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In vitro rumen degradation, fermentation, and methane production of four agro-industrial protein-rich co-products, compared with soyabean meal

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ABSTRACT

Soyabean is considered an unsustainable protein source for livestock feeds because of the large quantity of input and energy required to cultivate and process it. Other protein-based agro-industrial co-products that are less input-intensive, can mitigate methane (**CH_4**) production and may therefore be more sustainable options instead soyabean. The objective of this study was to compare the effect of replacing the same amount of protein (40 g/kg DM crude protein) as soyabean meal (**SBM**) with low-carbon local agro-industrial co-products, (brewers' spent grains, **BSG**; dried wheat distillers' grains, **WDG**; dried corn distillers' grains, **CDG** and corn steep liquor **CSL**), on in vitro rumen degradation, fermentation and gas and methane production. The study used a 72-hour in vitro gas production method with a basal substrate of dried, ground grass silage and wheat. Gas volumes were measured at ten different specific intervals, and CH_4 concentrations were analysed via gas chromatography. After 72 hours, in vitro DM degradability (**IVDMD**) and volatile fatty acid (**VFA**) concentrations were assessed. Gas and CH_4 production curve profiles were fitted to models to determine asymptote production, the extent of degradation in rumen

Abbreviations: A, asymptote of gas production mL; ADF, acid detergent fibre; B, rate constant /h, independent of time, influencing the fractional rate of degradation; BSG, substrate including brewers' spent grains; C, rate constant /0.5 h, decreasing over time, influencing the fractional rate of degradation; CDG, substrate including dried corn distillers' grains; CH_4, methane; CSL, substrate including corn steep liquor; DM, dry matter; $[H^+]$, hydrogen ion; NDF, neutral detergent fibre; EE, ether extract; GC, gas chromatography; GHG, greenhouse gas emissions; IVDMD, in vitro dry matter degradability; RoP, extent of degradation in rumen proper at given passage rate of 0.04 h and 0.025 h; SBM, substrate including soyabean meal; VFA, volatile fatty acids; WDG, substrate including dried wheat distillers' grains; WSC, water soluble carbohydrates; μ, fractional rate of degradation /h in the halfway 50 % of the asymptote.

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proper (**RoP**), and the fractional degradation rate (**μ**) (h^{-1}) in the halfway 50 % of the asymptote production. The IVDMD and estimated RoP at 0.04 h and 0.025 h were lower ($P<0.05$) for BSG compared to the other treatments, by 4.9-6.6 %; 5.8–9.9 %; 5.2–9.0 %. Gas and CH_4 yield (mL/g substrate and mL/g substrate degraded), and pH (SB = 6.77, BSG = 6.80, WDG = 6.74, CDG = 6.84, and CSL = 6.73; $P>0.05$), were not significantly affected by treatment. Butyrate and valerate were lower ($P<0.05$) for BSG compared to CSL, and caproate was lower ($P<0.001$) for BSG compared to the other treatments and in CSL compared to SBM. The results regarding degradability and VFAs concentrations of this study demonstrated that dried wheat distillers' grains, dried corn distillers' grains, and corn steep liquor have the potential to replace soyabean meal as protein sources for ruminants, but further reduction of CH_4 emissions as a result of such practice may not be expected. Although slightly less degradable, based on their nutrient composition and the fact they did not affect rumen fermentation characteristics, brewers' spent grains can still play a complementary role in ruminant diets, especially in regions where they are locally readily available.

1. Introduction

Soyabean meal is the most popular protein source for livestock feed, due to its high nutritional value and availability. However the sustainability of soyabean meal is under scrutiny (de Visser et al., 2014; Kebreab et al., 2016; Tallentire et al., 2018) due to concerns around land use and land degradation (deforestation) (Sasu-Boakye et al., 2014; Ferreira et al., 2016; Song et al., 2021), excessive water use (Ercin et al., 2012; Song et al., 2021), and long-distance supply chain (transportation) (Heron et al., 2018). Another sustainability concern for the livestock industry is the contribution of livestock production to methane (**CH_4**) emissions, most of which (39 % of all livestock emissions) is from enteric fermentation in the rumen of ruminant livestock, contributing ≈6 % of total global anthropogenic greenhouse gas (**GHG**) emissions (Gerber, 2013; Beauchemin et al., 2020). Therefore, it is critical that more sustainable sources of protein with lower carbon footprint potential for livestock feed are identified. The ideal alternative feedstuffs must support innovative and sustainable practices to optimize production and feed efficiency (FAO, 2018).

