Journal of Cleaner Production 454 (2024) 142166

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Red seaweed as an abundant, natural methanogenesis inhibitor for industrial biorefinery

Nicholas A. Tenci [a,**], Fariza Ammam [a], Nichola Austen [a,b], Wei E. Huang [a], Ian P. Thompson [a,*]

[a] University of Oxford, Department of Engineering Science, Parks Road, Oxford, OX1 3PJ, UK
[b] University of Oxford, Department of Biology, South Parks Road, OX1 3RB, UK

ARTICLE INFO

Handling Editor: Zhen Leng

ABSTRACT

Acidogenic fermentation offers a means to renewably refine biomass to volatile fatty acids from which industrial chemicals can be sustainably derived. Small molecules (bromoethane sulfonate) are used in the literature to inhibit methanogenesis and improve fatty acid yields but are impractical at industrial scale due to cost. The red seaweed *Asparagopsis taxiformis* has been shown to provide methanogenesis inhibition in the microbiome of the ruminant gut over short retention times (up to 72 h), but its suitability for bioreactor control has not been previously characterised. This study investigated the responses of both rumen fluid and anaerobic digester sludge to various *Asparagopsis* doses. In rumen fluid, doses of ≥5% *Asparagopsis* showed equivalent inhibition to bromoethane sulfonate at retention times under 30 days, producing VFA of both similar yields and qualities to this standard inhibitor. Digester sludge was not found to respond to *Asparagopsis* treatment at the levels tested, suggesting a specificity of treatment to microbial community. These data support the application of *Asparagopsis* as a scalable reactor control agent for rumen-driven fermenter configurations.

1. Introduction

Volatile fatty acids (VFA) produced from the acidogenic fermentation (AF) of biomass offer a sustainable alternative to petrochemicals as substrates for the chemical industry. VFAs have been demonstrated as substrates for bioproduction of polyhydroxyalkanoate bioplastics (PHA) (Mengmeng et al., 2009; Shen et al., 2014), oils (Christophe et al., 2012), waxes (Martin et al., 2023), and other chemicals. They are additionally used as additives in food, pharmaceuticals, and cosmetics, and as a carbon source for biological nutrient removal from wastewater (Agnihotri et al., 2022). AF is a modified form of anaerobic digestion (AD), where methanogenesis is inhibited to allow VFA to accumulate.

Methanogenesis can be inhibited by several means. Acidogenic and acetogenic bacteria exhibit a wider pH tolerance than do methanogenic archaea, which can be inhibited by reducing reactor pH to <6 (Wang et al., 2014). Inhibition can also be achieved through accumulation of degradation products such as VFA or ammonium (Shi et al., 2017; Siegert and Banks, 2005). Another common strategy for inhibiting methanogens is the use of dedicated methanogenesis inhibitor chemicals (Yin et al., 2016). The use of the 2-bromoethanesulfonate (BES) is particularly widespread in the literature (Tenci et al., 2023;Yin et al., 2016). BES is a structural analogue of Coenzyme M (CoM), a methanogen-specific cofactor. BES is typically understood to inhibit methanogenesis through competitive inhibition of this essential cofactor (Liu et al., 2011), which is required for the enzyme methyl-coenzyme M reductase (MCR) to catalyse the terminal step in all forms of methanogenesis (Liu and Whitman, 2008). BES accordingly demonstrates efficacy in a broad spectrum of methanogenic cultures, including anaerobic soils (Chidthaisong and Conrad, 2000), rumen fluid (Lazuka et al., 2015), and AD sludge (Tenci et al., 2023) and at low (typically millimolar) concentrations. Its broad efficacy, relatively low effective dose, and relative specificity for methanogenic archaea have contributed to the favoured status of BES as a laboratory scale methanogenesis inhibitor. Despite these considerable benefits, the high cost of BES (US $41/kg) (Baleeiro et al., 2022) precludes its wider use at industrial scale.

The red seaweed *Asparagopsis taxiformis* has recently gained attention as a potential feed additive to reduce carbon emissions from livestock. *Asparagopsis* has demonstrated an ability to inhibit methanogenesis in rumen fluid both *in vitro* (Kinley et al., 2016a; Machado et al., 2014), and *in vivo* (Li et al., 2016; Roque et al., 2021).

* Corresponding author. Department of Engineering Science, University of Oxford, Parks Road, Oxford, OX1 3PJ, UK.
** Corresponding author.
E-mail addresses: nicholas.tenci@eng.ox.ac.uk (N.A. Tenci), ian.thompson@eng.ox.ac.uk (I.P. Thompson).

https://doi.org/10.1016/j.jclepro.2024.142166
Received 11 October 2023; Received in revised form 30 March 2024; Accepted 7 April 2024
Available online 14 April 2024

The proposed active ingredient in this process is bromoform ($CHBr_3$), a halogenated methane analogue (Machado et al., 2016b). While the preliminary efficacy of *Asparagopsis* has been demonstrated, key questions remain unanswered. Current literature on the subject is mainly focused on agricultural applications and the timescales studied are based on the retention times of the ruminant digestive tract (<4 days) (Kinley et al., 2016a; Machado et al., 2018), with little known about its efficacy over the longer timescales relevant to AF. Studies have also worked exclusively in rumen fluid, which contains a microbiome distinct from those typically found in commercial acidogenic fermenters. While a 2015 analysis by Meat and Livestock Australia (Cotter et al., 2015) placed the cost of wild-harvested *Asparagopsis taxiformis* at $AU 200/kg, this same report claims that prices below $AU 5/kg may be feasible with commercial cultivation and further mentions that imported, commercially grown algae (of other species) can retail for as little as $AU 1.50/kg. Recent data show bulk quantities of various species of seaweed of several species may be obtained for just $US 0.5–0.8/kg (Alibaba.com, 2023a; Alibaba.com, 2023b). Assuming these lower price bands to be feasible with large scale cultivation, farmed *Asparagopsis* could prove a cheap and renewable future alternative to BES for reactor control, if its efficacy can be demonstrated.

This study presents, to the best of our knowledge, the first systematic evaluation of *Asparagopsis* as a potential bioreactor control agent for VFA production. Its inhibitory effect on methane production and related kinetics was quantified in both rumen fluid and in commercial AD sludge over a 70-day time course, using biomass from *Euphorbia tirucalli* as a substrate. These effects were evaluated against a standard, small-molecule inhibitor – BES. VFA production was quantified, and its temporal variation mapped to the corresponding biomethane data, to understand how carbon allocation shifted as inhibition waned and methanogenesis resumed. A mechanistic explanation for these results was sought through a metagenomic characterisation of the two microbial communities, both prior to treatment, and at key timepoints throughout the fermentation time course.

