatives thereof, may be used to produce oil/biodiesel (Christophe et al., 2012), polyhydroxyalkanoate bioplastics (Mengmeng et al., 2009) and other bulk chemicals conventionally derived from fossil carbon (Lee et al., 2018; Zhou et al., 2020). This bypasses the need to derive these petrochemically.

While the cultivation of dedicated crops can provide an abundant feedstock for this purpose, this approach is criticised for its use of arable land otherwise needed to feed a still growing population. Feedstocks that do not compete with conventional agriculture, or that derive from the by-products of existing processes, are therefore desirable. Crassulacean acid metabolism (CAM) plants employ a unique form of photosynthesis, distinct from the C3 and C4 pathways of most common food crops, which allows them to readily adapt to restricted water availability. They have thus attracted attention as potential AD substrates, due to their cultivability in semi-arid areas ill-suited to conventional agriculture (Buckland and Thomas, 2021; Mason et al., 2015). CAM biomass is also notable for its relatively low lignin content (Lueangwattanapong et al., 2020). Lignin, a major constituent of lignocellulosic biomass, is highly inhibitory to hydrolytic processes in the AD bioreactor. This naturally lends CAM biomass to refining. While CAM photosynthesis is relatively widespread, occurring in an estimated 16,000 species (Winter and Smith, 2012), two plants in particular, *Euphorbia tirucalli* (Indian tree-spurge) and *Opuntia ficus indica* (Prickly pear), are able to yield considerable biomass under severe water restriction, and thus merit further investigation (Krümpel et al., 2020; Mason et al., 2015).

Despite these advantages as a feedstock, the introduction of a protein rich co-substrate might nonetheless allow for process optimisation. This co-digestion approach was recently shown to improve biomethane yields from AD of *Opuntia*, with slaughterhouse wastewaters providing the high-protein co-substrate (Panizio et al., 2020).

Slaughterhouse by-products, such as blood, offal, and wastewaters have attracted considerable attention as potential AD substrates, with blood having recently been reviewed as a substrate for biomethane production (Nazifa et al., 2021). The low C/N ratios of these substrates makes them ill-suited to methanogenic AD in a mono-digestion mode, with several authors recommending their use as co-substrates with high-carbon, or low-strength substrates (Hejnfelt and Angelidaki, 2009; Jensen et al., 2014). Successful methanogenic AD of meat wastes in a co-digestion format has been demonstrated with high carbon substrates such as paunch grass (Astals et al., 2014), maize (Cuetos et al., 2013) and

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Opuntia (Panizio et al., 2020). Slaughterhouse wastes have been shown to greatly improve stability of AD of carbohydrate-rich feedstocks, due to the pH-buffering effect of the ammonium released from their degradation (Miramontes-Martínez et al., 2021).

Substrate concentrations are another key parameter for optimisation in AD. Under conventional methanogenic AD, substrate concentrations must be kept low to prevent an accumulation of degradation products, mainly fatty acids and ammonium, which can inhibit methanogenesis (Abbassi-Guendouz et al., 2012). Under acidogenic AD, however, methanogenesis is undesirable and high VFA concentrations are instead favourable, as they allow for simplified product extraction. This freedom allows acidogenic reactors to be optimised over a wider parameter space of substrate concentrations than when biogas is the desired product. Despite this, a balance must still be struck between the increased titres achievable at higher substrate loadings, and the reduced yields of VFA (per unit of substrate) which have been documented at higher substrate concentrations. This decline in yields at higher loadings has been attributed to factors such as reduced hydrolytic efficiency and product inhibition (Abbassi-Guendouz et al., 2012; Bermúdez-Penabad et al., 2017; Pratt et al., 2012).

The composition of the resultant VFA is a further consideration in acidogenic AD. Unlike the relatively uniform product of methanogenic AD, biogas, acidogenic AD yields a mixture of fatty acids of different carbon chain lengths, typically ranging in size from 2 to 6 carbon compounds. The composition of any VFA produced will be highly responsive to reactor parameters and must be considered in conjunction with total VFA titres and yields when evaluating the suitability of a given reactor configuration (Jiang et al., 2013; Tepari et al., 2020). From an economic perspective, longer chain fatty acids typically command higher market prices as bulk chemicals and are cheaper to extract due to their higher hydrophobicity. These however typically constitute only a minority of the VFA produced. VFA-rich digestates are often applied as carbon sources for biological nitrogen removal from wastewater. As denitrifying bacteria preferentially utilize shorter classes of VFA in this process, here acetate is most desirable (Elefsiniotis and Wareham, 2007). The desired VFA composition will thus depend on the specific needs of the intended downstream applications of the acids being produced.

The objective of this study is thus to understand whether co-feeding with pig blood has any effect on the quantity or quality of VFA produced from *Euphorbia*. Feedstocks were tested at various concentrations and ratios. While co-digestion did not significantly improve overall TVFA titres, VFA composition was changed markedly. Acetate production benefitted significantly from co-feeding with blood.

2. Materials and methods

2.1. Biomass and inoculum

Euphorbia tirucalli biomass was obtained from the Royal Botanic Garden, Kew, United Kingdom, in dried form. This dry material was ground in a blender (Waring, USA) and passed through a screen (no. 10 mesh - 1 mm) to ensure a maximum particle size of 1 mm. Food-grade, dried pig blood was obtained from Tongmaster Seasonings (Coatbridge, United Kingdom).

The inoculum used was anaerobic digestion sludge from a local biogas plant processing food waste and operating under mesophilic conditions (M&M Waste Solutions, Cassington, Oxford, United Kingdom). This was stored at 30 °C until used. The inoculum was deployed within 4 days of collection.

