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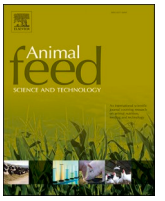

Effect of the garlic matrix and inclusion level on *in vitro* methane production and fermentation

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ABSTRACT

Garlic contains bioactive organosulphur compounds reported to be effective in reducing methane (**CH_4**) emissions, but garlic dietary supplements are inconsistent in reducing rumen CH_4 production, possibly due to matrix or inclusion level effects. To assess this, the effects of garlic matrix (freeze-dried, **FD**; garlic extract, **GE**), source (Chinese; Spanish) and inclusion level (30, 60, 120 mg extract/g DM) on gas, CH_4 and volatile fatty acid (**VFA**) production were measured using an in *vitro* gas production rumen fermentation system. Garlic extract was extracted from both sources, and this or FD garlic was incubated at the same oil inclusion levels in flasks with a dried, milled total mixed ration (**TMR**; forage:concentrate 50:50, DM basis) for 72 h, with strained rumen fluid and incubation medium (1:9, v/v). Flasks containing just TMR (control, **CON**) or no substrate (negative control) were also included. Gas pressure measured at intervals during the fermentation was converted to volume, and gas samples were analysed for CH_4 concentration using gas chromatography. Dry matter degradability and VFA concentrations in the medium were measured after 72 h. *In vitro* gas and CH_4 production was fitted to previously published models to obtain gas production kinetic characteristics. Data were analysed using linear mixed models, with processing method, origin, and their interaction as fixed, and run as random factors. Dry matter degradability (g/kg DM) was higher ($P < 0.001$) with all treatments (both FD and GE) for both Chinese and Spanish garlic across all inclusion rates (79.8 and 80.1) compared to CON (78.3). Total gas production (ml) was higher ($P < 0.05$) in the GE treatments than the CON (145.2) for both Chinese and Spanish garlic (164.1 and 169.1, respectively). Acetate:propionate ratio was lower ($P < 0.001$) for both garlic treatments and origin across all inclusion rates (2.4–2.9) compared with CON (3.0). Increasing the inclusion rate did not change *in vitro* CH_4 production compared with CON. The results suggest that the bioactive components in garlic, when presented in an extracted matrix, might enhance overall fermentation efficiency without directly mitigating CH_4 emissions. This finding implies that while garlic-based supplements might not consistently reduce CH_4 emissions, they could be used to improve feed efficiency and animal productivity in ruminant systems.

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1. Introduction

Among greenhouse gases, methane (**CH_4**) has a significant impact on global warming, with a global warming potential approximately 28 times greater than that of carbon dioxide (**CO_2**) over a 100-year period (Beauchemin et al., 2020; IPCC, 2021). Ruminant livestock production is a major contributor (20–25 %) to atmospheric CH_4, primarily through enteric fermentation (Thorpe, 2009; Beauchemin et al., 2020; Sari et al., 2022). While enteric CH_4 emissions contribute to global warming, they also represent a loss of energy from the animal's feed (Johnson and Johnson,1995; Knapp et al., 2014), reducing feed efficiency. Dietary manipulation has emerged as one of the most promising strategies for reducing CH_4 emissions from ruminants (Beauchemin et al., 2020; Honan et al., 2021). Among the various feed additives explored, garlic (*Allium sativum*) has gained considerable interest due to its rich composition of sulphur-containing bioactive compounds, such as allicin and diallyl disulphide, which have been shown to exhibit antimicrobial properties (Harris et al., 2001; Panthee et al., 2017; Nguyen et al.,2021). These compounds are hypothesised to inhibit specific rumen microorganisms, including methanogens, thereby reducing CH_4 production. Dietary supplementation with garlic products has been associated with reductions in CH_4 emissions and increases in propionate concentration (Kamel et al., 2008), an energy-efficient pathway in anaerobic fermentation that can offset some of the energy lost as CH_4 (Calsamiglia et al., 2007; Sari et al., 2022).