There is a growing interest in utilizing agro-industrial co-products as alternative protein sources (Suriyapha et al., 2022), due to their contribution to a circular economy (Sun et al., 2024). Brewers' spent grains are an excellent source of fibre, protein, and oil (Zeko-Pivac et al., 2022). Previous studies have reported that supplementing lactating dairy cows' diets with brewers' grains reduced CH_4 yield by 5.2 % (g/kg DM intake) (Moate et al., 2011), and in non-lactating beef cows by 10.2 % and 22.8 % for crossbreed Limousin and purebred Luing breeds, respectively (Duthie et al., 2015), potentially due to the relatively high oil concentration (≈10 % on a DM basis) (Santos et al., 2003; del Río et al., 2013). Additionally, other work showed that incorporating 72 g/kg DM of wet brewers' grains in finishing beef diets (Parmenter et al., 2019) or replacing soyabean- and corn-based concentrates with up to 200 g/kg DM brewers' grains in heifer diets (Hatungimana et al., 2020) did not negatively affect growth performance. The utilization of cereals within the biofuel sector has led to the emergence of distillers' grains (**DG**), which can be incorporated into ruminant diets as a protein, energy, and fibre source (Makkar, 2013). Like brewers grains, DG have a relatively high oil content (≈12 % on a DM basis) (Belyea et al., 2004; Liu, 2008), and have been studied for their potential to reduce enteric CH_4 production in beef cattle (Hünerberg et al., 2013a; b). However, different sources of DG i.e., corn- and wheat-based may have differential impacts on beef cattle CH_4 yield. Corn-dried DG demonstrate the potential to reduce CH_4 yield by up to 18 % (g/kg DM intake) but the same was not observed for dried wheat DG (Hünerberg et al., 2013a; b). Corn steep liquor (a co-product of wet-milling corn starch processing) is another good source of nutrients (crude protein; **CP**, amino acids, minerals, vitamins, polyphenols). Increasing the inclusion of wheat straw silage (450, 550, and 650 g/kg DM) ensiled with 9 g/kg DM corn steep liquor improved body weight and condition score of lactating buffaloes (Mahr-un-Nisa et al., 2004a; Mahr-un-Nisa et al., 2004b). However, in lamb diets, 100 g/kg DM of corn steep liquor resulted in lower digestibility and growth rates under isonitrogenous and isoenergetic diets probably due to its inclusion rate in the diet and consequently the total diets' chemical composition compared to the control diet of the study (Azizi-Shotorkhoft et al., 2016). The abovementioned agro-industrial co-products (brewers' spent grains, dried wheat- and corn-based DG, and corn steep liquor) have demonstrated potential as ruminant feed ingredients, with benefits to either improve growth and production, and/or mitigation of rumen methanogenesis.

Currently, there are no studies comparing the abovementioned protein-rich co-products simultaneously as a direct replacement for soyabean meal under the same basal diet. Existing literature focuses on the inclusion of each co-product in animal diets, mainly substituting other conventional protein or energy sources (Moate et al., 2011; Hünerberg et al., 2013a; b; Parmenter et al., 2018). Hence, it was hypothesised that the inclusion of these co-products to achieve specific dietary CP content would have similar effects of rumen fermentation and CH_4 production as soyabean meal over 72-h of in vitro incubation (on an isonitrogenous basis to that of soyabean meal). The objective of this study was therefore to assess the effect of brewers' spent grains, dried wheat DG, dried corn DG, and corn steep liquor on in vitro ruminal fermentation and degradability profiles, and enteric CH_4 production compared with soyabean meal, with all treatments being tested side-by-side, with the same basal diet.

2. Materials and methods

All animal procedures were conducted under the authority of the UK Animals (Scientific Procedures) Act, 1986, following prior

approval by the local animal welfare and ethical review body and local ethical clearance (DAS/C221Relivestock01RF).

2.1. Experimental design, dietary treatments, and in vitro gas production method

The in vitro gas production procedure was adapted from previously described methods (Theodorou et al., 1994; Mauricio et al., 1999; Sinclair et al., 2005). The ingredients (g/kg DM) of the experimental diets are presented in Table 1. All ingredients were dried and ground to < 2 mm apart from corn steep liquor which was kept in liquid form. In brief, the experimental treatments were based on a common basal diet of grass silage and wheat, with variations in the protein source: (i) Control, which included soyabean meal (**SBM**), and the same basal diet with soyabean meal replaced by (ii) brewers' spent grains (**BSG**), (iii) dried wheat distillers' grains (**WDG**), (iv) dried corn distillers' grains (**CDG**), and (v) corn steep liquor (**CSL**). The soyabean meal, brewers spent grains, dried corn and dried wheat DG, and corn steep liquor were included so that they provided 40 g/kg DM of CP. The incubation was conducted in triplicate flasks (volume 125 mL) for each treatment, and triplicate negative control flasks (no basal substrate) were included to correct for microbial residual gas production. Prior to each in vitro run, diet ingredients were weighed into each flask. The fermentation medium was made up according to Mauricio et al. (1999). Briefly, fermentation medium was made using deionised H_2O, a buffer solution (consisting of NH_4HCO_3, $NaHCO_3$, deionised H_2O), a micromineral solution (consisting of $Na_2HPO_4{\cdot}12\,H_2O$, KH_2PO_4, $MgSO_4{\cdot}7\,H_2O$, and deionised H_2O), a micromineral solution (consisting of $CaCL_2{\cdot}2\,H_2O$, $MnCL_2{\cdot}4\,H_2O$, $CoCl_2{\cdot}6\,H_2O$, and $FeCl_2{\cdot}6\,H_2O$), resazurin as the anaerobic indicator, and a reducing solution (consisting of Cysteine HCL, 1 M NaOH, $Na_2S{\cdot}9\,H_2O$, and deionised H_2O) in concentrations previously described (Mauricio et al., 1999). Rumen fluid was obtained, before feeding (7:00 AM), from donor cows fed at maintenance energy level a diet of grass hay/grass silage. Rumen fluid was strained through two layers of muslin cloth before being pooled and transferred to a large flask in a water bath set at 39 °C, where anaerobicity was maintained by flushing the flask with CO_2. The pH (6.8) of the rumen fluid was recorded. To each flask, 90 mL fermentation medium and 10 mL inoculum (strained, pooled rumen fluid) were added, and the flasks were sealed and incubated at 39 °C, with agitation before and after each reading (Theodorou et al., 1994; Mauricio et al., 1999). The in vitro run was conducted three times, and each data point represented the mean value of three within-run flask replicates.

