Protecting protein against ruminal degradation could contribute to reduced methane production

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Abstract

Ruminants have a low efficiency of nitrogen (N) utilization that has negative implications for animal production and the environment, but reducing the ruminal degradation of protein can help to reduce N losses. The objective of this study was to evaluate the inclusion of sunflower meal (SM) and sunflower seed (SS) protected against ruminal degradation in high-cereal diets on in vitro ruminal fermentation and CH₄ production. Samples of SS and SM were sprayed with a solution of malic acid 1 M (400 ml/kg sample) and dried at 150°C for 1 hr as a protective treatment. Four diets were formulated to contain either 13 (low) or 17 (high) g of crude protein (CP)/100 g dry matter (DM), and included SM and SS either untreated (13CON and 17CON diets) or treated as before described (13TR and 17TR diets). Diets were incubated in vitro with rumen fluid from sheep for 8 and 24 hr. The treatment did not affect ($p \geq 0.57$) total volatile fatty acid (VFA) production at any incubation time, but it reduced ($p < 0.05$) NH₃-N concentrations by 19.2 and 12.5% at 8 and 24 hr respectively. Both CH₄ production and CH₄/VFA ratio were lower ($p < 0.02$) in TR than in CON diets at 8 hr, but differences disappeared ($p > 0.05$) at 24 hr. The treatment increased the molar proportion of propionate ($p = 0.001$) and reduced that of isovalerate ($p = 0.03$) at 8 hr compared with CON diets, but only a reduction of isovalerate proportion ($p = 0.03$) was detected at 24 hr. There were no treatment x crude protein level interactions ($p > 0.05$) in any parameter, but high-protein diets had greater NH₃-N concentrations ($p < 0.001$) and lower VFA production ($p < 0.001$) than low-protein diets at 24 hr. The treatment reduced protein degradation, and CH₄ production was decreased by 4.6 and 10.8% for low- and high-protein diets, respectively, at short incubation times without affecting VFA production, thus improving fermentation efficiency and decreasing polluting emissions.

KEYWORDS

in vitro fermentation, methane, protein protection, sunflower meal, sunflower seed

1 | INTRODUCTION

Methane production in the rumen is a major concern worldwide due to its contribution to global warming. Dietary characteristics are one of the most important factors influencing CH₄ production,

and it is well known that diets rich in structural carbohydrates yield higher CH₄ emissions compared with those high in non-structural carbohydrates (Kumar et al., 2014). A recent in vitro study (Vanegas, González, & Carro, 2017) showed the generation of CH₄ from pure protein fermentation and that the response in

CH₄ production to protein supply differed with the basal substrate, being higher for a rapidly fermented substrate than for a slowly fermented one. In addition, the protection of sunflower protein against ruminal degradation by a combined treatment of malic acid and heat resulted in a reduction of in vitro CH₄ production that was attributed to reduced protein degradation (Vanegas, Carro, Alvir, & González, 2017; Vanegas, González, Alvir, & Carro, 2017). To our best knowledge the influence of including rumen-protected protein in diets representative of those fed in the practice on CH₄ production has not yet been investigated. The objective of this study was therefore to evaluate the inclusion of rumen-protected sunflower meal (SM) and sunflower seed (SS) in high-cereal diets on in vitro CH₄ production and ruminal fermentation. Both SM and SS are protein sources extensively used in practical feeding of ruminants, and a reduction in their ruminal protein degradation could increase protein utilization by the animal and reduce N environmental emissions.

2 | MATERIALS AND METHODS

Samples (500 g) of both SM and SS were ground (2 mm), weighed and sprayed with a solution of 1 M malic acid at a rate of 400 ml per

kg of substrate fresh matter, left at room temperature for 1 hr and subsequently dried at 150°C for 1 hr as a protective treatment (TR) against ruminal degradation (Vanegas, González, Alvir, et al., 2017). Four total mixed diets were formulated according to a 2 × 2 factorial arrangement of treatments. The diets had 13 (low) or 17 (high) g of crude protein (CP)/100 g dry matter (DM) and included SM and SS either untreated (13CON and 17CON diets) or treated as before described (13TR and 17TR diets). All feed ingredients were ground to pass a 1 mm screen and mixed. Ingredient and chemical composition of diets and chemical composition of SM and SS are shown in Table 1. Dry matter, ash, nitrogen and ether extract contents were determined according to the Association of Official Analytical Chemists (1999). Organic matter content was calculated as 100 minus ash content. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) analyses were performed as described by Van Soest, Robertson, and Lewis (1991). Sodium sulphite and heat-stable amylase were used in the sequential analysis of NDF, ADF and ADL, and they were expressed exclusive of residual ash.