Despite these promising findings, previous research using garlic products to decrease CH_4 emissions reported mixed results. Some studies using *in vitro* rumen gas production systems have demonstrated significant reductions in CH_4 yield with garlic supplementation. Garlic oil and garlic-derived diallyl disulphide (both incubated individually *in vitro* at an inclusion level of 30 mg/g substrate diet) decreased CH_4 yield by 73.6 % and 68.5 %, respectively, over 17 h, compared with a control diet (50:50 forage:concentrate; Busquet et al., 2005). When garlic powder was supplemented at a rate of 80 mg/g of substrate (60:40 forage:concentrate), CH_4 yield decreased by 21 % over 72 h *in vitro* incubation with buffalo rumen fluid, when compared with a control diet (Kongmun et al., 2010). Additionally, incubating 27.5 mg garlic oil/g substrate with buffered rumen fluid over 24 h decreased CH_4 yield by 29.4 and 17.6 % for a high forage or high concentrate diet, respectively, when compared with a control diet (Patra and Yu, 2015a). Others have reported minimal effect of supplemental garlic on *in vitro* gas and methane production, for example when incubating different garlic preparations (intact, freeze-dried, oven-dried, autoclaved) at 25 and 50 mg/g substrate for 24 h (Vargas et al., 2023). These inconsistencies could reflect different garlic preparations (the matrix), garlic types (with varying bioactive compound concentrations) or different conditions across different studies. The garlic matrix, i.e. whether it is added in fresh, freeze-dried, powdered or oil extract form, will affect the bioavailability and release rates of bioactive compounds. Differences in concentrations (and stability) of these compounds have been reported between freeze-dried garlic and garlic oil (Calsamiglia et al., 2007; Martins et al., 2016). The recovery of allicin from freeze or oven dried garlic cloves has been reported to be low (Nguyen et al., 2021), and allicin bioactivity is unstable upon storage (Amagase, 2006). Garlic oil, with its high concentration of volatile sulphur compounds and relatively high bioavailability, enables rapid interaction with methanogens, potentially resulting in immediate reductions in CH_4 production. In contrast, dried garlic powder releases its bioactive compounds more gradually, which may provide sustained effects on rumen fermentation over time (Calsamiglia et al., 2007; Kongmun et al., 2010; Vargas et al., 2023).

Studies indicate that higher inclusion rates of garlic products do not always lead to increased efficacy, suggesting that there may be an optimal inclusion rate that maximises benefits while minimising potential inhibitory effects on rumen fermentation (Busquet et al., 2005; Sari et al., 2022). Inconsistencies across studies are likely to be due to variations in factors such as the administered dose as well as substrate composition (Kamel et al., 2009). High doses of garlic oil and garlic powder have demonstrated the greatest reductions in CH_4 emissions, but the feasibility of these dose levels must be carefully evaluated for practical application at the farm level (Sari et al., 2022).

Previous research on the above factors has mainly been conducted in separate studies for each factor, which makes it difficult to determine the most effective combination. This study aims to address this by examining how garlic origin (Chinese or Spanish), preparation method (freeze-dried vs. garlic extract), and inclusion level influence total gas production, CH_4 production, dry matter degradability (**DMd**), pH, and the production of volatile fatty acids (**VFA**) using an *in vitro* gas production fermentation system.

2. Materials and methods

2.1. Garlic sample preparation

Two kilograms of each of Chinese and Spanish fresh garlic (*Allium sativum*) were obtained from a local supermarket in Reading, UK. Whole garlic bulbs (including skin) were chopped into small pieces (4 mm) and stored at −20 °C. A subsample (500 g) of each chopped variety was then freeze dried and milled to < 4 mm. A further subsample (100 g) was processed to extract garlic oil using a steam distillation method (Soltan et al., 2016). Briefly, finely chopped (undried) garlic was placed in a flask with water and heated to 100°C for 2 h. Water vapour arising from heating, (containing volatile compounds), was condensed and the concentrated extract collected. The amount of extract obtained from the known weight of garlic was recorded. Residue from the extraction process was dried and stored, to be added to fermentation flasks containing extract. The extract was diluted with ethanol to form a stock solution equivalent to the highest inclusion rate (120 mg/g DM) for each garlic type. For ease of pipetting it was decided that 20 µl diluted extract would be added to fermentation flasks, so this volume needed to hold the same amount of neat extract as 120 mg of freeze-dried garlic. The Chinese garlic contained 0.24 % extract (DM basis); 120 mg freeze dried Chinese garlic would contain (0.24/100) x 120 = 0.29 mg extract, so the stock solution for this extract needed to have 0.29 mg extract in 20 µl, or 14.5 mg extract/ml. Stock solutions for the other inclusion rates were also prepared in this way. For Spanish garlic a similar approach was used (freeze dried Spanish garlic contained 0.22 % extract, DM basis).