2. Materials and methods

2.1. Biomass and inoculum

AD sludge was obtained from an anaerobic digestion facility processing municipal food waste (Severn Trent Green Power, Cassington, UK). Rumen fluid was collected from a fistulated cow (The Centre for Dairy Research, University of Reading, UK). Both sludge and rumen fluid were passed through a 1 mm sieve to remove any large particulates before characterisation and use.

Euphorbia tirucalli biomass provided the substrate for anaerobic digestion. This was field grown in Kenya and dried at 80 °C for storage. Prior to use, the dried biomass was ground in a blender (Waring Pro® Stainless Steel Blender, 550 W) to ensure homogeneity.

Asparagopsis taxiformis biomass was obtained in dried form (seaExpert, Horta, Faial, Azores, Portugal). This was sourced from wild *Asparagopsis* grown in the coastal waters of the Azores, Portugal. Dried *Asparagopsis* was ground in a blender and passed through a 1 mm sieve to ensure consistent particle size.

2.2. Anaerobic batch assays

Anaerobic digestion experiments were performed in batch format in 250 mL vials with a 150 mL working volume. A standard culture medium was prepared as per Angelidaki and Sanders (2004), with the exclusion of sodium sulphide. This medium ensured an adequate supply of nutrients, vitamins, and trace minerals to promote robust microbial growth. A digestion master mix of medium, deionised water and the appropriate inoculum was prepared to a final inoculum concentration of 5 gVS/L, and the pH adjusted to 8.1. This bulk reaction mixture was aliquoted into vials containing the dried substrate. Adjustment to a mildly alkaline pH was both to allow for methanogenesis, and to allow for accumulation of VFA. Vials were sealed with rubber septa and aluminium crimp caps and inverted to mix their contents. The headspace gas was not purged to remove residual oxygen.

Each vial contained dried *E. tirucalli* biomass to give a 2:1 substrate to inoculum ratio (VS-basis). To test the efficacy of *Asparagopsis* as a methanogenesis inhibitor, *Asparagopsis* biomass was added to vials in five concentrations of 0.5, 1, 2, 5, and 10% (VS) of the *E. tirucalli* substrate. Three further positive control conditions were prepared with bromoethane sulfonate at concentrations of 5, 10, and 20 mM. A further condition containing *E. tirucalli,* but no inhibitor provided the negative (uninhibited) control. An 'inoculum blank' containing the digestion master mix but no substrate, was also prepared to account for the behaviour of the underlying inoculum. All conditions were prepared in triplicate. All conditions were incubated under mesophilic (37 °C) conditions, with constant agitation at 125 rpm. The experiment was terminated on day 70. This entire experimental setup was replicated for each of the two inocula tested. Neither inoculum source was purged of residual volatiles prior to the experiment.

2.3. Analytical methods

Determination of bulk biomass and inoculum parameters (total and volatile solids) was conducted as per standard methods (APHA, 1998), but with oven temperatures for determination of total solids (TS) set to 80 °C. Analysis of lignin, cellulose, and hemicellulose for *Euphorbia* and *Asparagopsis* were performed as described by van Soest et al. (1991).

The production of biogas was monitored by water displacement in a eudiometer. Gas volume data were corrected to standard temperature (273.15 K) and pressure (101.3 kPa) as previously described (Richards et al., 1991). The composition of biogas produced was analysed by Gas Chromatography with Thermal Conductivity Detection (GC-TCD) (Shimadzu GC-2010, Japan) with a Shincarbon capillary column. Injector, oven, and detector temperatures were 150, 75, and 200 °C, respectively, with helium providing the carrier gas.

At each timepoint, liquid samples of 1.5 mL were withdrawn using a syringe. The pH of liquid samples was measured upon sample collection with a pH probe (Thermoscientific, USA). Samples were frozen at −20 °C for later analysis of VFA, and for metagenomic analysis.

For analysis of VFA, samples were centrifuged at 15,500×*g* for 10 min to obtain a clarified supernatant. Supernatant samples were transferred to 1.5 mL GC vials and acidified by 1:1 dilution with 20% formic acid. VFA concentrations were quantified by Gas Chromatography-Flame Ionisation Detection (GC-FID) using a 30 m × 0.25 mm x 0.25 μm fused-silica column (ZB-FFAP). Temperatures of the injector and detector were set at 250 and 350 °C, respectively. The oven temperature was held for 2 min at 100 °C and then ramped at 8 °C/min to 150 °C, where it was held for a further 2 min. Acetic, propionic, isobutyric, butyric, isovaleric, valeric, and hexanoic acids were detected and quantified against standards. Total volatile fatty acid (TVFA) was calculated as the sum of these fatty acids.

Metagenomic analyses of inocula and reactor microbial communities were outsourced to an external provider (Genewiz Ltd., United Kingdom). The following protocol detailing the analytical procedures followed has been reproduced with their express permission:

DNA was extracted using NucleoMag DNA Microbiome Kit following manufacturer's instructions (Takara, San Jose, CA, USA). 16S-EZ rDNA next generation sequencing library preparations and Illumina MiSeq sequencing were conducted at Azenta, Inc. (South Plainfield, NJ, USA). Sequencing library was prepared using a MetaVx™ 16s rDNA Library Preparation kit (Azenta, Inc., South Plainfield, NJ, USA). Briefly, the DNA was used to generate amplicons that cover V3 and V4 hypervariable regions of bacteria and archaea16S rDNA. Indexed adapters were added to the ends of the 16S rDNA amplicons by limited cycle PCR. DNA libraries were validated and quantified before loading. The pooled DNA libraries were loaded on an Illumina MiSeq instrument according to

manufacturer's instructions (Illumina, San Diego, CA, USA). The samples were sequenced using a 2x 250 paired-end (PE) configuration. Image analysis and base calling were conducted by the Illumina Control Software on the Illumina instrument. Raw sequence data (.bcl files) generated from Illumina MiSeq was converted into FASTQ files and demultiplexed using Illumina's bcl2fastq 2.17 software.