2.2. Anaerobic batch assays

Synthetic basic medium was prepared according to the recommendations of Angelidaki and Sanders (2004), but with the omission of sodium sulphide. Medium was used to provide the requisite balance of micronutrients, trace metals, vitamins, and growth factors for robust microbial growth.

Batch assays were performed in 250 mL serum vials with a 150 mL working volume. The reaction mixture consisted of 20 mL inoculum sludge and 130 mL of microbial growth medium. Bromoethane sulfonate (BES - Sigma Aldrich, USA) was added to a final concentration of 10 mM to inhibit methanogenesis. The pH of the bulk reaction mixture was adjusted to 8.1 ± 0.1 before addition to serum vials into which the appropriate dried substrates had previously been added. Adjustment of initial pH to mildly alkaline was performed to provide a small amount of buffering capacity to allow VFA accumulation to occur. Five initial substrate concentrations were prepared: 100 % blood, co-digestions of 25/75 %, 50/50 %, and 75/25 % blood to *Euphorbia* on a volatile solids (VS) basis, and 100% *Euphorbia*. Each of these conditions was prepared to three initial substrate concentrations (ISC) of 10, 30, and 60 g-VS/L. Vials were sealed and capped with rubber septa and aluminium crimp-caps and mixed by inversion. In this experiment, the headspace gas was not purged to remove residual oxygen. A sludge-only control, with no added substrate, was prepared as a blank. As the residual volatiles were not purged from the inoculum, this blank was subtracted from all treatment conditions to control for the effect of residual volatiles in the inoculum.

A cellulose control (at 10 g-VS/L microcrystalline cellulose - Avicel, Sigma-Aldrich, USA) was used to validate inoculum activity. All sample and control conditions were prepared in triplicate. Vials were incubated under mesophilic (37 °C) conditions, with constant agitation at 125 rpm.

2.3. Analytical methods

The inoculum and both substrates were characterised by standard methodologies (APHA, 1998). Determinations of cellulose, hemicellulose, and lignin content were performed according to the methodologies of Rezende et al. (2018).

The biogas produced was measured by gas displacement of water in a eudiometer. Gas composition was measured using a gas chromatography device (Shimadzu – 2010, Shimadzu, Japan) equipped with a thermal conductivity (TCD) detector and 2 m × 0.53 mm 'ShinCarbon ST 80/100' (Restek) packed column. Helium provided the carrier gas at a flow rate of 3.39 mL/min. Injector, oven, and detector temperatures were set to 150, 75, and 200 °C, respectively. Biogas volumes were corrected to standard temperatures and pressures (273.15 K, 101.3 kPa) according to the method of Richards et al. (1991).

The pH of liquid samples was determined immediately upon sample collection using a pH probe (ThermoScientific, USA). To analyse VFA concentrations, liquid samples were centrifuged at 30,000 ×*g* for 12 min and the supernatant collected into 1.5 mL GC vials. Samples were acidified by 1:1 dilution in 10 % formic acid. VFA analysis was performed using a gas chromatography device (Shimadzu-2010) equipped with a flame-ionisation (FID) detector and 30 m × 0.25 mm × 0.25 μm fused silica column (nitroterephthalic acid-modified polyethylene glycol-phase) (ZB-FFAP). Injector and detector temperatures were 250 and 350 °C, respectively. The oven was set to 100 °C for 2 min, ramped at 8 °C/min to 150 °C, and held at 150 °C for 2 min. Acetic, propionic, isobutyric, butyric, isovaleric, valeric, and hexanoic acids were quantified against standards. Total VFA (TVFA) was calculated as the sum of these.

Concentrations of soluble protein were determined using a modified bicinchoninic acid (BCA) assay (Pierce Modified BCA Protein Assay Kit - ThermoScientific). Protein concentrations were provided by measurement of absorbance at 480 nm on a plate reader (Tecan, Switzerland) and quantified against a BSA standard curve.

Ammonium nitrogen was measured spectrophotometrically using a kit-based assay (HACH Lange - LCK303 kit) and DR2800 spectrophotometer.

2.4. Statistical analysis

All data herein are presented as means ± standard deviations of triplicate measurements. All statistical analyses were calculated, and all models fitted, in Microsoft Excel and in GraphPad PRISM. All figures presented herein were prepared in GraphPad PRISM.

3. Results and discussion

3.1. Characterisation of substrates and inoculum

3.1.1. Characterisation of substrates

The compositions of the two substrates used herein are given in Table 1. As substrates were obtained in dried form, TS/FW (Total Solids/Fresh Weight) values were not biologically relevant and have been omitted. It should be noted that while dried substrates were used, literature values for the total solids content of livestock blood sit in the range of ~10–20 %, or 100–200 gTS/L (Nazifa et al., 2021). Accordingly, the working concentrations used in this study (up to 60 g-VS/L) would all be achievable in practice through simple dilution of fresh substrate.

VS/TS gives the fraction of volatile, or degradable content in the solid fraction. A high VS/TS is a desirable trait in a feedstock as a greater proportion of the solid material is available for conversion to product (Li et al., 2013). The calculated VS/TS of *Euphorbia tirucalli* (82.55 ± 0.34 %) is close to literature values for other CAM plants – with literature values of 88.32 and 85.75 % for *Euphorbia virosa* stem and *Opuntia fragilis* cladodes, respectively (Lueangwattanapong et al., 2020). Notably in this study, the lignin content of *Euphorbia tirucalli* biomass was found to be just 3.65 %, considerably lower than literature values for other lignocellulosic biomass such as corn stover (18.8 %) (Yang et al., 2016) and wheat straw (18.52 %) (Zeng et al., 2013), and of the CAM plants agave (14.42 %), *Euphorbia virosa* (13.89 %), and pineapple (17.35 %) (Lueangwattanapong et al., 2020).