2.2. Feed analysis

The individual diet ingredients were analysed for chemical composition [DM; ash; CP; starch; water-soluble carbohydrates (**WSC**); Neutral detergent fibre (**NDF**); Acid detergent fibre (**ADF**); Gross energy (**GE**)] following previously described methods. Specifically, feedstuff samples were oven-dried at 100°C for DM (988.05) and for ash content by combustion at 600°C (942.05) (AOAC, 2012). Kjeldahl method (AOAC, 2012) was used for N analysis and CP was determined based on N × 6.25. Furthermore, NDF and ADF was determined based on Roberston and Van Soest (1981) and Mertens (2002). Feedstuffs were also analysed for starch and WSC content on a continuous flow autoanalyzer system according to previously described methods (Smith et al., 1964; Fuller, 1967; MacRae and Armstrong, 1968). Oil content was determined by the "Wiebul" acid hydrolysis method, and ether extract (**EE**) content by direct solvent extraction (Soxhlet, 1879).

2.3. Gas measurements, gas sampling, and gas production estimates

Gas pressure was measured at 2, 4, 6, 8, 10, 12, 24, 32, 48, and 72 h using a headspace gas pressure transducer (Bailey and Mackey Ltd, Birmingham, UK), and gas pressure readings (psi; Tracker 200; Data Track Process Instruments, UK) were used to calculate gas volume using the equation of Mauricio et al. (1999), that considers the diffusion of gas into the liquid phase:

$$\text{Gas volume (mL)} = (\text{gas pressure}^2 \times 0.082362) + (\text{gas pressure} \times 3.697378) + 0.179947$$

The gas volume was corrected for negative control flasks at each time point and expressed as cumulative gas yield (mL/g substrate) (Mauricio et al., 1999). To describe the degradation pattern of the feeds, the cumulative gas yield curve profiles were fitted to a previously published exponential model (France, 1993):

Table 1
Ingredient composition of the experimental diets.

Ingredients, g/kg DM	Dietary treatments[a]				
	SBM	BSG	WDG	CDG	CSL
Grass silage	558	558	558	558	558
Wheat	296	237	233	227	318
Soyabean meal	79	0	0	0	0
Brewer's spent grain	0	133	0	0	0
Wheat-dried distillers' grains	0	0	128	0	0
Corn-dried distillers' grains	0	0	0	134	0
Corn steep liquor	0	0	0	0	53

[a] SBM = Substrate including soyabean meal; BSG = Substrate including brewers' spent grains; WDG = Substrate including dried wheat distillers' grains; CDG = Substrate including dried corn distillers' grains; CSL = Substrate including corn steep liquor.

$$G = A\{1 - \exp[-b(t-T) + c\left(\sqrt{t} - \sqrt{T}\right)]$$

where ***G*** (mL) denotes total gas accumulation, ***A*** (mL) denotes the asymptotic value of *G*, ***t*** (h) is the incubation time, ***T*** (h) is the lag time to occur before degradation, ***b*** (h^{-1}) is a rate constant independent of time and influencing the fractional rate of degradation, ***c*** ($h^{-1/2}$) is a rate constant decreasing over time and influencing the fractional rate of degradation. Whilst the influence of *b* is independent of time, the influence of *c* decreases with time. The value of *c* therefore influences the shape of the gas profile; if *c* is negative the resulting gas profile is sigmoidal, whilst a positive *c* indicates that the growth rate is initially faster than exponential, and finally, if *c* is zero an exponential growth curve is described. The associated extent of digestion with the extent of degradation in rumen proper (**RoP**) at the given passage rate at 0.04 h and 0.025 h and the fractional rate of degradation (h^{-1}) in the halfway 50 % of the Asymptote (**μ**) were determined following the equations described by France et al. (1993).

2.4. CH_4 production estimates

Before sample injection, a five-point (25 000, 50 000, 75 000, and 100 000 ppm) standard curve for CH_4 was conducted, to allow calculation of CH_4 concentrations of samples using peak area. Following the gas pressure readings and for each reading time point, a sample (10 mL) of gas was collected from each flask using a two-way valve on the pressure transducer and was manually injected into a gas chromatograph (**GC**; Bruker 450 GC) via the port valve to determine the CH_4 concentration. Full column and GC conditions were previously reported (Munoz et al., 2012). Gas components were separated on a CH_4-packed Poropak N column (1.2 m length, 2 mm i.d. Varian Inc., Walnut Creek, CA), and CH_4 was detected using a flame ionization detector. The CH_4 concentrations were applied to calculated gas volumes to obtain CH_4 volume. For each reading time point, the CH_4 volumes were expressed as cumulative CH_4 yield was measured in mL/g substrate. Similar to the gas dataset, cumulative CH_4 yield curve profiles were fitted based on the model described by France (1993).