2.2. In vitro experiments

All animal procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1996. The *in vitro* rumen gas production system used a method adapted from (Theodorou et al., 1994; Mauricio et al., 1999). The treatments comprised of two types of garlic (Chinese and Spanish), two preparations (freeze dried, **FD**, and garlic extract plus extraction residue, **GE**) incubated at three inclusion rates (30, 60 or 120 mg/g DM) with 1.0 g dried, milled (< 2 mm) total mixed ration (**TMR**) basal substrate in Wheaton flasks. The TMR comprised of (g/kg DM); maize silage (403), grass silage (206), concentrate blend (300), sodagrain (77), distillers wheatfeed (94), minerals (5.4), salt (3.6), limestone (1.8), calcium salts of palm oil distillate (6.9), urea (1.8) with the concentrate blend comprising (g/kg DM): cracked wheat (180), rapeseed (185), soya meal (170), molassed sugar-beet feed (145), wheat distillers (140), soya hulls (120), molasses (40), minerals (20). Inclusion rates were achieved so that FD treatments were added at 30, 60 or 120 mg/g DM, and the GE treatments were added via the 20 µl stock solutions, to provide an equivalent amount of neat extract given the oil content of the FD garlic, as outlined above. For the GE treatment flasks, dried residue from oil extraction was included at equivalent weights to that of the FD samples (so 30, 60 and 120 mg/g). Also, to address the presence of ethanol in the extract stock solutions, 20 µl just ethanol was added to the FD treatment flasks. This ensured that the treatments were comparable across inclusion rates, and that the only difference between them was the matrix. TMR-only flasks were also prepared, as well as negative control flasks (no TMR substrate). Each treatment/control was incubated in triplicate. To each flask 90 ml fermentation medium (Mauricio et al., 1999) and 10 ml strained (through two layers of cheesecloth) rumen fluid obtained from a fistulated dry dairy cow at the Centre for Dairy Research was added, after which the flasks were sealed and incubated at 39°C. Rumen fluid was obtained before feeding, and the cow was fed a maintenance diet of chopped straw/grass silage with *ad libitum* access to a mineral lick block. At 2, 4, 6, 8, 10, 12, 24, 32, 48 and 72 h the gas pressure was recorded using a headspace pressure transducer (Bailey and Mackey Ltd., Birmingham) and recorder (psi; Tracker 200; Data Track Process Instruments, UK), and a 10 ml sample of gas at each time point was removed for CH_4 analysis via a two-way valve on the pressure transducer. At 72 h the lids were removed and the pH of the flask contents recorded before a subsample of fluid was taken and stored at −20°C for volatile fatty acid (**VFA**) analysis. Flask contents were filtered through pre-weighed sintered glass crucibles (grade 1) and the residue was oven dried at 100°C for 4 h before reweighing to obtain dry residue weight, so that DMd could be calculated by difference. Each fermentation run for each garlic source was run on three separate occasions, with triplicate flasks for each treatment within each run.

Methane concentration of gas samples taken was measured using gas chromatography (**GC**) following manual injection of gas samples. Prior to measurement, a five-point (25 000, 50 000, 75 000 and 100 000 ppm) standard curve was conducted on the GC (Bruker 450 GC, Bruker, Germany) fitted with a port valve and plumbed into the injector. Gas components were separated on a CH_4-packed Poropak N column (1.2 m, 2 mm internal diameter, Varian Inc., Walnut Creek, CA) and CH_4 detected using a flame ionisation detector. Full column and GC conditions were reported previously (Munoz et al., 2012).

2.3. Volatile fatty acid analysis

Subsamples of 72 h incubation medium were thawed, and to 1.2 ml thawed sample, 0.3 ml internal standard (25 mM 2-ethylbutyric acid in 25 % w/v metaphosphoric acid) was added and allowed to stand for 30 min. Samples were centrifuged at 13,000 rpm for 10 min at 4°C before 0.5 ml supernatant was transferred to a GC vial containing 0.8 ml distilled water. A series of standards (containing acetic, propionic, butyric, vaccenic, isobutyric, isovaleric and caproic acids) were prepared similarly. VFA in 1.5 µl standards and samples were separated using GC (Agilent 7890B) equipped with a flame ionisation detector and a 30 m column (30 m, 0.25 mm internal diameter, 10 m guard; Stabilwax-DA). The oven temperature started at 70°C and increased by 24 °C/min to a temperature of 190°C. Then it increased by 90 °C/min to 235°C and held for 0.5 min. The injector and detector temperatures were set at 220°C and 250°C, respectively, and split injection was employed (16:1). Hydrogen was used as a carrier gas, at a constant flow of 2.0 ml/min. Results for each VFA were recorded on a mM basis.

2.4. Diet component analysis

The individual diet components were analysed for their chemical composition, including DM, ash, CP, starch, water-soluble carbohydrates (**WSC**), NDF, ADF, and gross energy, following established methods. Feed samples were oven-dried at 100°C to determine DM (AOAC 988.05) and ashed by combustion at 600°C (AOAC 942.05; AOAC, 2012). Nitrogen content was measured using the Kjeldahl method (AOAC, 2012), and CP was calculated as N × 6.25. Neutral detergent fibre and ADF were analysed based on methods by Roberston and Van Soest (1981) and Mertens (2002). Oil content was assessed by the "Wiebul" acid hydrolysis method.

2.5. Data analysis

Gas pressure readings (psi) were used to calculate the gas volume using the equation according to Mauricio et al. (1999). Gas volume (ml) was adjusted to take into account blank volume (negative control) at each time point, and expressed as ml/g fermented/h. Methane concentrations obtained using the GC were applied to the gas volumes to calculate CH_4 volume (ml/g fermented/h). pH values were converted to hydrogen ion concentration ($\mathbf{[H^+]}$) prior to statistical analysis and then means from the model were log converted to calculate pH. Dry matter degradability (after 72 h) was calculated using the total weight of substrate added at time 0 and the dry weight of residue after 72 h. Gas and CH_4 volumes were fitted to previously published models of gas production (France et al., 1993). Gas and CH_4 model parameters, VFA concentrations, $[H^+]$ and DMd were analysed using linear mixed models in Minitab

(Minitab Statistical Software version 20.2), using processing method (FD or GE), source (Chinese or Spanish), inclusion rates (30, 60 or 120 mg extract/g DM) and their interaction as fixed, and batch run as random, factors. Tukey's test was conducted for multiple comparisons among treatment means when there was a significant effect at $P > 0.05$ of any treatments.