2.4. Statistical analysis and modelling

All experimental data herein are presented as the mean ± standard deviation of triplicate samples, unless otherwise stated. Model parameters are quoted with their associated standard errors. Data processing was performed in Microsoft Excel and GraphPad Prism 9.5.0 (GraphPad Prism version 9.5.0 for Windows, 2023). Biomethane yields were calculated based on total reactor volatiles (calculated as the sum of substrate, inoculum, and inhibitor volatile solids). Biomethane yield data were fitted using the following Modified Gompertz kinetic model, as previously demonstrated by Lueangwattanapong et al. (2020):

Modified Gompertz Model: $Y = A \exp[-\exp[(\mu_m e/A)(\lambda - t) + 1]]$

In which, Y = cumulative methane yield at time 't', A = maximum theoretical methane yield, t = time, λ = lag phase, μ_m = maximum methane production rate. This model was fitted in GraphPad Prism 9.5.0 to the methane yield data for each substrate to extract the above kinetic parameters for each condition. These parameters provided a standardised measure of the defining features of methane production: lag, rate, and yield. They were thus used as the basis by which the inhibition phenomenon was compared across treatment conditions.

3. Results

3.1. Characterisation of feedstock and inocula

Table 1 details the bulk characteristics of feedstocks and inocula in this experiment.

The volatile content of *E. tirucalli* in this study was 83.3 ± 0.3%. This is comparable to values of 87.1–88.4 reported by (Krümpel et al., 2020). *Asparagopsis* was found to have an organic content of just 52.94 ± 0.03%. This implies an ash content of 47.06%, which is consistent with values reported for oven-dried *Asparagopsis* of 47.3–49.5% ash (Regal et al., 2020). AD sludge and rumen fluid inocula had volatiles contents of 60.3 ± 0.2, and 61.3 ± 0.2 %, respectively, consistent with previous literature values (Lueangwattanapong et al., 2020; Tenci et al., 2023).

Table 1
Bulk characteristics of substrates and inocula used in this experiment. All data are given as means ± standard deviations of triplicate measurements.

Characteristic	Substrates		Inocula	
	Euphorbia	*Asparagopsis*	AD sludge	Rumen Fluid
TS/FM (%)	N/A	N/A	4.81 ± 0.08	2.48 ± 0.03
VS/TS (%)	83.3 ± 0.3	52.94 ± 0.03	60.3 ± 0.2	61.3 ± 0.2
Ash/TS (%)	16.7 ± 0.3	47.06 ± 0.03	39.7 ± 0.2	38.7 ± 0.2
Cellulose (%TS)	27.50	5.8	–	–
Hemicellulose (% TS)	2.78	12.1	–	–
Lignin (% TS)	5.32	1.25	–	–
TVFA (g/L)	–	–	0.28 ± 0.05	8.80 ± 0.03

*TS – Total solids, FM – Fresh matter, VS – Volatile solids, TVFA – Total volatile fatty acids.

3.2. Response of biomethane production to Asparagopsis and BES treatment

Methane yields are given in Fig. 1. Uninhibited conditions (shown in red) showed characteristic, sigmoidal methane production curves for both rumen fluid (Fig. 1 A, C1) and AD sludge (Fig. 1 B, D1). All three concentrations of BES tested showed near complete inhibition of methane production in both inocula (Fig. 1 A, B), with only trace amounts of methane registered, and only at later timepoints.

For *Asparagopsis*-inhibited conditions, biomethane production was sigmoidal at all treatment levels, and a Modified Gompertz model (Fig. 1 C2, D2) was accordingly fit to facilitate comparison of dose-response. With rumen fluid, methanogenesis appeared to respond in a dose-dependent fashion to *Asparagopsis* treatment. Negligible inhibition was observed at doses of 0.5–2% (of substrate VS) *Asparagopsis*, with these curves broadly corresponding to the negative control. At the 10% inhibition level, theoretical maximum methane yields were notably reduced relative to the negative control (170 ± 10 vs. 250 ± 10 ml/gVS). However, it was in the lag phase that the inhibition phenomenon manifested itself most clearly; conditions treated with 5 and 10% *Asparagopsis* demonstrated a considerably extended lag phase, while also ultimately resuming a sigmoidal pattern of methane production. The Modified Gompertz parameter lambda (λ -Table 2) measures the lag phase prior to initiation of methane production and allows a standardised comparison of this observed inhibitory effect. In rumen-fed conditions fed 0–2% *Asparagopsis*, λ ranged from 7 to 11 days, while 5% and 10% doses saw lag phases of 30.3 and 32 days, respectively. Such a dramatic shift in lag phase was not registered in conditions inoculated with AD sludge, with lag increasing from 6.4 to just 11.6 days between the uninhibited control and 10% *Asparagopsis* treatment with this inoculum.

3.3. Response of VFA production to Asparagopsis and BES treatment

Fig. 2 details production of total volatile fatty acids (TVFA). Uninhibited conditions for both rumen fluid and AD sludge showed an initial spike in VFA concentrations, which then dropped as methanogenesis rose, ultimately converging to near zero. BES treated conditions in both rumen fluid and AD sludge (Fig. 2 A, B) also saw a rapid initial acidification, with titres remaining at or near maximum concentrations for the duration of the experiment.

Rumen conditions treated with 0.5–2% *Asparagopsis* clustered with the uninhibited control (Fig. 2C). 5 and 10% *Asparagopsis*-inhibited rumen conditions maintained high concentrations to day 30 – corresponding to the lag phase (λ) seen in Gompertz models for these conditions (Table 2). Beyond this point, VFA concentrations fell consistently for the remainder of the experiment. All *Asparagopsis*-treated AD sludge conditions (Fig. 2D) clustered with the uninhibited control.

As day 30 was the longest retention time at which *Asparagopsis*-treated rumen conditions behaved like those of BES, an analysis of both VFA yields and compositions is provided for this timepoint (Fig. 2E and F). VFA yields with 5% Asparagopsis and 5 mM BES were found to be equal, and both significantly higher than the uninhibited control. The composition of the VFA produced at this timepoint (Fig. 2F) is also similar, showing an acetic acid and propionic acid-rich fermentation, with smaller amounts of C4–C6 compounds. In the uninhibited control, a substantial reduction in C2 and C4 compounds can be seen - corresponding to the resumption of methanogenesis.