2.5. In vitro dry matter degradability, pH, and volatile fatty acids analysis

At 72 h and after the final gas measurement and sampling, pH of each flask was measured and recorded, transformed to hydrogen ion concentration **[H^+]** before statistical analysis ([H^+] = 10^-pH). Flask contents were then filtered through pre-dried sintered glass crucibles, and this flask residue was then oven-dried at 100 °C before being weighed. In vitro dry matter degradability (**IVDMD**) was calculated based on the dry weight of the residue. In addition, after the final gas measurement (72 h), the flask liquid filtrate was

Table 2
Chemical composirimental diets (g/kg DM unless stated).

item[a]	Diet ingredients[b]						
	Grass silage	Wheat	Soyabean meal	Brewers' spent grains	Dried wheat DG	Dried corn DG	Corn steep liquor
Dry matter (g/kg)	930	930	961	920	853	849	450
Organic matter	844	986	932	965	950	907	840
Gross energy (MJ/kg DM)	17.3	18.2	18.4	21.3	21.0	21.0	32.0
Crude protein	167	94.8	509	273	281	283	381
NDF	472	128	113	541	147	153	-
ADF	339	35.4	92.0	271	90.3	34.5	-
Oil	50.9	28.7	23.7	110	65.5	179	-
Ether extract	45.7	22.1	9.47	81.9	42.9	160	-
Starch	9.02	752	21.6	24.4	21.5	38.6	19.7
WSC	7.75	38.1	118	6.03	67.6	48.5	171
Ash	156	14.0	68.1	35.1	50.4	92.8	160

Feed composition	**Dietary treatments**[c]				
	SBM	**BSG**	**WDG**	**CDG**	**CSL**
Dry matter (g/kg)	933	929	919	918	930
Organic matter	897	898	896	889	883
Gross energy (MJ/kg DM)	17.7	18.1	18.1	18.1	20.4
Crude protein	172	164	166	168	178
NDF	333	394	337	336	327
ADF	222	252	226	217	215
Oil	41.6	53.8	47.6	65.5	40.4
Ether extract	35.2	45.0	39.4	57.8	35.0
Starch	246	201	195	197	265
WSC	26.4	15.3	24.3	21.5	37.9
Ash	103	102	104	111	117

[a] DM = Dry matter; NDF = Neutral detergent fibre; ADF = Acid detergent fibre; WSC = Water soluble carbohydrates.
[b] *DG* = Distillers' grains.
[c] *SBM* = Substrate including soyabean meal; BSG = Substrate including brewers' spent grains; WDG = Substrate including dried wheat distillers' grains; CDG = Substrate including dried corn distillers' grains; CSL = Substrate including corn steep liquor.

collected and stored at −20 °C until analysed for volatile fatty acids (**VFA**) according to Lowman et al. (1999). Volatile fatty acids were separated using an Agilent 7890B GC system equipped with an HP-FFAP column (30 m/0.25 mm/0.25 μm with 10 m guard, Agilent) and a flame ionization detector. Acetic, propionic, butyric, valeric, iso-butyric, iso-valeric, and caproic (Sigma Aldrich Co.) as well as 25 mM 2-Ethylbutyric acid were used as external standards.

2.6. Statistical analysis

Statistical analysis of model parameters and their functions was conducted using IBM SPSS, version 29.0 (Armonk, NY, USA). At each time measurement point, cumulative gas yield (mL/g substrate) and cumulative CH_4 yield (mL/g substrate) data analysis was performed using repeated measures analysis of variance to the including dietary treatment (SBM, BSG, WDG, CDG, CSL), incubation time, and dietary treatment × incubation time as fixed factors and run as random factor. Data analysis of pH, $[H^+]$, IVDMD, VFAs, gas yield (mL/g substrate), CH_4 yield (mL/g substrate), and the results from the model estimates was performed using linear mixed effects model and using the restricted maximum likelihood (**REML**) including dietary treatment (SBM, BSG, WDG, CDG, CSL) as fixed factor and run as a random factor. Fisher's Least Significant Difference (LSD) test was used for pairwise comparisons where the effect of treatment was significant ($P<0.05$).

3. Results

3.1. Chemical composition of the dietary treatments

The CP contents ranged between 509 and 273 (g/kg DM) with soyabean meal having the highest (Table 2). The GE was higher for the corn steep liquor and as for the treatments it was similar apart from CSL. Both NDF and ADF content were numerically higher for the brewers' spent grains and BSG treatment (by 14.5 – 17.0 % for NDF and by 10.3 – 14.7 % for ADF). Oil and EE content was higher for dried corn DG followed by brewers' spent grains. For the dietary treatments oil content ranged from 40.4 to 65.5 g/kg DM and EE content ranged from 35.0 to 57.8, with CDG having numerically higher content of both (by 17.9–38.3 % for oil and by 22.1 – 39.4 % for EE) compared with the other treatments. Starch and WSC content were numerically higher for corn steep liquor and CSL treatment (by 7.2 – 26.4 % for starch and by 30.3 – 59.6 % for WSC) followed by SBM treatment.

3.2. In vitro dry matter degradability, pH, and volatile fatty acids

The IVDMD (g/kg) was lower ($P<0.001$) for BSG compared to the other treatments (Table 3). It was also lower ($P<0.001$) for CDG compared to CSL. By fitting the data to the model, RoP set at 0.04 h ($P=0.016$) and 0.025 h ($P=0.005$) was also lower for BSG compared to the other treatments. When RoP from rumen was set at 0.025 h WDG was lower ($P=0.005$) than CSL. The vessel pH (transformed to $[H^+]$), did not differ ($P>0.05$) between treatments. Butyrate ($P=0.022$) and valerate ($P=0.033$) concentrations were lower for BSG compared with CSL, while caproate ($P<0.001$) was lower for BSG compared to the other treatments. The ratio of acetate:

Table 3
The extent of degradation in the rumen proper for a given rate of passage at 0.04 h and 0.025 h (RoP), pH, and the concentration of the volatile fatty acids (VFA, mM) of the five dietary treatments incubated with rumen fluid.