3. Results

3.1. Effect of garlic matrix and inclusion rates on gas production, methane (CH_4) production, and dry matter degradability (DMd)

The analysed nutrient composition results of the ingredients are given in Table 1. The nutrient composition analysis revealed distinct differences among the different garlic preparations. There were differences in composition between Chinese and Spanish garlic for both FD and post-extract garlic residue. Chinese garlic contained more DM compared to Spanish across both FD and residue samples. Organic matter was similar between sources but there was less in the residue compared to FD. Crude protein, total oil and fibre content differed between sources, with Spanish garlic appearing to contain more CP than Chinese garlic, and Chinese containing more total oil and NDF/ADF than Spanish garlic. The extraction process appeared to reduce CP and total oil content, as residue samples were lower CP and oil concentrations than FD samples. Gross energy values were higher in the residue samples than in the FD samples, with Chinese garlic having marginally higher gross energy content than Spanish garlic.

The effect of garlic matrix (freeze-dried or GE plus extraction residue) and varying inclusion rates (30, 60, 120 mg extract/g DM) on total gas production, CH_4 emission, and DMd after 72 h of *in vitro* incubation is presented in Table 2 (for Chinese garlic) and 3 (for Spanish garlic). The Chinese garlic matrix affected total (ml) and cumulative (ml/g DM) gas production, with the GE matrix resulting in higher ($P < 0.001$) gas production than the FD matrix, and both were higher than the Control (Table 2). Matrix did not seem to affect total volume of gas produced following fermentation of the Spanish variety ($P = 0.185$; Table 3). Increasing inclusion rate (across both preparations and origins) led to a linear decrease ($P < 0.001$) in lag time, and increase ($P < 0.05$) in total (ml) and cumulative (ml/g DM) gas production. For Chinese garlic there was an interaction ($P < 0.05$) between matrix and inclusion rate for all gas production measures, as the FD preparation decreased gas production (Table 2).

Matrix had an effect on CH_4 production; the FD matrix of both varieties resulted in lower ($P < 0.05$) total (ml) and cumulative (ml/g DM) CH_4 production than the GE matrix, which increased CH_4 production compared with the Control (Tables 2 and 3). There was no effect of matrix on CH_4 lag time for both varieties ($P = 0.519$ and 0.864 for Chinese and Spanish varieties, respectively). Increasing the inclusion rate resulted in a linear decrease ($P < 0.001$) in lag time for CH_4 production for both varieties and preparations. There was also a linear decrease ($P < 0.05$) in cumulative CH_4 production (ml/g dDM) for both varieties. A matrix by inclusion rate interaction ($P < 0.001$) was observed for total and cumulative CH_4 production for the Chinese garlic. An increase in inclusion rate of Chinese FD resulted in decreases in total and cumulative CH_4, whereas an increase in GE inclusion resulted in increases in these measures.

Dry matter degradability was higher ($P < 0.001$) with the garlic treatments compared to the Control (Tables 2 and 3). There was no effect ($P > 0.05$) of matrix of both varieties on DMd, but increasing inclusion rate across all preparations and varieties resulted in a linear increase ($P < 0.001$) in DMd.

3.2. Effect of garlic matrix and inclusion rates on in vitro volatile fatty acid (VFA) profiles

The effect of garlic matrix and inclusion rates on VFA production is reported in Tables 4 and 5. Garlic matrix had no effect on *in vitro* vessel pH or the concentration and proportion of vessel VFAs for both varieties of garlic ($P > 0.05$; Tables 4 and 5), and increasing inclusion rate had little impact on VFA concentrations. However increasing inclusion rate did affect VFA proportions; linear and quadratic increases ($P < 0.05$) in propionate proportion were observed for both garlic varieties, which, together with linear and quadratic decreases ($P < 0.01$) in acetate proportion contributed to a linear/quadratic decline ($P < 0.05$) in acetate:propionate ratio.

Increasing inclusion of Chinese garlic preparations resulted in a decrease ($P < 0.05$) in the proportion of iso-butyrate, iso-valerate and caproate (Table 4). With Spanish garlic, proportional increases, ($P < 0.05$) in butyrate and decreases in iso-butyrate were observed, while iso-butyric acid showed no significant change ($P > 0.05$; Table 5).

Table 1
Nutritional composition of the fermented substrates (total mixed ration, TMR, freeze-dried and freeze-dried post-extraction residue of Spanish and Chinese garlic; g/kg DM unless otherwise stated).

Composition	Total Mix Ration	Garlic		Post extraction garlic residue	
		Spanish	Chinese	Spanish	Chinese
DM (g/kg)	923	983	988	932	929
OM	840	940	943	890	884
Ash (g/kg)	83.0	43.0	45.5	42.5	45.5
CP (g/kg DM)	154	184	147	161	149
Total Oil (%)	3.91	0.35	0.40	0.38	0.60
NDF (g/kg DM)	359	97	128	102	132
ADF (g/kg DM)	236	106	110	104	109
Gross energy (MJ/kg DM)	17.4	16.9	16.5	17.1	17.3

DM = Dry matter; OM = Organic matter; CP = Crude protein; NDF = Neutral detergent fibre; ADF = Acid detergent fibre.