Total Organic Carbon (TOC) and Total Kjeldahl Nitrogen for *Euphorbia* biomass were calculated as 40.80 and 1.18 %, respectively, giving a C:N ratio of 34.60. This is a moderate C:N ratio, falling near the range of 20–30 widely used for conventional anaerobic digestion (Wang et al., 2012).

Pig blood was calculated to have a VS/TS of 96.37 ± 0.03 %, in agreement with the value of 95.79 % (Kovács et al., 2013). TOC and TKN values of 34.10 and 14.80 %TS, respectively gave a low C:N ratio of just 2.3.

Table 1
Biomass characteristics of substrates and inoculum. All values are means ± standard deviations of triplicate measurements.

Characteristic[a]	*Euphorbia*	Blood	AD sludge
TS/FM (%)	N/A	N/A	4.77 ± 0.13
VS/TS (%)	82.55 ± 0.34	96.37 ± 0.03	64.75 ± 0.10
Cellulose (%TS)	27.94	–	–
Hemicellulose (%TS)	3.81	–	–
Lignin (%TS)	3.65	–	–
Crude Protein (%TS)	7.38	92.63	3.69
Total Organic Carbon (%TS)	40.80	34.10	33.50
Total Kjeldahl Nitrogen (%TS)	1.18	14.82	0.59
Carbon to Nitrogen Ratio (*w*/w)	34.58	2.30	56.78
Initial pH	–	–	8.02
Acetic acid (g/L)	–	–	0.17 ± 0.02
Propionic-hexanoic acids (g/L)	–	–	0.00 ± 0.00

[a] TS, FM, and VS, refer to total solids, fresh matter, and volatile solids, respectively.

3.1.2. Characterisation of inoculum

Characteristics of the AD sludge inoculum are provided in Table 1. AD sludge was found to have a total solids content of 4.77 ± 0.13 %, of which 64.75 ± 0.10 % was volatile solids. TOC and TKN values of 33.50 and 0.59 % gave a C:N ratio of 56.78. This high ratio is within expectation for a commercial AD facility processing municipal food waste, which is a homogeneous feedstock, the C/N ratio of which can vary widely depending on its constituent materials (Salangsang et al., 2022). The inoculum was additionally found to have low concentrations of VFA (at 0.17 ± 0.02 g/L acetic with no C3-C6 acids detectable), and an initial pH of 8.02, consistent with the low VFA concentrations sought in methanogenic reactors.

3.2. Acidogenic anaerobic digestion of Euphorbia and blood by AD sludge

3.2.1. Acidification profiles of Euphorbia, blood, and co-digestions thereof

Reactor pH and concentrations of VFA and soluble protein over the 16-day time-course are shown in Fig. 1. Blood-rich and *Euphorbia*-rich digestions generated distinct profiles of VFA accumulation, the former showing more rapid initial VFA production in days 0–5 than the latter,

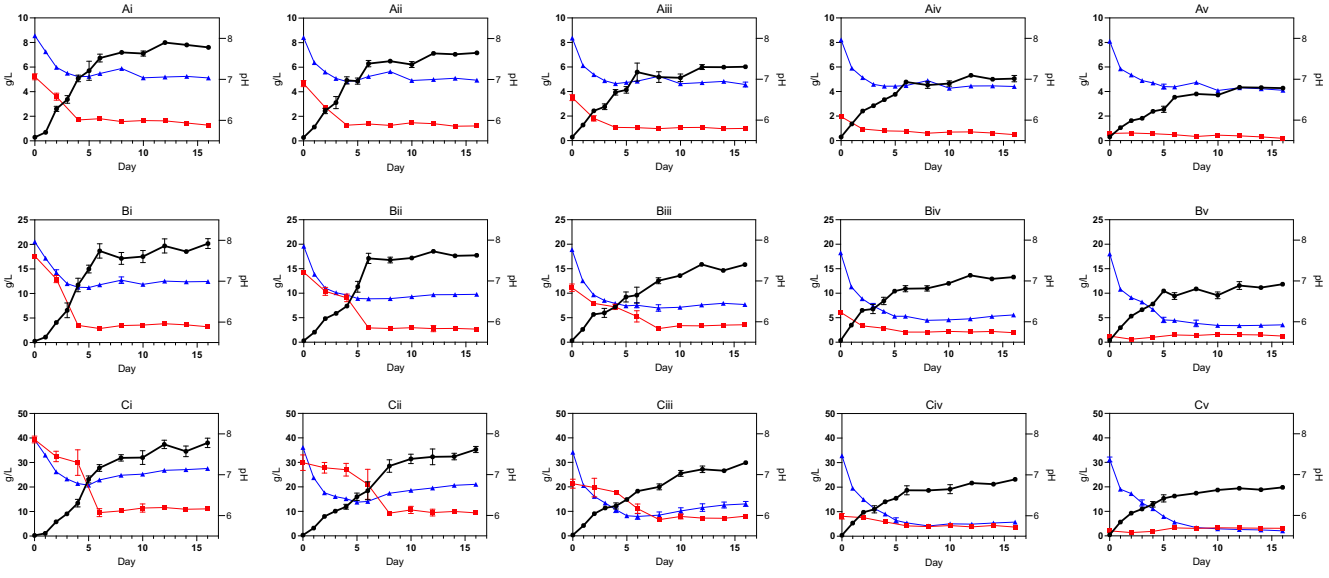


Fig. 1. Acidification time-courses of *Euphorbia* and blood by AD sludge in mono- and co-digestion formats. Total volatile fatty acid concentrations (g/L), soluble protein concentrations (g/L), and pH are denoted by ●, ■, and ▲, respectively. All values are given as means ± standard deviations of triplicate measurements. A, B, and C denote initial substrate concentrations of 10, 30, and 60 g-VS/L. Substrate ratios (*Euphorbia* to blood of 0:100, 25:75, 50:50, 75:25 and 100:0 (% VS)) are indicated by i, ii, iii, iv, and v, respectively.