Parameters[a]	Dietary treatments[b]						
	SBM	BSG	WDG	CDG	CSL	SEM	*P-value*[c]
pH	6.77	6.80	6.74	6.84	6.73	0.14	0.800
H^+	1.72E−7	1.62E−7	1.82E−7	1.68E−7	1.88E−7	0.00	1.000
IVDMD (g/kg)	895[ab]	838[c]	892[ab]	881[b]	897[a]	1.11	<0.001
RoP at 0.04 h	36.7[a]	33.8[b]	35.9[a]	36.7[a]	37.5[a]	1.83	0.016
RoP at 0.025 h	49.0[ab]	45.2[c]	47.7[b]	48.3[ab]	49.7[a]	1.64	0.005
VFA (mM)							
Acetate	75.0	71.1	71.4	70.2	74.8	5.31	0.085
Propionate	29.1	28.1	29.7	29.4	29.0	1.80	0.188
Iso-Butyrate	1.33	1.22	1.24	1.20	1.28	0.06	0.187
Butyrate	17.5[ab]	15.8[b]	16.6[ab]	15.9[b]	17.7[a]	0.34	0.022
Iso-Valerate	2.38	2.10	2.14	2.09	2.25	0.14	0.102
Valerate	2.28[ab]	2.12[b]	2.27[ab]	2.19[ab]	2.38[a]	0.13	0.033
Caproate	0.45[b]	0.38[c]	0.47[b]	0.47[b]	0.50[a]	0.02	<0.001
A:P	2.53[a]	2.58[a]	2.40[b]	2.39[b]	2.53[a]	0.03	0.002
(Ac+Bu)/Pr	3.09[b]	3.18[a]	2.96[c]	2.93[c]	3.14[ab]	0.02	0.002

[a] SBM = Substrate including soyabean meal; BSG = Substrate including brewers' spent grains; WDG = Substrate including dried wheat distillers' grains; CDG = Substrate including dried corn distillers' grains; CSL = Substrate including corn steep liquor.

[b] IVDMD = In vitro dry matter degradability; RoP = Extent of degradation in rumen proper for given rate of passage at 0.04 h and 0.025 h (France, 1993); $[H^+]$ were calculated with the following formula: $[H^+] = 10^{\wedge}$-pH; A:P = Acetate:Propionate; (Ac+Bu)Pr = (Acetate + Butyrate)/Propionate.

[c] Significances were declared at $P<0.05$. Significant differences between dietary treatments within variable are indicated with different superscript letters according to the Fisher's Least Significant Difference (LSD) test ($P<0.05$).

propionate was higher (P=0.002) for SBM, BSG, and CSL compared to WDG and CDG. The (acetate + butyrate)/propionate ratio was higher (P=0.002) for BSG compared to the other treatments and lower for WDG and CDG.

3.3. Gas yield

The cumulative gas yield (mL/g substrate) over the 72-h incubation period did not differ between treatments (P>0.05) (Table 4). Similarly, there was no significant treatment by time interaction (P>0.05) (Fig. 1(a); Supplementary Table S1). Rate constant b (h^{-1}), independent of time influencing the fractional rate of degradation was higher (P<0.001) for SBM, BSG and CSL compared with the other treatments (Table 4). Rate constant c ($h^{-1/2}$), decreased with time and influencing the fractional rate of degradation, was higher (P<0.001) for WDG and CDG compared with the rest. In contrast, SBM, BSG, and CSL resulted in a longer fermentation lag time (P<0.001) compared with WDG and CDG. Additionally, μ was higher (P<0.001) in SBM and CSL compared to the other treatments.

3.4. CH_4 yield

The cumulative CH_4 yield (mL/g substrate) was not affected by treatment (P>0.05) (Table 5). There was no significant treatment by time interaction (P>0.05; Fig. 1(b); Supplementary Table S2). Rate constant c ($h^{-1/2}$), decreased with time and influencing the fractional rate of degradation, was lower (P=0.033) for SBM compared with BSG, WDG, and CDG, and higher for WDG compared with SBM, BSG, and CSL (Table 5). Lag time was (h) higher (P=0.018) for SBM, BSG, and CSL compared with WDG and CDG.

4. Discussion

4.1. Effect of the agro-industrial co-products on in vitro dry matter degradability and fermentation characteristics

Utilization of agro-industrial co-products fosters a more resilient and eco-friendlier agro-industrial (Wagh et al., 2024) and livestock production system. Relative to this, agro-industrial co-products such as brewers' spent grains and dried corn DG that were tested in the present study, can be used as protein- and oil-rich feedstuffs and can replace protein sources that raise concerns about their sustainability, such as soyabean meal (FAO, 2018). The co-products tested in this study were selected because they represent potential alternatives to soyabean meal for livestock rations on the basis of their crude protein content and their wide commercial availability in specific regions.

The BSG treatment diet resulted in a much lower IVDMD and RoP compared with the other treatments, which could be due to several factors. The BSG had 15.5 % higher NDF and 11.9 % higher ADF content compared to SBM, and these dietary constituents can negatively affect digestibility. The contents of NDF and ADF in BSG used in this particular study is likely to be the main reason for the lower degradability observed and modelled in vivo via RoP. Faccenda et al. (2019) found lower apparent DM digestibility in situ when dried brewers' grains were included in 117 g/kg DM and partially replaced corn and soyabean meal in crossbreed steers. Despite the fact that BSG degradability in the present in vitro study was shown to be lower, in vivo work shows that feeding brewers' grains in lactating dairy cows (at 259 g/kg DM inclusion rate) as a partial substitution of wheat grain and solvent-extracted canola meal (Moate et al., 2011) and in non-lactating pregnant cows (at 226 g/kg DM inclusion rate) as a replacement of grass silage in a barley-straw diet (Duthie et al., 2015), did not affect performance. It should also be considered that the composition of these co-products varies. For instance, brewers' spent grains in our study had similar NDF (541 g/kg DM) and ADF (271 g/kg DM) content compared to Duthie et al. (2015) (553 and 279 g/kg DM, for NDF and ADF, respectively), while in contrast the content in Moate et al. (2011) study the NDF content was 305 g/kg DM and in Faccenda et al. (2019) it was 675 g/kg DM. A lower IVDMD might typically lead to a reduction in

Table 4
In vitro gas yield and associated kinetics parameter estimates of the five dietary treatments incubated with rumen fluid.