3.4. Microbial communities of initial inocula and community response to Asparagopsis treatment

The relative abundance of microbial communities (at phylum level) in the fresh inoculum sources is provided in Table 3. Rumen reactor communities were also characterised at day 25 with and without *Asparagopsis* treatment at the maximum treatment level (10%). Among

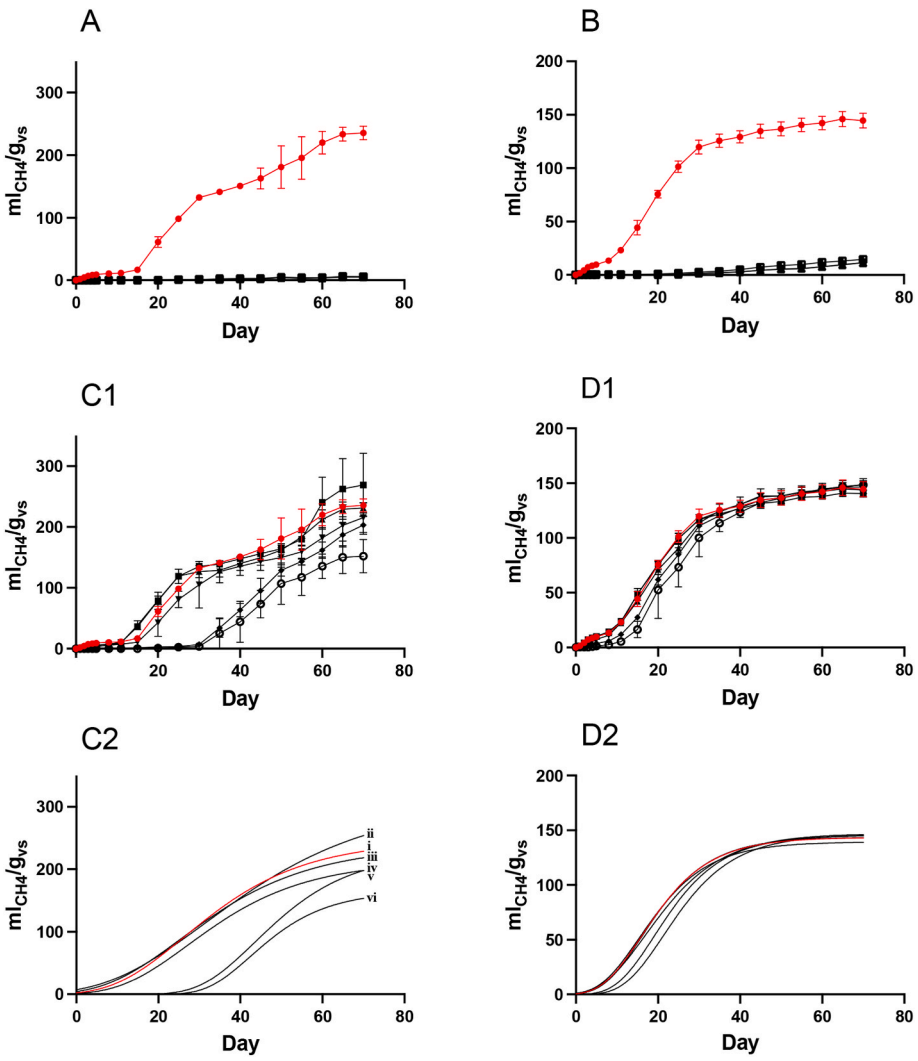

Fig. 1. Biomethane yields from *Euphorbia* biomass under inhibition with *Asparagopsis* and bromoethane sulfonate. Panels **A** and **B** show biomethane production from rumen fluid and AD sludge, respectively, under BES inhibition. **C1** and **D1** show biomethane production from rumen fluid and AD sludge, respectively, under *Asparagopsis* inhibition. **C2**, and **D2** show Modified Gompertz models of best fit obtained from the data in **C1** and **D1**. The uninhibited control and *Asparagopsis* treatments of 0.5, 1, 2, 5, and 10% are indicated by ●, ■, ▲, ▼, ◆ and, ○ respectively, or by i, ii, iii, iv, v, and vi in **C2**. BES concentrations of 5, 10, and 20 mM are indicated by □, ◭, and ▽.

the firmicutes, the families *Lachnospiraceae*, *Ruminococcaceae*, and *Veillonellaceae* were depleted in treated conditions relative to the control, while *Streptococcaceae* were enriched. As expected, the methanogenic phylum *Euryarchaeota* was substantially depleted at day 25 between untreated and *Asparagopsis*-treated conditions.

4. Discussion

4.1. Efficacy of Asparagopsis as a methanogenesis inhibitor in rumen and AD sludge inocula

Asparagopsis was found to provide effective inhibition of methanogenesis at levels of 5% and 10% VS in rumen fluid up to day 30 but was not found to effectively inhibit methanogenesis in AD sludge at any of the concentrations tested (Fig. 1, Table 2). BES was included in this study as a positive control against which the inhibitory efficacy of *Asparagopsis* could be tested and was found to provide effective inhibition of methanogenesis at all concentrations tested, and in both inocula, to experiment termination on day 70 (Fig. 1). BES is a pure compound typically understood to inhibit the terminal step of methanogenesis through competitive inhibition of CoM (Liu et al., 2011). *Asparagopsis* conversely contains many known antimethanogenic compounds, though its antimethanogenic activity is generally attributed to its bromoform content (Machado et al., 2016b). Halomethanes, such as bromoform, have been reported to inhibit methanogenesis by reacting with key co-factors, such as vitamin B_{12} which is required for CoM-methyltransferase to catalyse methyl group transfer onto CoM (Glasson et al., 2022; Wood et al., 1968; Romero et al., 2023). They have also been shown to directly inhibit the terminal reduction step, in which this methyl group is then reductively released from CoM as CH_4, by binding the cofactor F430 (nickel tetrapyrrole) of MCR (Glasson et al., 2022; Romero et al., 2023).

The efficacy of *Asparagopsis* was clear in rumen fluid, but only a very slight shift in Gompertz lag phase was seen for biomethane production in AD sludge when treated with 10% *Asparagopsis* (from 6.4 to 11.6 days – Table 2). This suggests the minimum effective inhibitory dose of *Asparagopsis* to be much higher in AD sludge than rumen fluid – beyond the concentrations tested in this study. This observation has important practical ramifications for biorefinery application of *Asparagopsis*. It suggests that any use of *Asparagopsis* in sludge-driven systems would require a very large fraction of the substrate volatiles to be provided by *Asparagopsis* – shifting its role from inhibitor to co-substrate. Aside from the practical issues of needing to cultivate such large quantities of

Table 2
Kinetic parameters derived from Modified Gompertz Models of methane production by AD sludge and rumen fluid at varying levels of *Asparagopsis* inhibition.