Table 2
Effect of garlic (Chinese variety) matrix (freeze-dried vs garlic extract plus residue) and inclusion dose (30, 60 and 120 mg extract equivalent/g DM; linear, LIN; quadratic, QUAD; cubic, CUB relationship) on *in vitro* gas and methane production and DM degradability, after 72 h incubation with a total mixed ration diet.

	Control	Freeze-dried			Extract plus residue			SEM	*P*[a]					
		30 mg/g	60 mg/g	120 mg/g	30 mg/g	60 mg/g	120 mg/g		Matrix	Dose	Matrix*Dose	LIN	QUAD	CUB
Gas Production														
Lag time (h)	2.8	3.0	2.4	2.1	2.8	2.5	1.8	0.45	0.203	< 0.001	0.440	< 0.001	0.043	0.045
Total production (ml)	143	144	144	136	150	159	184	14.0	< 0.001	0.029	0.002	0.004	0.956	0.918
Cumulative gas production (ml/g DM)	141	142	142	132	146	154	174	15.2	< 0.001	< 0.001	< 0.001	0.006	0.836	0.803
Cumulative gas production (ml/g dDM)	181	181	180	165	186	192	215	22.1	< 0.001	0.378	< 0.001	0.090	0.908	0.963
Methane Production														
Lag time (h)	6.0	6.2	5.3	4.5	5.9	5.4	4.1	0.78	0.519	< 0.001	0.807	< 0.001	0.169	0.315
Total production (ml)	11.4	11.6	10.6	8.4	11.7	12.0	12.5	1.40	< 0.001	0.223	< 0.001	0.071	0.340	0.700
Cumulative methane production (ml/g DM)	12.6	12.9	12.0	9.1	13.0	13.4	13.8	0.66	< 0.001	0.123	< 0.001	0.050	0.157	0.872
Cumulative methane production (ml/g dDM)	16.1	16.4	15.1	11.3	16.5	16.8	17.1	0.97	< 0.001	0.062	< 0.001	0.017	0.202	0.776
Digestibility														
DM (g/kg)	782	788	793	803	786	802	811	1.36	0.370	< 0.001	0.720	< 0.001	0.740	0.447

DM = Dry matter, dDM = digested DM

[a] Significance of effect of matrix, dose, matrix by dose interaction, and linear, quadratic or cubic effect of dose response

Table 3
Effect of garlic (Spanish variety) matrix (freeze-dried vs garlic extract plus residue) and inclusion dose (30, 60 and 120 mg extract equivalent/g DM; linear, LIN; quadratic, QUAD; cubic, CUB relationship) on *in vitro* gas and methane production and DM degradability, after 72 h incubation with a total mixed ration diet.

	Control	Freeze-dried			Extract plus residue			SEM	*P*[a]				
		30 mg/g	60 mg/g	120 mg/g	30 mg/g	60 mg/g	120 mg/g	.	Matrix	Dose	Matrix*Dose	LIN	QUAD	CUB
Gas Production														
Lag time (h)	2.6	2.4	2.1	1.6	2.4	2.3	1.8	0.13	0.223	< 0.001	0.798	< 0.001	0.655	0.981
Total production (ml)	148	154	164	164	164	173	170	17.4	0.185	< 0.001	0.865	0.008	0.062	0.766
Cumulative gas production (ml/g DM)	148	155	165	163	165	174	173	13.2	0.159	< 0.001	0.860	0.012	0.068	0.810
Cumulative gas production (ml/g dDM)	189	195	206	201	209	217	213	15.5	0.150	0.094	0.848	0.058	0.084	0.859
Methane Production														
Lag time (h)	5.2	5.4	5.0	4.1	5.3	5.1	4.1	0.77	0.864	< 0.001	0.933	< 0.001	< 0.001	0.323
Total production (ml)	10.5	11.1	11.3	9.0	12.0	12.7	11.4	1.17	< 0.001	0.106	0.519	0.529	0.020	0.887
Cumulative methane production (ml/g DM)	9.2	9.8	9.8	7.9	10.6	11.2	9.1	0.97	< 0.001	< 0.001	0.622	0.096	0.003	0.959
Cumulative methane production (ml/g dDM)	11.8	12.4	12.2	9.7	13.5	13.9	11.2	1.20	< 0.001	< 0.001	0.603	0.029	0.003	0.988
Digestibility														
Dry Matter (g/kg)	784	794	801	811	791	801	812	0.89	0.819	< 0.001	0.935	< 0.001	0.338	0.736

DM = Dry matter, dDM = digested DM

[a] Significance of effect of matrix, dose, matrix by dose interaction, and linear, quadratic or cubic effect of dose response

Table 4
Effect of garlic (Chinese variety) matrix (freeze-dried vs garlic extract plus residue) and inclusion dose (30, 60 and 120 mg extract equivalent/g DM; linear, LIN; quadratic, QUAD; cubic, CUB relationship) on *in vitro* volatile fatty acid (VFA) production (mM and %), after 72 h incubation with a total mixed ration diet.