Parameters[a]	Dietary treatments[b]					SEM	*P-value*[c]
	SBM	BSG	WDG	CDG	CSL		
Gas yield (mL/g substrate)	192	178	190	181	192	3.20	0.050
b (h^{-1})	0.06^{ab}	0.06^{ab}	0.05^{c}	0.05^{c}	0.07^{a}	0.002	<0.001
c ($h^{-1/2}$)	−0.19^{a}	−0.17^{c}	−0.12^{d}	−0.12^{d}	−0.19^{ab}	0.02	<0.001
Lag time (h)	2.30^{a}	2.05^{ab}	1.52^{c}	1.31^{c}	2.18^{a}	0.41	<0.001
A (mL)	201	188	204	193	201	4.09	0.116
Gas yield (mL/g substrate degraded)	225	224	229	219	224	4.36	0.708
μ (h^{-1})	0.044^{a}	0.041^{b}	0.038^{c}	0.040^{bc}	0.045^{a}	0.002	<0.001

[a] SBM = Substrate including soyabean meal; BSG = Substrate including brewers' spent grains; WDG = Substrate including dried wheat distillers' grains; CDG = Substrate including dried corn distillers' grains; CSL = Substrate including corn steep liquor.

[b] Gas yield (mL/g substrate) = 72-h cumulative gas yield per gram of substrate;; b = rate constant (h^{-1}), independent of time, influencing the fractional rate of degradation (France, 1993); c = rate constant ($h^{-1/2}$), decreasing over time, influencing the fractional rate of degradation (France, 1993); A = Asymptote of gas production (mL) (France, 1993); Gas yield (mL/g substrate degraded) (France, 1993); μ = fractional rate of degradation (h^{-1}) in the halfway 50 % of the Asymptote (France, 1993).

[c] Significances were declared at P<0.05. Significant differences between dietary treatments within variable are indicated with different superscript letters according to the Fisher's Least Significant Difference (LSD) test (P<0.05).

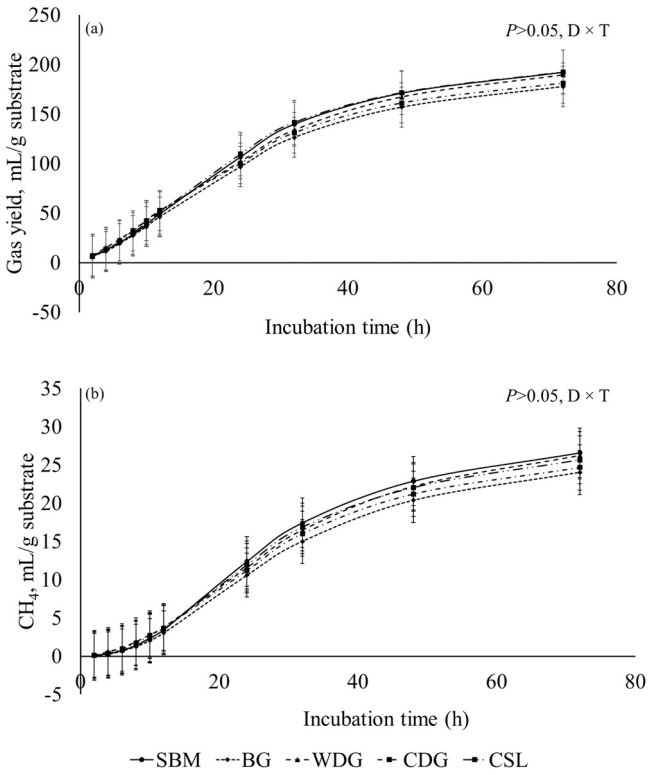

Fig. 1. (a) Gas yield (mL/g substrate) and (b) CH_4 yield (mL/g substrate) of dietary treatments (D) (basal substrate including soyabean meal; SBM, basal substrate including brewers' spent grains; BSG, basal substrate including wheat-dried distillers' grains; WDG, basal substrate including corn-dried distillers' grains; CDG, basal substrate including corn steep liquor; CSL) at the different incubation time measurements (T). Error bars within rows represent the standard error of means. The significance level for the D × S was not significant ($P>0.05$).

Table 5
In vitro CH_4 yield and associated kinetics parameter estimates of the five dietary treatments incubated with rumen fluid.