Four sheep (67.2 kg ± 2.31 kg body weight), each provided with a permanent rumen cannula, were used as rumen fluid donors for the in vitro incubations. Sheep were fed a mixed diet of grass hay and a commercial concentrate (1:1) at a daily rate of 42 g/kg body weight^{0.75} distributed in two equal meals. Animals were managed

TABLE 1 Chemical composition and ingredients of experimental diets used as substrates for in vitro incubations and chemical composition of sunflower seed and sunflower meal either untreated (CON) or treated (TR) to protect the protein against ruminal degradation

Item	Experimental diets ^a				Sunflower seed ^b		Sunflower meal ^b	
	13CON	13TR	17CON	17TR	CON	TR	CON	TR
Chemical composition (g/kg dry matter)								
Organic matter ^c	961	960	952	951	969	970	927	932
Crude protein	131	130	170	169	179	171	359	337
Neutral detergent fibre	325	322	369	366	292	271	490	466
Acid detergent fibre	127	125	165	166	159	147	275	261
Acid detergent lignin	26.2	25.6	39.0	40.2	53.8	48.7	88.2	84.5
Ether extract	85.3	85.7	83.7	85.3	467	459	12.9	20.2
Non-structural carbohydrates ^d	420	422	329	331	31.9	69.0	65.1	109
Ingredients (g/kg fresh matter)								
Soft wheat grains ^e	469	464	349	329				
Maize grains	156	155	116	110				
Straw	150	150	150	150				
CON-sunflower seed	150	–	150	–				
TR-sunflower seed	–	153	–	153				
CON-sunflower meal	75.0	–	235	–				
TR-sunflower meal	–	79.6	–	259				

^a13CON, 13TR, 17CON and 17TR: Diets with two levels of crude protein (130 and 170 g/kg dry matter) and containing either untreated (CON) or treated (TR) sunflower seed and sunflower meal to protect protein against ruminal degradation. ^bSunflower seed and meal were either untreated (CON) or treated with a solution 1M malic acid (400 ml/kg) and dried at 150°C for 1 hr to protect protein against ruminal degradation (TR). ^cCalculated as 1000 – ash. ^dCalculated as 1000 – (ash + crude protein + neutral detergent fibre + ether extract). ^eA wheat variety with greater starch and lower protein than hard wheat.

in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes) in line with European legislation (Directive 2010/63/UE), and experimental procedures were approved by the Animal Experimentation Ethics Committee of the Comunidad Autónoma de Madrid (Approval number PROEX 035/17).

Samples (300 mg DM) of each diet were accurately weighed into glass vials (115 ml). Ruminal contents from each sheep were obtained immediately before the morning feeding, strained through 4 layers of cheesecloth and the fluid mixed with a culture medium (1:4 ratio; vol/vol) at 39°C. The medium of Goering and Van Soest (1970) was changed by replacing the $(\text{NH}_4)\text{HCO}_3$ with NaHCO_3 and excluding the trypticase to obtain a N-free medium. Each vial was filled with 30 ml of the mixture under CO_2 flushing before being capped and incubated at 39°C. For each diet, a total of 16 vials were incubated, four with the ruminal fluid from each sheep (inoculum); therefore, there were four replicates per experimental treatment and sampling time. After 8 hr of incubation, gas production was determined in 2 vials per diet and inoculum using a pressure transducer (Wider eager Wide Range Pressure Meter; Sper Scientific LTD, Scottsdale, AZ, USA) and a plastic syringe. A gas sample (15 ml) was removed from each vial and stored in a vacuum airtight tube (Terumo Europe N.V., Leuven, Belgium) until CH_4 analysis by gas chromatography according to Martínez, Ranilla, Tejido, Ramos, and Carro (2010). Vials were uncapped, the pH was measured (Crisson Basic 20 pHmeter, Crisson Instruments, Barcelona, Spain) and 5 ml of vials content were added to 5 ml of 0.5 M HCl and kept frozen (-20°C) until volatile fatty acid (VFA) and $\text{NH}_3\text{-N}$ analysis following the procedures described by Ranilla, Carro, López, Newbold, and Wallace (2001). The rest of the vials were processed as before described after 24 hr of incubation.

At each incubation time, data from the 2 vials incubated for each diet and inoculum were averaged before statistical analysis (4 values for each diet). The amount of organic matter apparently fermented (OMAF) in each vial was calculated from VFA production following the equation proposed by Demeyer (1991): $\text{OMAF (mg)} = 162 \times [\mu\text{mol acetate}/2] + [\mu\text{mol propionate}/2] + \mu\text{mol butyrate}$. Data for each incubation time were analysed independently as a factorial model using the PROC MIXED of SAS (SAS Institute Inc, 2012). The effects of treatment, CP level and the treatment $\times$ CP level interaction were considered fixed, and inoculum effect was considered random. Significance was declared at $p < 0.05$ and p values between 0.05 and 0.10 were considered as a trend.