	Control	Freeze-dried			Extract plus residue			SEM	P^{a}					
		30 mg/g	60 mg/g	120 mg/g	30 mg/g	60 mg/g	120 mg/g	.	Matrix	Dose	Matrix*Dose	LIN	QUAD	CUB
pH	6.7	6.6	6.6	6.6	6.7	6.7	6.7	0.10	0.306	0.727	0.896	0.954	0.280	0.817
Acetic acid (mM)	38.4	39.4	43.9	36.1	43.5	39.3	38.7	3.79	0.757	0.192	0.271	0.433	0.048	0.811
Propionic acid (mM)	13.0	13.6	15.7	15.4	15.1	13.8	15.5	1.40	0.948	0.063	0.319	0.013	0.346	0.671
Butyric acid (mM)	8.9	9.3	9.8	9.5	9.8	9.0	9.7	1.19	0.942	0.540	0.587	0.308	0.491	0.439
Valeric acid (mM)	1.2	1.2	1.4	1.1	1.3	1.2	1.2	0.10	0.808	0.292	0.362	0.917	0.063	0.896
iso-Butyric acid (mM)	0.60	0.61	0.68	0.56	0.67	0.60	0.58	0.06	0.923	0.185	0.310	0.249	0.066	0.750
iso-Valeric acid (mM)	1.0	1.0	1.1	0.9	1.1	1.0	1.0	0.12	0.857	0.080	0.291	0.081	0.048	0.775
Caproic acid (mM)	0.93	1.0	1.1	0.62	1.0	1.0	0.83	0.19	0.528	< 0.001	0.308	0.014	0.017	0.807
Total VFA (mM)[2]	64.0	66.1	73.6	64.2	72.6	65.9	67.4	5.59	0.853	0.375	0.299	0.842	0.096	0.722
Acetic acid (% VFA)	60.0	59.7	59.5	56.1	59.8	59.6	57.3	1.03	0.170	< 0.001	0.306	< 0.001	0.001	0.283
Propionic acid (% VFA)	20.4	20.7	21.2	24.2	20.7	21.0	23.1	1.50	0.097	< 0.001	0.189	< 0.001	0.002	0.450
Butyric acid (% VFA)	13.8	13.8	13.5	14.7	13.8	13.7	14.3	1.47	0.787	0.101	0.862	0.060	0.113	0.503
Valeric acid (% VFA)	1.8	1.8	1.9	1.7	1.8	1.8	1.8	0.11	0.695	0.527	0.588	0.442	0.230	0.733
iso-Butyric acid (% VFA)	0.93	0.93	0.92	0.87	0.93	0.91	0.87	0.02	0.667	< 0.001	0.899	< 0.001	0.003	0.623
iso-Valeric acid (% VFA)	1.6	1.6	1.5	1.4	1.6	1.5	1.4	0.07	0.745	< 0.001	0.817	< 0.001	0.008	0.710
Caproic acid (% VFA)	1.5	1.5	1.5	0.9	1.5	1.5	1.3	0.31	0.371	< 0.001	0.391	0.004	0.056	0.523
Acetic:Propionic ratio	3.0	2.9	2.8	2.4	2.9	2.9	2.5	0.20	0.057	< 0.001	0.076	< 0.001	< 0.001	0.186

[a] Significance of effect of matrix, dose, matrix by dose interaction, and linear, quadratic or cubic effect of dose response

Table 5
Effect of garlic (Spanish variety) matrix (freeze dried vs garlic extract plus residue) and inclusion dose (30, 60 and 120 mg extract equivalent/g DM; linear, LIN; quadratic, QUAD; cubic, CUB relationship) on *in vitro* volatile fatty acid (VFA) production (mM and %), after 72 h incubation with a total mixed diet.