likely due to differences in substrate degradability. Differences in the availability of soluble substrate between sample groups can be seen in the soluble protein concentrations between reactors. Soluble protein concentrations rapidly declined over the first week as substrate was acidified. In conditions where soluble protein accounted for a large proportion of substrate volatiles at day 0, this depletion of soluble substrate accounted for the fast accumulation of VFA observed. Other studies have also reported maximal VFA titres at short retention times (<7 days) when a large proportion of substrate volatiles were present in a soluble form (Zhang et al., 2005). Also of note in Fig. 1 is the decoupling of VFA concentrations from pH. At each substrate concentration, blood-rich mono-digestions showed both the highest TVFA concentrations and yet also the most alkaline pH. This observation is likely the result of the release of ammonium from protein degradation, with NH_3 and NH_4^+ providing a buffer pair which checks the acidifying effect of VFA release in protein-rich digestions (refer to endpoint ammonium concentrations in Table 2). This and other ammonium-mediated buffering mechanisms have been implicated in improved AD efficiency at moderate C/N ratios (Miramontes-Martínez et al., 2021).

3.2.2. Total VFA production

Total VFA concentrations (titres) at experiment termination at day 16 are given in Table 2. Endpoint VFA titres were highest in blood mono-digestions and showed a monotonic decrease with increasing *Euphorbia* fraction. This decreasing trend extended to VFA yields (Fig. 2C). This monotonic, linear decrease in yields with increasing *Euphorbia* content, visualised in Fig. 2C indicates an absence of synergistic effects of co-digestion on overall VFA yields.

Declining yields accompanied increased substrate concentrations in all sample groups in this study. Blood mono-digestions gave VFA yields of 0.65 ± 0.01, 0.58 ± 0.03, and 0.54 ± 0.03 g-VFA/g-VS at substrate concentrations of 10, 30, and 60 g/L-VS, respectively, while the corresponding *Euphorbia* digestions yielded 0.38 ± 0.01, 0.35 ± 0.01, and 0.29 ± 0.01 g/L-VS. The above values additionally demonstrate that VFA yields were affected more by substrate composition than by substrate concentration. The discrepancy between the yields of blood and *Euphorbia* is again likely explained by the relative availability of soluble substrate at day 0, rather than a preference of acidification at low C/N ratios. Reactors fed blood at 60 g/L-VS had soluble protein concentrations at day 0 of 39 ± 1 g/L, as compared to just 2.1 ± 0.3 g/L for those fed equivalent amounts of *Euphorbia*. This disparity in soluble substrate likely accounts for a large part of the observed difference in yields.

Product-inhibition by VFA may also have influenced observed yields. The acidification of amino acids, whether by Stickland or alternative mechanisms, released ammonium which raised reactor pH. This partially compensated for the acidifying effect of VFA release. At low pH, VFAs are disproportionately present in their undissociated forms and more easily traverse the cell membrane of microbes, inhibiting growth and leading to greater product inhibition than at higher pH (Babel et al., 2004). The higher pH detected in the protein-fed digestions may thus have lessened VFA product-inhibition. In *Euphorbia*-fed conditions, lacking the compensatory effect of ammonium release (see Table 2 and Fig. 2), higher VFA toxicity may have contributed to lower yields. It should be noted that acidogenic reactors are often run at a mildly acidic pH of ~6 to minimise VFA losses to methanogenesis. In this study, methanogenesis was inhibited by BES (see Table 2 gas data), which masked the yield improvements a mildly-acidic pH might have otherwise provided. Endpoint gas data in Table 2 also show that at each substrate concentration, *Euphorbia*-fed conditions produced more biogas, as CO_2, than did blood-fed conditions. This "mis"-allocation of carbon to biogas may additionally account for the lower VFA yields of *Euphorbia*-fed conditions.

Co-digestion of *Euphorbia* with blood did not produce the increase in total VFA yields expected, which conflicts with some evidence in the literature for its benefits to anaerobic fermentation. Several mechanisms have been proposed to explain synergistic interactions between co-

Table 2
Batch reactor endpoint parameters at day 16. All values are given means ± standard deviations of triplicate measurements.