Inoculum	*A. taxiformis* dose	Model parameters			Model R^2
		A (ml/gVS)	μ_m (ml/gVS/d)	λ (d)	
AD Sludge	Control	143 ± 1	5.6 ± 0.2	6.4 ± 0.4	0.993
	0.5%	139.4 ± 0.9	5.4 ± 0.1	5.8 ± 0.3	0.997
	1%	147 ± 1	5.1 ± 0.1	6.4 ± 0.4	0.996
	2%	143.6 ± 0.6	5.68 ± 0.09	6.7 ± 0.2	0.999
	5%	145 ± 1	5.7 ± 0.2	9.8 ± 0.4	0.997
	10%	146 ± 2	5.6 ± 0.3	11.6 ± 0.8	0.986
Rumen fluid	Control	250 ± 10	5.4 ± 0.3	9 ± 1	0.974
	0.5%	310 ± 30	4.8 ± 0.3	8 ± 2	0.934
	1%	240 ± 10	4.8 ± 0.3	7 ± 1	0.965
	2%	210 ± 10	4.8 ± 0.4	11 ± 2	0.949
	5%	230 ± 10	6.3 ± 0.3	**30.3 ± 0.8**	0.987
	10%	170 ± 10	5.8 ± 0.7	**32 ± 2**	0.938

*A-methane production potential, μ_m – maximum production rate, λ – lag phase. BES-inhibited conditions were omitted as these failed to produce sigmoidal response data.

seaweed, on a process level too, its high ash content (Table 1) would also mean VFA yields would suffer on a TS basis, reducing reactor efficiency. Sludge-driven systems could circumvent this ash limitation by extracting bromoform for direct application, but this would entail additional process costs.

The minimum effective inhibitory concentration of *Asparagopsis* in rumen fluid this study was 5% of substrate VS. Machado et al. (2016a) and Kinley et al. (2016b) found *Asparagopsis* at 2% of substrate VS to be effective at inhibiting methanogenesis over short timescales in rumen fluid. This reduced potency might have arisen from differences in processing and storage. In this study, *Asparagopsis* was dried in the dark at ambient temperature. The dried, ground biomass was stored in the dark in a sealed container at ambient temperature (~21 °C). The biomass in Kinley et al. (2016b) was instead freeze-dried, and then stored at −20 °C. Tan et al. (2023) found the bromoform content of freeze-dried *Asparagopsis* powder to be temperature sensitive, finding bromoform levels to show good stability over 24 weeks at −20 °C and 4 °C, but that they depleted by approximately half at over this same timeframe, when stored at 25 °C.

In rumen fluid, the effect of *Asparagopsis* treatment began to abate after day 30. (Fig. 2C). Halomethanes, such as chloroform, are known to be anaerobically degradable (Cappelletti et al., 2012; Nijenhuis and Kuntze, 2016; van Beelen and van Keulen, 1990) and methanogens of the genus *Methanosarcina* have been demonstrated to effectively dehalogenate both chloroform and bromoform (Mikesell and Boyd, 1990). Metagenomic analyses (Table 3) confirmed the presence of the family *Methanosarcinaceae* in rumen-driven reactors. The existence of anaerobic mechanisms to degrade bromoform is relevant if *Asparagopsis* is to be considered for biorefinery applications requiring continuous dosing, as chronically exposed anaerobic microbiomes may acclimate to its presence. Under these conditions, enrichment of bromoform-degrading subpopulations, or of methanogenic species less susceptible to bromoform inhibition could effectively restore stable methanogenesis despite the presence of *Asparagopsis*.

4.2. Effect of Asparagopsis treatment on volatile fatty acid production and biorefinery

Volatile fatty acid titres in rumen conditions with *Asparagopsis* treatment showed a clear divergence between uninhibited controls and treatments of 5 and 10% *Asparagopsis* at retention times beyond 15 days (Fig. 2C). From t = 0 to t = 30 days, 5 and 10% *Asparagopsis*-treated rumen conditions demonstrated acidification profiles largely analogous to BES-treated rumen conditions (Fig. 2A), suggesting the appropriateness of *Asparagopsis* as an alternative to BES at retention times of ≤30 days. 5% *Asparagopsis* treatment and 5 mM BES treatment produced comparable VFA yields at day 30 (Fig. 2E). Moreover, the compositions of the VFA at t = 30 was found to be near identical between these conditions (Fig. 2F), further supporting the use of *Asparagopsis* as a BES-substitute.

Rumen fluid-driven AF is not commonly employed at industrial scale, though it has been successfully demonstrated at laboratory scale (Lazuka et al., 2015; Liang et al., 2022). Particular attention has been paid to the potential of rumen microbes to improve hydrolysis of recalcitrant lignocellulosic biomass (Meyer et al., 2022; Wang et al., 2018). While these lab-scale systems used BES as the antimethanogenic agent, given the above results, *Asparagopsis* could conceivably be used in its place.

At an industrial scale, the transient, ≤30-day inhibition provided by *Asparagopsis* could be used to establish initial control of the bioreactor, allowing for VFA accumulation, which can then provide sustained product-inhibition of methanogenesis. An alternative system could be envisioned based on the findings of Karekar and Ahring (2023). This paper suggested that a methanogenic (rumen) consortium could be supplemented with homoacetogens to compete for the hydrogen-scavenging function typically played by methanogens. One-time BES inhibition of the methanogens allowed homoacetogens to become established, whereafter they appeared to prevent the reestablishment of methanogenesis. Such a system could be envisioned at industrial scale but with *Asparagopsis* playing the role of transient inhibitor: establishing the system initially, and if required, periodically returning it to equilibrium if methanogens reappear. Halogenated hydrocarbons, such as bromoform, may however also modify the activity of microbial species other than methanogens, including homoacetogens (Liu et al., 2011), and this system would need to be experimentally validated to ensure that *Asparagopsis* does not also inhibit this key community member.

4.3. Interplay of Asparagopsis and rumen microbial community

It is necessary to acknowledge that the microbiome of any given bioreactor will depend not just on the initial inoculum, but also on the parameters under which the reactor is run. Substrate composition, loading rates, pH, and the concentrations of degradation products such as VFA and ammonium will all affect community structure. Despite these caveats, AD sludge inoculum is taken here to provides a reasonable approximation of the microbiome of an industrial-scale AD plant. In this study, *Asparagopsis* was not found to provide significant methanogenesis inhibition in sludge-fed reactors. The most likely explanation is that the concentrations tested (up to 10% *Asparagopsis*), were below the minimum inhibitory concentration (MIC) for sludge. A slight shift in lag phase was registered at the 5 and 10% dose levels (Table 2), suggesting the MIC could lie just beyond this point.