	Control	Freeze-dried			Extract plus residue			SEM	P^{a}					
		30 mg/g	60 mg/g	120 mg/g	30 mg/g	60 mg/g	120 mg/g		Matrix	Dose	Matrix*Dose	LIN	QUAD	CUB
pH	6.7	6.6	6.6	6.6	6.6	6.6	6.6	0.11	0.109	< 0.001	0.441	< 0.001	0.002	0.032
Acetic acid (mM)	46.2	47.6	47.7	42.8	46.2	49.1	47.9	1.91	0.148	0.107	0.088	0.464	0.027	0.327
Propionic acid (mM)	15.4	16.3	16.5	16.9	15.6	16.9	18.2	0.74	0.480	< 0.001	0.212	< 0.001	0.598	0.625
Butyric acid (mM)	9.8	10.0	10.2	9.9	9.8	10.4	11.3	0.94	0.077	0.041	0.073	0.007	0.915	0.500
Valeric acid (mM)	1.4	1.5	1.5	1.5	1.4	1.6	1.6	0.14	0.959	0.360	0.917	0.161	0.371	0.684
iso-Butyric acid (mM)	0.72	0.74	0.74	0.66	0.71	0.75	0.74	0.05	0.184	0.063	0.056	0.161	0.031	0.179
iso-Valeric acid (mM)	1.3	1.3	1.3	1.3	1.3	1.3	1.5	0.18	0.442	0.563	0.661	0.175	0.776	0.866
Caproic acid (mM)	1.1	1.1	1.2	1.2	1.1	1.2	1.1	0.20	0.767	0.711	0.990	0.604	0.365	0.709
Total VFA (mM)[2]	75.9	78.6	79.2	74.2	76.1	81.2	82.2	3.70	0.184	0.170	0.104	0.223	0.104	0.389
Acetic acid (% VFA)	60.9	60.6	60.3	57.6	60.7	60.4	58.2	0.54	0.098	< 0.001	0.339	< 0.001	< 0.001	0.168
Propionic acid (% VFA)	20.3	20.8	20.9	22.9	20.5	20.8	22.1	0.70	0.116	< 0.001	0.446	< 0.001	0.041	0.518
Butyric acid (% VFA)	12.9	12.7	12.8	13.3	12.9	12.8	13.8	0.92	0.248	< 0.001	0.665	0.005	0.046	0.815
Valeric acid (% VFA)	1.9	1.9	1.9	2.0	1.9	1.9	1.9	0.12	0.448	0.763	0.824	0.324	0.806	0.973
iso-Butyric acid (% VFA)	1.0	0.93	0.94	0.88	0.93	0.93	0.89	0.03	0.810	< 0.001	0.414	< 0.001	0.017	0.012
iso-Valeric acid (% VFA)	1.7	1.7	1.7	1.7	1.7	1.7	1.8	0.18	0.666	0.683	0.947	0.362	0.403	0.912
Caproic acid (% VFA)	1.4	1.5	1.5	1.5	1.5	1.5	1.4	0.24	0.585	0.953	0.886	0.842	0.622	0.905
Acetic:Propionic ratio	3.0	2.9	2.9	2.5	3.0	2.9	2.7	0.08	0.113	< 0.001	0.486	< 0.001	0.028	0.446

[a] Significance of effect of matrix, dose, matrix by dose interaction, and linear, quadratic or cubic effect of dose response

4. Discussion

The unexpected increase in total gas production observed in this study across all garlic preparations, along with the decreased lag phase, most probably reflected the combined effects of differences in fermentable substrate when compared with the TMR, and also garlic bioactive compounds present (such as organosulphur compounds allicin, diallyl disulphide, diallyl trisulphide, and others; Sari et al., 2022). Both the FD garlic and extraction residue contained more organic matter on an as weighed basis, so direct replacement of the TMR with this increased organic matter available for fermentation in each of the treatment flasks, enhancing microbial activity. A similar reason could explain the lack of effect of garlic on in vitro gas production in the study of Vargas et al. (2023), where direct replacement of the basal diet with the dried garlic (with a high organic matter content) would have provided the rumen microbes with sufficient substrate, regardless of any antimethanogenic activity.

Although it is generally assumed that bioactive compounds in garlic are antimicrobial, there are suggestions that in some cases garlic oil could sometimes stimulate microbial growth and fermentation (Klevenhusen et al., 2011), possibly via improving organic sulphur availability for microbial protein synthesis (McSweeney et al., 2009). The increased gas production was not observed with the Spanish variety in the present study. However this did not contain as much oil as the extract from the Chinese variety (as demonstrated by the difference in total oil content between the FD and residue, in Table 1), so it is assumed had a lower organosulphur content. Unfortunately organosulphur compound profiles of the garlic preparations were not measured in this study. The relatively low oil yield in the Spanish garlic extract suggests that diethyl ether extraction may not have been equally effective across different garlic sources, potentially due to structural or compositional variation (Lanzotti, 2006). Additionally, lower CP content in the defatted residue indicates that some peptides/proteins were co-extracted, which may have altered nutrient availability and fermentation (Amagase, 2006; Calsamiglia et al., 2007).

The inconsistent effects of garlic supplementation on CH_4 production observed in this study are consistent with the variability reported in the literature (Kamel et al., 2008; Vargas et al., 2023). While FD garlic significantly reduced CH_4 at moderate inclusion levels when compared with the control diet, GE plus extraction residue (prepared from the same garlic) increased CH_4 compared to the control. One possible explanation for the differential effects on CH_4 production observed for different matrices could be that any bioactive compounds which supressed methanogenesis in the FD matrix may have been denatured by the extraction process (Subroto et al., 2021). This, coupled with the improved fermentability of substrate, could explain the higher CH_4 production (ml/g DM) observed with GE. Another possibility is that any antimicrobial action of bioactive compounds in GE were overcome during the 72 h incubation in this study. It has been reported that the rumen microbial ecosystem can adapt to the bioactive compounds present in garlic oil over longer periods of time, both *in vivo* (Ohene-Adjei et al., 2008) and *in vitro* (Patra and Yu, 2015b), either via increasing methanogen diversity or degradation of bioactive components. Given that our extraction process may have led to some degree of bioactive compound degradation, it is unclear which of these mechanisms were active in our study.