Parameters[a]	Dietary treatments[b]					SEM	*P-value*[c]
	SBM	BSG	WDG	CDG	CSL		
CH_4 yield (mL/g substrate)	26.6	24.1	26.3	24.7	25.7	0.88	0.201
b (h^{-1})	0.07	0.06	0.06	0.06	0.07	0.004	0.054
c ($h^{-1/2}$)	−0.27[a]	−0.24[b]	−0.20[c]	−0.22[bc]	−0.26[ab]	0.02	0.033
Lag time (h)	4.86[a]	4.58[a]	3.36[b]	3.42[b]	4.57[a]	0.65	0.018
A (mL)	28.2	26.1	28.8	26.7	27.3	0.80	0.193
CH_4 of total gas (%)	13.8	13.5	13.9	13.6	13.2	0.22	0.191
CH_4 yield (mL/g substrate degraded)	31.5	31.1	32.3	30.3	30.5	0.83	0.535
μ (h^{-1})	0.043	0.039	0.037	0.039	0.042	0.002	0.063

[a] SBM = Substrate including soyabean meal; BSG = Substrate including brewers' spent grains; WDG = Substrate including dried wheat distillers' grains; CDG = Substrate including dried corn distillers' grains; CSL = Substrate including corn steep liquor.

[b] CH_4 yield (mL/g substrate) = 72-h cumulative CH_4 yield per gram of substrate; b = rate constant (h^{-1}), independent of time, influencing the fractional rate of degradation (France, 1993); c = rate constant ($h^{-1/2}$), decreasing over time, influencing the fractional rate of degradation (France, 1993); A = Asymptote of CH_4 production (mL) (France, 1993); CH_4 yield (mL/g substrate degraded) (France, 1993); μ = fractional rate of degradation (h^{-1}) in the halfway 50 % of the Asymptote (France, 1993).

[c] Significances were declared at $P<0.05$. Significant differences between dietary treatments within variable are indicated with different superscript letters according to the Fisher's Least Significant Difference (LSD) test.

asymptotic gas volume, as gas is generated during the fermentation of carbohydrates to acetate and butyrate (Getachew et al., 1998). Contrary to expectations, the results in the present study suggest that although there was no difference between treatments for the asymptotic gas production, the production for BSG is 6.5 % lower, but not significantly, than that of SBM. For the BSG, the observed CH_4 yield exhibited a pattern similar to the asymptotic gas yield; underscoring the need for a better understanding of the underlying mechanisms governing gas production and CH_4 emissions when agro-industrial co-products are used as animal feed.

The main rumen fermentation products are VFA, gases, and microbial biomass. Alterations in the VFA concentrations could be

attributed to nutrients content (fibre, fat, WSC, and starch), but also to feed particle size and basal diet composition which can modify fermentation patterns and VFA concentrations (Kononoff et al., 2003; Ellis et al., 2008; Patra, 2013). Although BSG and CDG contained more EE, the concentrations of the VFAs were not significantly affected. Increasing dietary fat content increases proportion of propionate to acetate (Patra, 2013), and diets rich in starch and WSC content may also support the growth of microorganisms that increase propionate concentrations (Brossard et al., 2004; Ellis et al., 2008). Notably, the propionate to acetate ratio was lower for the WDG and CDG treatment only, a significant change considering its implications for improving dietary energy utilisation efficiency (Lin et al., 2020). This shift is significant for productivity, as butyric and propionic acid are key drivers of increased milk production (Seymour et al., 2005). As for the CSL, the observed changes in VFA concentrations could be attributed to the liquid form of corn steep liquor. Caproate was the only VFA that differed compared to SBM. Although caproate is present in lower concentrations in rumen fluid compared to short-chain VFAs, it interacts with butyrate metabolism (Kristensen and Harmon, 2005) and has been shown to inhibit hepatic gluconeogenesis in vitro (Chow and Jesse, 1992). The differences in VFA results between the present study and in vivo studies could be further attributed to variations in basal diet ingredients and their inclusion rates, the static condition of in vitro studies compared to in vivo where VFAs are also absorbed, the lack of passage rate of feed, and potential differences in microbial biomass synthesis (Dijkstra et al., 2005). For instance, the results of the present in vitro study differ from in vivo studies such as Duthie et al. (2015), given the differences in dietary inclusion rates of brewers' spent grains (included at a lower level of 226 vs 133 g/kg DM in the present study) and the fact that in vitro studies cannot entirely mimic the rumen environment. Overall, the results regarding the in vitro degradability and the VFAs concentrations that were evaluated in the present study highlight dried wheat distillers' grains, dried corn distillers' grains, and corn steep liquor are promising alternatives to soyabean meal protein sources for ruminant diets. Brewers' spent grains had lower degradability in this study, but other work (in vivo) has not observed any adverse effects on animal performance and productivity (Moate et al., 2011; Duthie et al., 2015).

4.2. *Effect of the agro-industrial co-products on CH_4 yield*

The inclusion of various agro-industrial co-products in livestock diets holds promise for mitigating enteric CH_4 emissions, due to their distinctive chemical composition and bioactive compounds, including lipids, polyphenols, saponins, etc. (Salami et al., 2019; Vargas et al., 2020; Vastolo et al., 2022).