Only methanogens of the family *Methanobacteriaceae* were detected in the initial rumen inoculum. At day 25, the untreated reactor was found to contain both *Methanobacteriaceae* and *Methanosarcinaceae* and both methanogenic families were detected at low levels in *Asparagopsis*-treated samples.

Treatment was found to significantly perturb other community members (Table 3). Perhaps the most noteworthy community level changes were among the firmicutes, where the families *Lachnospiraceae*,

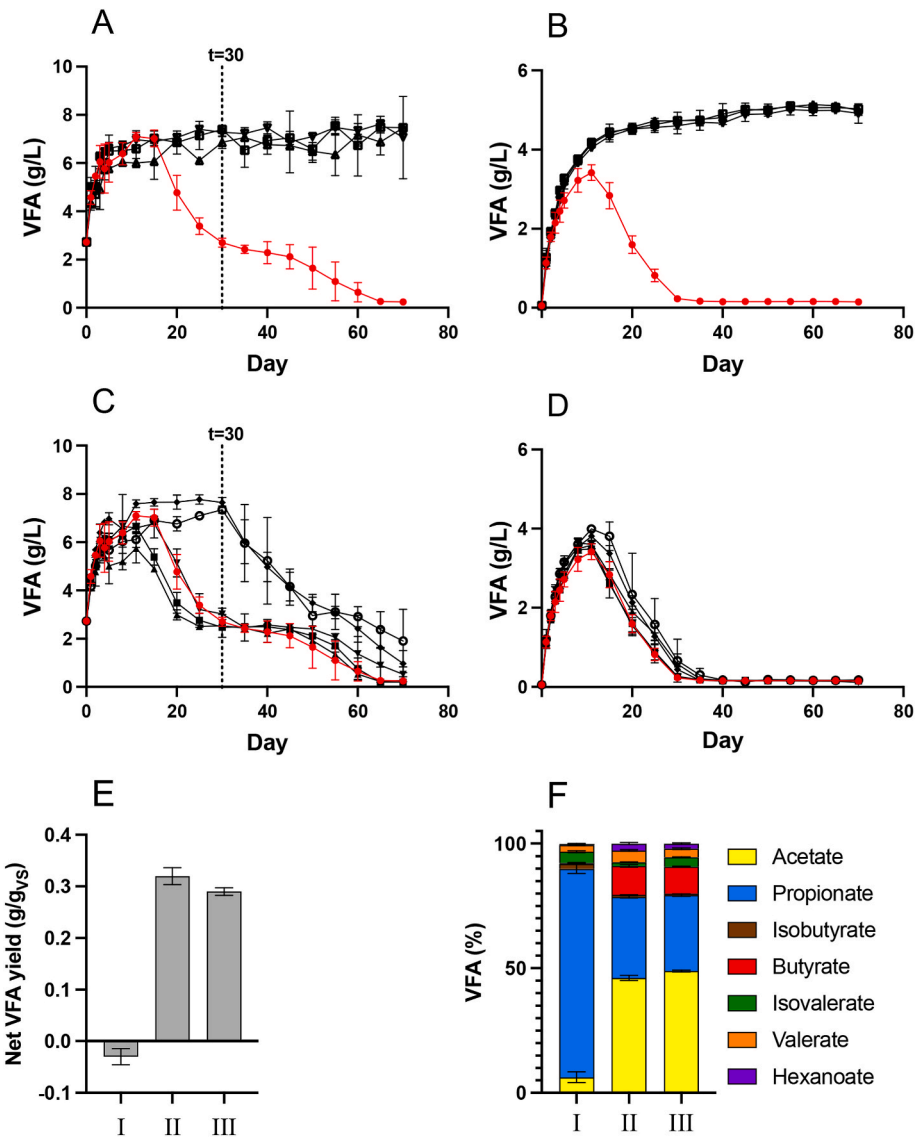

Fig. 2. Volatile fatty acid production from *Euphorbia* under treatment with *Asparagopsis* and bromoethane sulfonate. Panels **A** and **B** show observed VFA titres in rumen fluid and AD sludge, respectively, treated with BES. **C** and **D** show VFA titres in rumen fluid and AD sludge, respectively, when treated with *Asparagopsis*. Panel **E** shows the VFA yield between days 0 and 30 in rumen fluid with no treatment (I), and treatments of 5% *Asparagopsis* (II) and 5 mM BES (III). Panel **F** gives the composition of VFA in these same treatment conditions at day 30. Uninhibited controls and *Asparagopsis* treatments of 0.5, 1, 2, 5, and 10% are indicated by ●, ■, ▲, ▼, ◆ and, ○ respectively. BES concentrations of 5, 10, and 20 mM are indicated by □, ◭, and ⧨.

Ruminococcaceae, and *Veillonellaceae* were depleted in treated conditions, while *Streptococcaceae* were greatly enriched. *Lachnospiraceae* and *Ruminococcaceae* are hydrolytic, specialising in fibrolysis, or the degradation of the recalcitrant, structural components of lignocellulosic biomass (Biddle et al., 2013). These communities are also fermentative, producing VFAs - butyrate in particular (Vacca et al., 2020). Depletion of key fibrolytic/fermentative community members did not produce a reduced VFA yield as might be expected. However, the low lignin content of the *Euphorbia* substrate used in this study may have masked this effect. Testing with a higher-lignin biomass source should be done to determine whether *Asparagopsis* treatment interferes with the fibrolytic capacity of rumen fluid.

Veillonellaceae, which were slightly depleted, are known propionate producers (Kishimoto et al., 2006). Interestingly, Machado et al. (2016a) found *Asparagopsis* to increase the ratio of propionate to acetate over short timescales. Despite quite considerable shifts in community structure in *Asparagopsis* treated samples, VFA composition was nearly identical to that produced through BES inhibition, suggesting from an industrial perspective that these community level shifts are not of concern as VFA quality is equal to that produced through standard inhibitory processes. While the depletion of methanogens can most likely be attributed to bromoform, it is unknown whether this, or other compounds present in *Asparagopsis*, is responsible for these broader community-level perturbations. Further investigation would be required to determine this. Despite these considerable shifts in reactor community, at day 30 *Asparagopsis* produced inhibition in rumen fluid that was quantitatively and qualitatively equivalent to BES.