Initial substrate concentration (g-VS/L)	*Euphorbia*: blood (%VS)	pH	TVFA (g/L)	Acetic acid (g/L)	TVFA yield (g-VFA/g-VS)[a]	Ammonium (g/L)	Cumulative biogas produced (mL)	Headspace gas composition (CH_4, CO_2%v/v)	
10	0:100	7.04 ± 0.02	7.6 ± 0.1	4.07 ± 0.06	0.65 ± 0.01	2.1 ± 0.1	66 ± 2	4.6 ± 0.6,	57.7 ± 0.6
	25:75	6.98 ± 0.02	7.2 ± 0.2	3.9 ± 0.1	0.62 ± 0.02	1.6 ± 0.1	88.9 ± 0.8	5.6 ± 0.6,	66 ± 5
	50:50	6.87 ± 0.06	6.0 ± 0.1	3.5 ± 0.1	0.52 ± 0.01	1.08 ± 0.08	103 ± 3	3.7 ± 0.6,	73 ± 1
	75:25	6.82 ± 0.04	5.1 ± 0.3	3.1 ± 0.2	0.44 ± 0.02	0.6 ± 0.1	115.5 ± 0.8	1.3 ± 0.6,	79.9 ± 0.5
	100:0	6.73 ± 0.03	4.28 ± 0.04	2.50 ± 0.05	0.38 ± 0.01	0.08 ± 0.04	127 ± 2	n. d.	82 ± 3
30	0:100	6.99 ± 0.01	20 ± 1	8.4 ± 0.3	0.58 ± 0.03	6.0 ± 0.4	248 ± 3	n. d.	96.4 ± 0.6
	25:75	6.67 ± 0.04	17.7 ± 0.4	9.5 ± 0.3	0.51 ± 0.01	4.5 ± 0.1	302 ± 3	n. d.	95 ± 3
	50:50	6.42 ± 0.05	15.9 ± 0.4	9.0 ± 0.5	0.46 ± 0.01	2.7 ± 0.1	347 ± 3	n. d.	99 ± 2
	75:25	6.17 ± 0.03	13.3 ± 0.4	8.1 ± 0.2	0.39 ± 0.01	1.4 ± 0.1	407 ± 4	n. d.	99 ± 7
	100:0	5.93 ± 0.03	11.8 ± 0.2	7.0 ± 0.4	0.35 ± 0.01	0.08 ± 0.03	412 ± 5	n. d.	90 ± 10
60	0:100	7.15 ± 0.01	38 ± 2	11.8 ± 0.4	0.54 ± 0.03	10.5 ± 0.2	502 ± 8	n. d.	91 ± 5
	25:75	6.76 ± 0.02	35 ± 1	15.4 ± 0.8	0.51 ± 0.02	7.8 ± 0.3	640 ± 4	n. d.	87.5 ± 0.4
	50:50	6.28 ± 0.06	29.9 ± 0.6	15.2 ± 0.4	0.43 ± 0.01	5.1 ± 0.3	670 ± 20	n. d.	95 ± 5
	75:25	5.85 ± 0.02	23.2 ± 0.5	13.2 ± 0.2	0.34 ± 0.01	2.0 ± 0.4	680 ± 7	n. d.	99 ± 9
	100:0	5.62 ± 0.03	19.8 ± 0.2	10.9 ± 0.7	0.29 ± 0.01	n. d.	760 ± 50	n. d.	93 ± 7

[a] VS and TVFA refer to volatile solids and total volatile fatty acid, respectively.

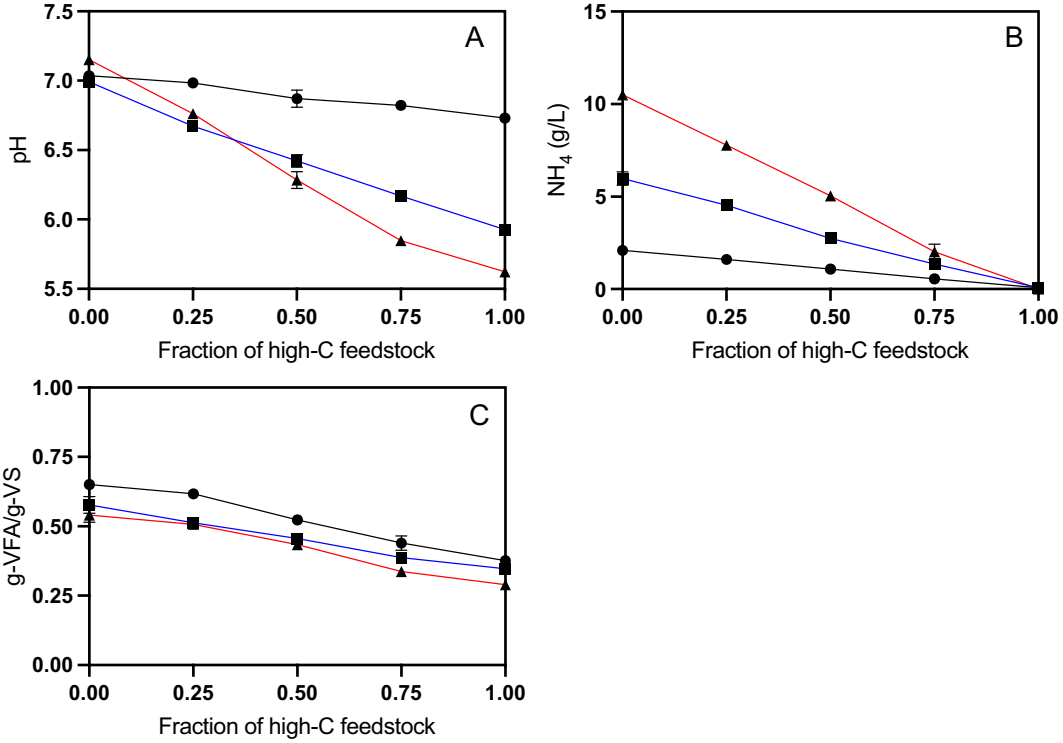


Fig. 2. Endpoint (day 16) reactor parameters. Panels are as follow: A) Reactor pH, B) ammonium concentrations, C) TVFA yields (volatile solids basis – g-VFA/g-VS). Initial substrate concentrations of 10, 30 and 60 g-VS/L are denoted by ●, ■, and ▲.