Regarding enteric CH_4 yield, previous research reported that higher levels of fat result in a decrease in enteric CH_4 production (Patra, 2013). For every 10 g/kg DM of increase in dietary lipid content, CH_4 yield can be decreased by up to 3.8 % (Martin et al., 2010). In this experiment, the varying fat content of the different dietary treatments was between 35 and 58 g/kg DM. The CH_4 yield (mL/g substrate) exhibited only a nominal reduction across treatments, with reductions of 9.39 % for BSG, 1.13 % for WDG, 10.15 % for CDG, and 3.38 % for CSL when compared to SBM. The proportionate difference in the EE content between BSG and SBM was 0.98 %, resulting in a 1.3 % CH_4 (mL/g substrate degraded) nominal reduction for every increase of added fat by one proportional unit. Higher inclusion rates of brewers' grains in in vivo studies resulted in significantly lower CH_4 production from cattle (Moate et al., 2011; Duthie et al., 2015). More specifically, the partial substitution of wheat grain and solvent-extracted canola meal with brewers' grains (259 g/kg DM) significantly reduced CH_4 yield by 5.2 % (g/kg DM intake), and CH_4 intensity by 9.05 % (g/L milk) (Moate et al., 2011). Replacing grass silage with brewers' grains in barley-straw diets in two different beef breeds (crossbreed Limousin and purebred Luing) reduced CH_4 yield (g/kg DM intake) by 10.2 % and 22.8 %, and yield (g/kg $BW^{0.75}$) by 16.7 % and 24.3 % (Duthie et al., 2015). In these studies, the EE difference between the diets treated with brewers' grains and the control diets was 2.5 % and 1.7 %. This difference resulted in CH_4 reductions of 4.08 % and 13.4 % (g/kg DM intake) per unit increase in added fat for the two breeds, respectively (Moate et al., 2011; Duthie et al., 2015). The CH_4 mitigation in these studies could be associated with the higher inclusion levels used and the different chemical composition of the diets (e.g. fat content) compared to our study. In vitro, studies also reported that brewers' grains can reduce CH_4 production in a linear trend when included as an additive in increased levels (0, 20, 40, and 60 g/kg DM) by 11.1 %, 27.2 %, and 37.0 %, respectively (Sina and Preston, 2021) or when tested against other agro-industrial brewery waste by-products (Bekele et al., 2024). This observation contrasts this present study in spite of the fact that higher concentrations of brewers' grains were used (133 g/kg DM), possibly due to the different ingredients of the diets, due to the increasing inclusion rate, and due to the different composition of the co-products. However, these results confirm the potential of brewers' grains to reduce CH_4 emissions in experimental approaches other than the ones used in the present study. Regarding the effect of DG on CH_4 production, the results of this in vitro study did not confirm a possible CH_4 mitigation potential. The EE content difference between CDG and SBM was 2.26 %, while between WDG and SBM was only 0.42 % and the % reduction of CH_4 yield (mL/g substrate degraded) per added fat was the highest in the CDG compared to the SBM (1.7 %). Keomanivong et al. (2017) studied in vitro rumen fluid collected from steers fed diets consisting of coarse-rolled or fine-rolled corn, along with two levels of dried corn DG (200 and 400 g/kg DM) with solubles and found that CH_4 production was unaffected by the treatments. Besides, it should not be overlooked that in vivo studies included dried wheat and corn DG at much higher levels compared to the present study (400 g/kg DM), supporting the hypothesis of these co-products as CH_4 mitigators (Hünerberg et al., 2013a; b). The % CH_4 yield (g/kg DM intake) per added fat for the corn-based DG treatment from these two studies ranged between 5.3 – 6.3 % (Hünerberg et al., 2013a; b). More specifically, Hünerberg et al. (2013a) investigated the effect of replacing 35 % of DM barley grain and 5 % of DM canola meal with dried wheat-based DG with solubles and corn-based DG with solubles in growing heifer diets. In Hünerberg et al. (2013a), the addition of 24 g/kg DM corn oil in a diet that also included dried wheat DG reduced CH_4 yield (g/kg DM intake) by 16.6 % while a diet with dried corn DG led to a reduction in CH_4 yield (g/kg DM intake) by 15.0 %. The significant decrease in CH_4 could be attributed to the liquid-added oil in the diet and not the wheat DG inclusion. Therefore, EE content is an important parameter to consider alongside inclusion rate when evaluating CH_4 mitigation potential of agri-food industry co-products. As for the corn steep liquor, to our knowledge, there are no

studies that evaluated its effect on enteric CH_4 production in vitro or in vivo. Existing studies have predominantly focused on assessing this co-product as a high-energy and protein feedstuff in ruminant diets, particularly in digestibility and performance trials (Mahr-un-Nisa et al., 2004a; Mahr-un-Nisa et al., 2004b; Azizi-Shotorkhoft et al., 2016). In our study, the lack of effect on CH_4, compared with the SBM, is consistent with the lack of difference in the dietary chemical composition.

These findings underscore the significance of co-products like brewers' grains and DG in potentially mitigating CH_4 emissions from ruminants. As previously stated, the present study included the co-products at much lower rates compared to the literature, since the main hypothesis was to evaluate the tested co-products as alternative protein sources to soyabean meal. The contrasting outcomes from various studies indicate the complexity of using agro-industrial co-products as CH_4 mitigators, as their efficacy may be affected by the composition (fat content) and presented bioactive components, the different dietary inclusion rates, and animals' basal diet, the different animal species, as well as the rumen microbiome. Discrepancies between in vitro and in vivo trials could also become apparent due to the feedstuff processing and 72-hour retention time condition, which would typically be longer than the retention time of the same feed in the rumen. In in vitro studies, feedstuffs undergo fine grinding, while the longer retention time may overestimate the degradability of the feed (Macome et al., 2017). These methodological disparities emphasize the importance of interpreting in vitro results cautiously, recognizing the inherent limitations in replicating the dynamic and peculiar conditions of the rumen in live animals.

5. Conclusion

Based on the degradability and fermentation parameters' results of this study, the dried wheat distillers' grains, dried corn distillers' grains, and corn steep liquor, could be included in ruminant rations as alternative protein sources to soyabean meal. The tested co-products might reduce the embodied carbon footprint of animal feed, but the potential to further reduce emissions by acting as methane mitigators was not confirmed in the present in vitro study.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare no conflict of interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2024.116151.

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