5. Conclusion

Asparagopsis was found to provide effective methanogenesis inhibition in rumen fluid at concentrations at or above 5% of substrate (VS-basis). Complete inhibition persisted to day 30, suggesting the suitability of *Asparagopsis* over timescales relevant for acidogenic fermentation. *Asparagopsis* was not found to provide effective inhibition of methanogenesis in AD sludge inoculum at the treatment levels tested. In rumen

Table 3
Phylum-level community structure of initial inocula and of rumen-fed conditions at day 25 (relative abundance - %). Selected microbial families seen to respond to *Asparagopsis* treatment are included under their respective phyla.

Phylum- *Family*	Sludge (t = 0)	Rumen (t = 0)	Rumen (t = 25)	Rumen (t = 25) + *Asparagopsis*
Firmicutes	69.58	60.56	59.53	45.53
Lachnospiraceae	1.56	11.41	14.72	5.5
Ruminococcaceae	0.34	13.82	15.01	5.02
Veillonellaceae	N.D.	11.35	10.31	6.52
Streptococcaceae	0.3	N.D.	0.52	13.67
Bacteroidetes	15.31	25.67	11.81	17.83
Prevotellaceae	N.D.	13.65	0.15	0.77
Actinobacteria	0.53	7.23	5.27	4.91
Thermotogae	6.2	N.D.	N.D.	N.D.
Synergistetes	4.09	0.13	5.98	5.6
Proteobacteria	0.57	1.57	12.6	21.7
Enterobacteriaceae	N.D.	0.06	10.58	16.79
Euryarchaeota	0.02	1.04	2.4	0.17
Methanosarcinaceae	N.D.	N.D.	1.78	0.02
Methanobacteriaceae	N.D.	1.04	0.61	0.06

fluid, *Asparagopsis* produced VFA both of equal quantity and quality to reactors inhibited with bromoethane sulfonate. These results suggest *Asparagopsis* could find use as an alternative control agent in rumen-driven acidogenic reactor systems.

CRediT authorship contribution statement

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

Declaration of competing interest

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

Nicholas A. Tenci reports financial support was provided by Rotary International and East X LLP.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Prof. Chris K Reynolds and David Humphries (University of Reading) and Severn Trent Green Power (Cassington, Oxfordshire, UK) for providing the rumen fluid and AD sludge inocula, respectively. Nicholas A. Tenci would like to thank East X LLP. and Rotary International (Grant number: GG2128217) for their financial support of this research.

References

Agnihotri, S., Yin, D.-M., Mahboubi, A., Sapmaz, T., Varjani, S., Qiao, W., Koseoglu-Imer, D.Y., Taherzadeh, M.J., 2022. A glimpse of the world of volatile fatty acids production and application: a review. Bioengineered 13, 1249–1275.
Alibaba.com, 2023a. *Eucheuma Cottonii* Dried Seaweed from Vietnam Sea. (Accessed 11 October 2023).
Alibaba.com, 2023b. Wholesale High Quality Dried Sargassum Seaweed. (Accessed 11 October 2023).
Angelidaki, I., Sanders, W., 2004. Assessment of the anaerobic biodegradability of macropollutants. Rev. Environ. Sci. Biotechnol. 3, 117–129.
APHA, 1998. Standard Methods for the Examination of Water and Wastewater. American Water Works Association and Water Environment Federation.
Baleeiro, F.C.F., Kleinsteuber, S., Sträuber, H., 2022. Recirculation of H2, CO2, and ethylene improves carbon fixation and carboxylate yields in anaerobic fermentation. ACS Sustain. Chem. Eng. 10, 4073–4081.
Biddle, A., Stewart, L., Blanchard, J., Leschine, S., 2013. Untangling the genetic basis of fibrolytic specialization by *Lachnospiraceae* and *Ruminococcaceae* in diverse gut communities. Diversity 5, 627–640.
Cappelletti, M., Frascari, D., Zannoni, D., Fedi, S., 2012. Microbial degradation of chloroform. Appl. Microbiol. Biotechnol. 96, 1395–1409.
Chidthaisong, A., Conrad, R., 2000. Specificity of chloroform, 2-bromoethanesulfonate and fluoroacetate to inhibit methanogenesis and other anaerobic processes in anoxic rice field soil. Soil Biol. Biochem. 32, 977–988.
Christophe, G., Lara Deo, J., Kumar, V., Nouaille, R., Fontanille, P., Larroche, C., 2012. Production of oils from acetic acid by the oleaginous yeast *Cryptococcus curvatus*. Appl. Biochem. Biotechnol. 167, 1270–1279.
Cotter, J., Glass, R., Black, J., Madden, P., Davidson, T., 2015. A marginal abatement cost analysis of practice options related to the NLMP program. Meat & Livestock Australia Limited.
Glasson, C.R.K., Kinley, R.D., de Nys, R., King, N., Adams, S.L., Packer, M.A., Svenson, J., Eason, C.T., Magnusson, M., 2022. Benefits and risks of including the bromoform containing seaweed *Asparagopsis* in feed for the reduction of methane production from ruminants. Algal Res. 64, 102673.
Karekar, S.C., Ahring, B.K., 2023. Reducing methane production from rumen cultures by bioaugmentation with homoacetogenic bacteria. Biocatal. Agric. Biotechnol. 47, 102526.
Kinley, R.D., de Nys, R., Vucko, M.J., Machado, L., Tomkins, N.W., 2016a. The red macroalgae *Asparagopsis taxiformis* is a potent natural antimethanogenic that reduces methane production during *in vitro* fermentation with rumen fluid. Anim. Prod. Sci. 56, 282–289.
Kinley, R.D., Vucko, M.J., Machado, L., Tomkins, N.W., 2016b. *In vitro* evaluation of the antimethanogenic potency and effects on fermentation of individual and combinations of marine macroalgae. Am. J. Plant Sci. 7, 2038–2054.
Kishimoto, A., Ushida, K., Phillips, G.O., Ogasawara, T., Sasaki, Y., 2006. Identification of intestinal bacteria responsible for fermentation of gum Arabic in a pig model. Curr. Microbiol. 53, 173–177.
Krümpel, J., George, T., Gasston, B., Francis, G., Lemmer, A., 2020. Suitability of *Opuntia ficus-indica* (L) Mill. and *Euphorbia tirucalli* L. as energy crops for anaerobic digestion. J. Arid Environ. 174, 104047.
Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D.P., O'Donohue, M., Hernandez-Raquet, G., 2015. Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. Bioresour. Technol. 196, 241–249.
Li, X., Norman, H.C., Kinley, R.D., Laurence, M., Wilmot, M., Bender, H., de Nys, R., Tomkins, N., 2016. *Asparagopsis taxiformis* decreases enteric methane production from sheep. Anim. Prod. Sci. (58), 681–688.
Liang, J., Fang, W., Chang, J., Zhang, G., Ma, W., Nabi, M., Zubair, M., Zhang, R., Chen, L., Huang, J., Zhang, P., 2022. Long-term rumen microorganism fermentation of corn stover *in vitro* for volatile fatty acid prodution. Bioresour. Technol. 358, 127447.
Liu, H., Wang, J., Wang, A., Chen, J., 2011. Chemical inhibitors of methanogenesis and putative applications. Appl. Microbiol. Biotechnol. 89, 1333–1340.
Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125 (1), 171–189.
Lueangwattanapong, K., Ammam, F., Mason, P.M., Whitehead, C., McQueen-Mason, S.J., Gomez, L.D., Smith, A.C., Thompson, I.P., 2020. Anaerobic digestion of Crassulacean Acid Metabolism plants: exploring alternative feedstocks for semi-arid lands. Bioresour. Technol. 297, 122262.
Machado, L., Magnusson, M., Paul, N.A., de Nys, R., Tomkins, N., 2014. Effects of marine and freshwater macroalgae on *in vitro* total gas and methane production. PLoS One 9, e85289.
Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N., 2016a. Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on *in vitro* fermentation and methane production. J. Appl. Phycol. 28, 1443–1452.
Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N., 2016b. Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity *in vitro*. J. Appl. Phycol. 28, 3117–3126.
Machado, L., Tomkins, N., Magnusson, M., Midgley, D.J., de Nys, R., Rosewarne, C.P., 2018. *In vitro* response of rumen microbiota to the antimethanogenic red macroalga *Asparagopsis taxiformis*. Microb. Ecol. 75, 811–818.
Martin, L.K., Huang, W.E., Thompson, I.P., 2023. The effect of nutrient limitation on bacterial wax ester production. Bioresour. Technol. Rep. 22, 101423.
Mengmeng, C., Hong, C., Qingliang, Z., Shirley, S.N., Jie, R., 2009. Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation. Bioresour. Technol. 100, 1399–1405.
Meyer, G., Okudoh, V., van Rensburg, E., 2022. A rumen based anaerobic digestion approach for lignocellulosic biomass using barley straw as feedstock. S. Afr. J. Chem. Eng. 41, 98–104.
Mikesell, M.D., Boyd, S.A., 1990. Dechlorination of chloroform by *Methanosarcina* strains. Appl. Environ. Microbiol. 56, 1198–1201.
Nijenhuis, I., Kuntze, K., 2016. Anaerobic microbial dehalogenation of organohalides - state of the art and remediation strategies. Curr. Opin. Biotechnol. 38, 33–38.
Regal, A.L., Alves, V., Gomes, R., Matos, J., Bandarra, N.M., Afonso, C., Cardoso, C., 2020. Drying process, storage conditions, and time alter the biochemical composition and bioactivity of the anti-greenhouse seaweed *Asparagopsis taxiformis*. Eur. Food Res. Technol. 246, 781–793.