substrates, which may explain this observation. VFA and ammonium produced from protein degradation may loosen the structure of lignocellulosic biomass, increasing its degradability (Saritpongteeraka et al., 2014), with "Ammonia Fibre Expansion" (AFEX) having been elsewhere demonstrated as a biomass pre-treatment in its own right (Lau et al., 2010). Similarly, digestion of protein-rich meat waste has benefitted from the addition of high-C biomass, as it dilutes the long chain fatty acids (LCFA) and ammonium, often present in these wastes, both of which can inhibit reactor processes (Astals et al., 2014; Jensen et al., 2014). The relatively low concentration of lignin in the *Euphorbia* (3.65 %) and of LCFA in the blood used in this experiment may explain the observed lack of cooperativity between these substrates as these compounds are integral to the above mechanisms of cooperativity.

In a similar experiment to this, Zhang et al. (2016) digested excess sludge (high-N) with tall fescue biomass (high-C). VFA yields in co-digestions were significantly higher than for mono-digestions, indicating a strong, synergistic relationship between feedstocks. Saritpongteeraka et al. (2014) digested pig manure (high-N) with palm oil residues (high-C) and found a more muted effect, with an overall trend of declining yields with increased fraction of the lignocellulosic (high-C) feedstock, which provide a closer parallel to this study.

Co-digestion is also held to aid AD by keeping C:N ratios within the 'ideal' range of 20–30 (Parkin and Owen, 1986). This provides sufficient nitrogen for microbial growth, while avoiding potentially toxic accumulation of ammonium from excessive protein degradation. *Euphorbia* was found to have a TKN of 1.18 % and moderate C/N ratio of 34.58 (Table 1). There may thus have been sufficient endogenous nitrogen in the *Euphorbia* to allow robust microbial growth. With a non-limiting amount of nitrogen already present, the growth benefits of protein supplementation would have been limited.

It is notable that *Euphorbia* addition was not seen to increase VFA yields from blood by relieving ammonium toxicity, given the high ammonium concentrations seen (Table 2) in blood-rich digestions. At neutral to mildly acidic pH, ammonium nitrogen is primarily present in its less-toxic, ionic (NH_4^+) form, rather than as ammonia (NH_3). Had these digestions been instead carried out under alkaline conditions, the need to moderate ammonia toxicity may have seen co-digestions outperform blood mono-digestions.

3.2.3. *Responses of individual VFA titres over the co-digestion parameter space*

The time dependent composition of VFA produced is given in Fig. 3. Acidification was primarily acetogenic at earlier timepoints (<5 days), with a shift to mixed-acid fermentation at later timepoints, with greater proportions of C3, 4, and 5 acids seen in all conditions.

Acetic acid represented a majority of the produced VFA in all conditions tested at day 5 and remained the most abundant individual species in all conditions by day 16. Higher protein (blood) content, longer retention time, and higher substrate concentrations were all associated with an increased fraction of longer-chain fatty acids. In just one condition, blood mono-digestion at a substrate concentration of 60 g/L-VS, C4 compounds (isobutyric and butyric acids) represented a greater fraction of TVFA by day 16 than did C2 (Fig. 3). The response of endpoint fatty acid titres to substrate composition and concentration is summarised in Fig. 4. For all fatty acids, higher substrate concentrations gave higher titres. Titres of C4 and C5 compounds (isobutyric, butyric, isovaleric, and valeric acids) assumed the same monotonic, linear response shown by total VFA with the highest titres seen in blood, and lowest in *Euphorbia* mono-digestions. Valeric and hexanoic acids demonstrated the inverse of this trend, with highest titres seen in *Euphorbia* mono-digestions, though the biological or economic importance of this observation is limited by the low concentrations of these species.

Acetic acid was the most abundant individual VFA in all conditions. Importantly, it was also the only fatty acid whose generation was found to benefit from co-digestion, (Fig. 4B). The strength of this synergistic effect was found to be dependent on substrate concentration, with a more pronounced effect at higher concentrations. At the highest substrate concentrations (60 g/L substrate VS), all three co-digestion ratios significantly outperformed the mono-digestions, with the highest acetic