The increased DMd across all garlic treatments indicates no negative effect of garlic on feed digestibility and potentially improving overall efficiency (Patra and Yu, 2015a; Vargas et al., 2023). However, the observed increase in DMd in this study may also be partly explained by the differences in nutrient density between the garlic treatments and the TMR. Since garlic and its residue contained more organic matter on an as-weighed basis than the TMR, substituting part of the TMR with these supplements likely provided a more fermentable substrate, enhancing microbial activity and improving digestibility. *In vivo* studies that supplemented *ad libitum* diets with garlic without replacing other diet components have reported less pronounced effects on nutrient digestibility (Yang et al., 2007; Chaves et al., 2008), and *in vitro* incubation of garlic oil in the presence two different basal diets (medium vs high concentrate mix ration) resulted in differences in fermentation measures (Mateos et al., 2013). This suggests that the impact of garlic on digestibility may depend on both its bioactive properties, and also the overall composition of the basal diet.

Both matrices increased the proportion of propionate, and reduced the acetate: propionate ratio. This might be expected with the FD preparation, where a decreased CH_4 production may have resulted in greater hydrogen availability for propionate synthesis (Lan and Yang, 2019), but does not explain the change in propionate concentration observed after in vitro incubation of the GE preparation. It is possible that the increased propionate proportion for the GE preparation could have been related to the matrix of that preparation and its role in nutrient availability to micro-organisms, which might have affected VFAs independently of changes in methanogenesis. Previous *in vitro* studies reported that garlic preparations do not always lead to consistent changes in propionate, suggesting a matrix-dependent effect (Patra and Yu, 2015a; Vargas et al., 2023). In addition, Dey et al. (2021) reported that garlic oil reduced *in vitro* CH_4 production but did not always alter VFA proportions, supporting an alternative hypothesis for the FD treatment, that CH_4 suppression may involve alternative microbial shifts. This highlights the complexity of fermentation responses to different matrices, warranting further investigation into the specific microbial shifts responsible for CH_4 suppression with FD garlic treatments.

Increasing the inclusion of Chinese or Spanish garlic preparations resulted in different effects on other VFA concentrations. Iso-valerate, iso-butyric acid, and caproate are branched-chain volatile fatty acids (BCVFAs) and straight-chain VFAs which are products of amino acid deamination and carbohydrate fermentation. The decrease in iso-valerate and caproate with Chinese garlic preparations suggests a potential suppression of protein fermentation pathways, possibly due to the antimicrobial properties of this garlic source's organosulphur compounds. These compounds are known to modify microbial populations by inhibiting certain proteolytic bacteria in the rumen (Busquet et al., 2005; Patra and Yu, 2015a). By contrast, the increase in butyrate concentration/proportion with Spanish garlic suggests stimulation of butyrogenic fibrolytic bacteria such as *Butyrivibrio fibrisolvens*. These results point to source- and matrix-specific effects on rumen microbial activity.

The results of this study indicate that while garlic supplementation, particularly in extract form, has the potential to enhance rumen fermentation by increasing gas production and DMd, its efficacy in reducing CH_4 production is inconsistent. This inconsistency may be attributed to differences in garlic matrices, processing methods, and the bioavailability of key organosulphur compounds. Future

research should focus on elucidating the mechanisms by which garlic and its bioactive compounds affect rumen microbiota and CH_4 production *in vivo*, including investigations into longevity of any effects. The processing method, such as freeze-drying or oil extraction, should be carefully considered to maximise the concentration and stability of the active compounds responsible for the desired effects.

In conclusion, the addition of garlic preparations Soltan et al. (2016) had no overall effect on *in vitro* CH_4 production, but increased total gas production, DMd, and decreased the acetate-to-propionate ratio when compared with a control TMR. However, when comparing the different preparations (FD garlic or GE plus residue), FD garlic generated less CH_4 than garlic extract plus residue from the same sample, thereby demonstrating a matrix effect, or that the extraction process had a negative impact on bioactive compounds. There was little difference in fermentation response between the two garlic origins included in this study (Chinese and Spanish). The improved DMd and decreased acetate-to-propionate ratio showed promising effects on digestibility and efficiency in rumen fermentation, and this study demonstrates the importance of conserving the bioactivity of antimethanogenic compounds when preparing extracts of feed supplements.

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CRediT authorship contribution statement

Nurul Fitri Sari: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - reviewing and editing. **Sokratis Stergiadis**: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Writing - original draft, Writing - reviewing and editing. **Partha P Ray**: Conceptualization, Investigation, Supervision. **Caroline Rymer**: Conceptualization, Investigation, Supervision. **Les A Crompton**: Data curation, Formal analysis, Investigation, Methodology**. Kirsty E Kliem**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - original draft, Writing - reviewing and editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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