Richards, B.K., Cummings, R.J., White, T.E., Jewell, W.J., 1991. Methods for kinetic analysis of methane fermentation in high solids biomass digesters. Biomass Bioenergy 1, 65–73.

Romero, P., Belanche, A., Jiménez, E., Hueso, R., Ramos-Morales, E., Salwen, J.K., Kebreab, E., Yáñez-Ruiz, D.R., 2023. Rumen microbial degradation of bromoform from red seaweed (*Asparagopsis taxiformis*) and the impact on rumen fermentation and methanogenic archaea. J. Animal Sci. Biotechnol. 14, 133.

Roque, B.M., Venegas, M., Kinley, R.D., de Nys, R., Duarte, T.L., Yiang, X., Kebreab, E., 2021. Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. PLoS One 16, e0247820.

Shen, L., Hu, H., Ji, H., Cai, J., He, N., Li, Q., Wang, Y., 2014. Production of poly (hydroxybutyrate-hydroxyvalerate) from waste organics by the two-stage process: focus on the intermediate volatile fatty acids. Bioresour. Technol. 166, 194–200.

Shi, X., Lin, J., Zuo, J., Li, P., Li, X., Guo, X., 2017. Effects of free ammonia on volatile fatty acid accumulation and process performance in the anaerobic digestion of two typical bio-wastes. J. Environ. Sci. 55, 49–57.

Siegert, I., Banks, C., 2005. The effect of volatile fatty acid additions on the anaerobic digestion of cellulose and glucose in batch reactors. Process Biochem. 40, 3412–3418.

Tan, S., Harris, J., Roque, B.M., Askew, S., Kinley, R.D., 2023. Shelf-life stability of *Asparagopsis* bromoform in oil and freeze-dried powder. J. Appl. Phycol. 35, 291–299.

Tenci, N.A., Ammam, F., Huang, W.E., Thompson, I.P., 2023. Anaerobic co-digestion of *Euphorbia tirucalli* with pig blood for volatile fatty acid production. Bioresour. Technol. Rep. 21, 101333.

Vacca, M., Celano, G., Calabrese, F.M., Portincasa, P., Gobbetti, M., De Angelis, M., 2020. The controversial role of human gut Lachnospiraceae. Microorganisms 8, 573.

van Beelen, P., van Keulen, F., 1990. The kinetics of the degradation of chloroform and benzene in anaerobic sediment from the river Rhine. Hydrobiol. Bull. 24, 13–21.

van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Wang, K., Yin, J., Shen, D., Li, N., 2014. Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: effect of pH. Bioresour. Technol. 161, 395–401.

Wang, S., Zhang, G., Zhang, P., Ma, X., Li, F., Zhang, H., Tao, X., Ye, J., Nabi, M., 2018. Rumen fluid fermentation for enhancement of hydrolysis and acidification of grass clipping. J. Environ. Manag. 220, 142–148.

Wood, J.M., Scott Kennedy, F., Wolfe, R.S., 1968. The reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. Biochemistry 7, 1707–1713.

Yin, J., Yu, X., Zhang, Y., Shen, D., Wang, M., Long, Y., Chen, T., 2016. Enhancement of acidogenic fermentation for volatile fatty acid production from food waste: effect of redox potential and inoculum. Bioresour. Technol. 216, 996–1003.