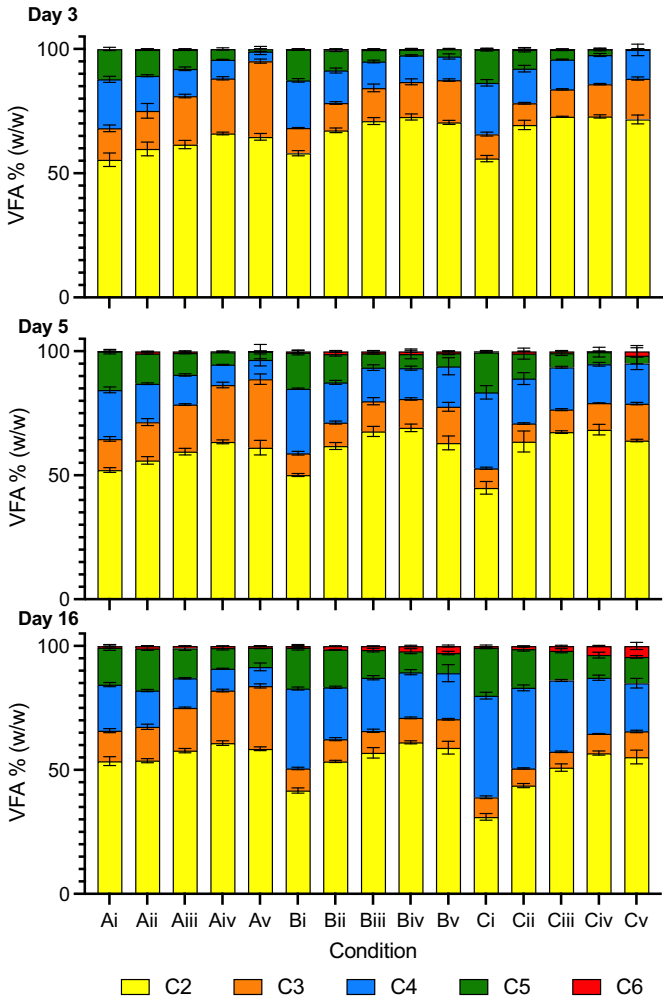


Fig. 3. Time dependence of VFA product spectra. VFA profiles are shown at days 3, 5, and 16 (endpoint). Initial substrate concentrations of 10, 30 and 60 g-VS/L are denoted by A, B, and C, respectively. Substrate ratios (*Euphorbia* to blood of 0:100, 25:75, 50:50, 75:25 and 100:0 (% VS)) are indicated by i, ii, iii, iv, and v.

acid titres found in digestions of 75 % blood with 25 % *Euphorbia* (15.4 ± 0.8 g/L acetic acid). This synergistic relationship is further examined in Fig. 5 in which a quadratic model was fit to acetic acid titres. As substrate concentration increased, so too did the absolute magnitude of the coefficient ß$_2$, defining an increasingly pronounced relationship between co-digestion and acetic acid titre. Conversely, reducing substrate concentration towards 10 g/L caused this quadratic relationship to decay, ß$_2$ to reduce, and the relationship to approach linearity. Co-substrate benefits thus increase with increased substrate concentration, perhaps due to dilution of toxic degradation products (e.g. ammonium) or due to the pH buffering effect of ammonium on acid release, both of which become more pronounced at higher substrate concentrations.

When attempting to understand these findings mechanistically, it is important to recognise that the major constituents of *Euphorbia* and blood i.e., carbohydrate and protein, respectively, are acidified for the most part via independent pathways. In *Euphorbia,* structural carbohydrates (cellulose and hemicellulose) represented 31.75 % of total solids (Table 1). AF of carbohydrate proceeds mainly via pyruvate, which represents a central node from which acetate, propionate, and butyrate may all be derived (Zhou et al., 2018). Protein accounted for 92.63 % of total solids in blood, but just 7.38 % in *Euphorbia* (Table 1). Acidification of protein occurs primarily via Stickland (redox) mechanisms, in which pairs of amino acids (AAs) act as electron donors and acceptors. Fixed stoichiometries for protein conversion to VFA have been proposed (Ramsay and Pullammanappallil, 2001), which assume VFA profile to be a product mainly of protein composition, independent of reactor parameters. Imbalances in Stickland redox pairs, the availability of alternative Stickland pathways for some AAs, and non-Stickland degradation reactions add nuance to this picture. Several studies, including that of (Tepari et al., 2020), have shown VFA product spectra from protein to be responsive to reactor parameters such as pH.

In this study, the early fermentation was primarily acetic acid-rich, which has precedent in the literature (Saritpongteeraka et al., 2014). This shifted to a more mixed-acid fermentation at later timepoints, after which significant differences in VFA composition became apparent. In Figs. 3 and 4, VFA composition can be seen to change as co-feedstock ratios change. This is partially a result of the different metabolic pathways used, from mostly Stickland reactions for blood, to pyruvate-mediated mechanisms for *Euphorbia*. A higher abundance of longer chain fatty acids has been reported with increased protein content (Saritpongteeraka et al., 2014) and in mono-digestions of high-protein substrates (Yu et al., 2018). This mirrors results seen in Fig. 3, showing abundant C4 and C5 compounds in digestions of 75 % and 100 % blood.

This observation will also have been influenced by the relative sensitivities of these pathways to shifting reactor parameters such as pH and ammonium. Several studies have shown pH to be the dominant parameter in defining VFA composition (Bermúdez-Penabad et al., 2017; Horiuchi et al., 2002). The relative pH stability of blood digestions due to ammonium release thus likely also contributed to the divergence of blood and *Euphorbia*-derived VFA profiles. However a detailed, mechanistic exploration of the underlying pathways is beyond the scope of this study.

Having demonstrated that co-digestion of *Euphorbia* with blood can provide increased yields of acetic acid, future investigation should aim to explore the underlying mechanisms of this synergy. Disentangling the relative contributions of pH, ammonium, and substrate composition through active buffering and controlled ammonium dosing would allow for greater control of product spectra. It would also do to test whether these results generalise to other CAM feedstocks, such as *Opuntia*, and other protein-rich co-substrates. Of particular interest for future investigation would be to test co-substrates whose sources might geographically co-localise with existing or potential areas of CAM cultivation.

4. Conclusion

The co-substrates *Euphorbia tirucalli* and pig blood were subjected to acidogenic fermentation in batch format at various mixing ratios and substrate concentrations. Higher fractions of pig blood gave greater overall titres of VFA, likely due to a higher fraction of the substrate being present in suspension. Greater fractions of blood, higher substrate concentrations, and longer retention times were all associated with an increased fraction of C4 and C5 fatty acids. Maximal titres of acetic acid were detected in co-digestions of 75:25 and 50:50 blood: *Euphorbia*, significantly outperforming monodigestions. Co-digestion thus offers great benefit where acetic acid is the desired product.

CRediT authorship contribution statement

Nicholas A. Tenci: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Fariza Ammam:** Conceptualization, Methodology, Data curation. **Wei E. Huang:** Supervision. **Ian P. Thompson:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal

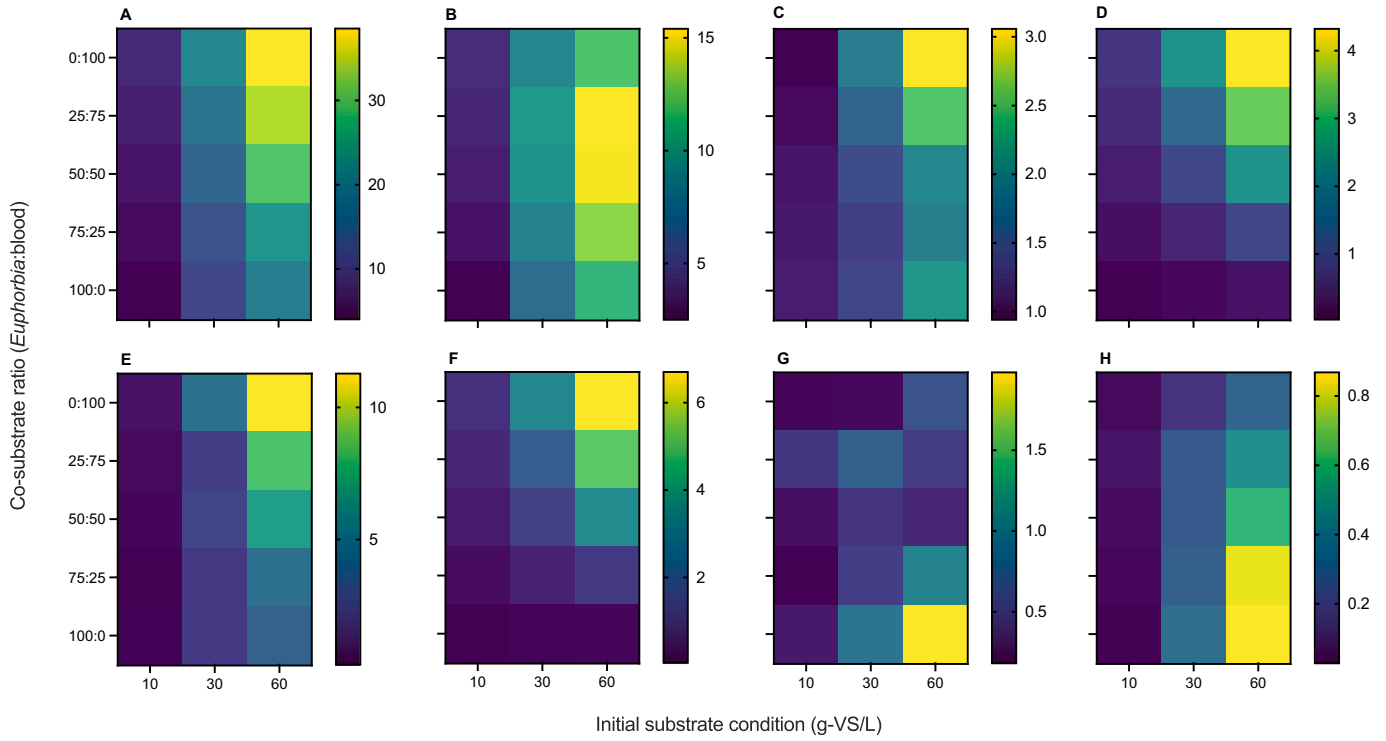
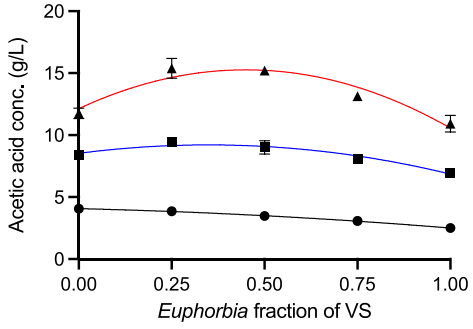


Fig. 4. Total endpoint (day 16) VFA titres. VFAs shown are A) Total VFA, B) Acetic, C) Propionic, D) Isobutyric, E) Butyric, F) Isovaleric, G) Valeric, and H) Hexanoic acid. Heatmap shows mean of triplicate measurements, all VFA concentrations are given in g/L.



	Initial substrate concentration (g-VS/L)		
	10	30	60
$ß_0$	4.07 [3.94, 4.20]	8.53 [8.09, 8.98]	12.13 [11.24, 13.03]
$ß_1$	-0.72 [-1.34, -0.10]	3.89 [1.78, 5.99]	13.95 [9.70, 18.20]
$ß_2$	-0.84 [-1.43, -0.25]	-5.56 [-7.58, -.3.54]	-15.51 [-19.59, -11.44]
Model R^2	0.9698	0.8594	0.8649

Fig. 5. Quadratic model of endpoint acetic acid titre responses to substrate composition. Initial substrate concentrations of 10, 30 and 60 g-VS/L are denoted by ●, ■, and ▲. Model parameters are given below with their respective 95 % confidence intervals.

relationships which may be considered as potential competing interests: Nicholas A. Tenci reports financial support was provided by East Alpha.

Data availability

Data will be made available on request.

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