3 | RESULTS AND DISCUSSION

The combined malic acid heat treatment was selected because its efficacy for protecting protein from ruminal degradation was proved in previous *in situ* and *in vitro* studies (Arroyo et al., 2011, 2013; Díaz-Royón, Arroyo, Sánchez, & González, 2016; Vanegas, González, Alvir, et al., 2017; Vanegas, Carro, et al., 2017). Malic acid, as other acids, causes denaturation of proteins, reduces its solubility and provides

effective protection against degradation by ruminal microorganisms. The treatment caused only minor changes in chemical composition of both SS and SM (Table 1), and the slight decreases observed in CP, NDF, ADF and ADL content in TR samples compared with the untreated samples might be due to a dilution effect associated with adding malic acid in the treatment.

The experimental diets were formulated to contain a high proportion of cereal grains (62.2 and 45.2% for low- and high-protein diets respectively) because Vanegas, González, et al. (2017) observed that the contribution of protein fermentation to CH_4 production was greater with a rapidly fermentable substrate (starch) than with a slowly fermentable substrate (cellulose). Diets with two different CP levels were formulated to analyse the possible interaction between treatment and CP level. Finally, the study examined the differences between diets at two incubation times (8 and 24 hr), as sunflower protein is rapidly degraded in the rumen (Arroyo et al., 2013) and the influence of treatment might become less marked as incubation time increased.

There were no effects ($p > 0.05$) of either treatment or CP level on final pH (values ranged from 7.02 to 7.08, and from 6.63 to 6.71 at 8 and 24 hr of incubation respectively). As shown in Table 2, no treatment $\times$ CP level interactions were observed for any determined parameter, excepting a trend for CH_4 /total VFA ratio at 8 hr incubation ($p = 0.09$) and for total VFA at 24 hr ($p = 0.088$). After 8 hr incubation, the fermentation of diets including rumen-protected SS and SM resulted in lower $\text{NH}_3\text{-N}$ concentration ($p = 0.01$) compared with CON diets, which is consistent with the hypothesis that treatment reduced sunflower protein degradation. The lower proportions of isovalerate and valerate observed in TR diets also suggest a reduction of protein degradation, as these VFA are only generated in the degradation of branched-chain amino acids (Wallace & Cotta, 1988).

Fermentation of TR diets produced lower CH_4 production ($p = 0.02$) compared with CON diets, but total VFA was similar ($p = 0.57$). The lack of differences in total VFA production indicates that the treatment of SS and SM did not negatively affect their DM fermentation, and that the reduced CH_4 production observed in TR diets was not a consequence of lower diet fermentability. In fact, there were no differences ($p = 0.59$) between TR and CON diets in the estimated amount of OMAF. Previous *in vitro* studies with protein as the only substrate (Vanegas, González, et al. (2017)) or replacing non-protein N with protein-N with different substrates (Carro, López, Valdés, & González, 1999; Vanegas, González, et al. (2017)) showed that CH_4 generation in ruminal fermentation was partly due to protein degradation, presumably due to the hydrogen generation in the fermentation of carbon skeletons resulting from amino acid deamination. Values of $\text{NH}_3\text{-N}$ concentrations and those of CH_4 production at both incubation times were positively correlated ($p < 0.001$) for both low-protein ($r = 0.797$; $n = 16$) and high-protein ($r = 0.892$; $n = 16$) diets. A positive relationship between $\text{NH}_3\text{-N}$ and CH_4 may just reflect different amounts of organic matter fermented in the vials, but in this study there were no differences in the OMAF between CON and TR diets; therefore, it seems that the lower CH_4 production observed for TR diets was due to the reduction in protein degradation.

TABLE 2 *In vitro* ruminal fermentation of diets containing sunflower seed and sunflower meal, either untreated (CON) or treated (TR) against ruminal degradation, and two crude protein levels after 8 and 24 hr of incubation

Incubation time and item	Experimental diets ^a				SEM	<i>p</i> value		
	13CON	13TR	17CON	17TR		TR	Crude protein level	TR x Crude protein level
8 hr								
Gas (ml)	30.7	31.7	27.2	27.5	1.85	0.75	0.07	0.86
CH ₄ (ml)	1.53	1.46	1.67	1.49	0.045	0.02	0.09	0.26
NH ₃ -N (mg/l)	78.0	62.4	85.5	69.7	3.01	0.01	0.09	0.98
Total volatile fatty acid (VFA; mmol)	0.421	0.421	0.443	0.427	0.0138	0.57	0.33	0.59
Individual VFA (mol/100 mol)								
Acetate	51.4	51.2	52.8	51.3	0.57	0.18	0.22	0.28
Propionate	31.9	33.0	31.0	33.2	0.34	0.001	0.30	0.14
Butyrate	12.7	12.4	12.3	12.1	0.27	0.37	0.21	0.67
Isobutyrate	0.25	0.11	0.17	0.06	0.106	0.28	0.57	0.91
Isovalerate	1.34	1.10	1.46	1.19	0.099	0.03	0.32	0.90
Valerate	2.34	2.19	2.29	2.14	0.072	0.07	0.52	0.99
Acetate/propionate (Ac/Pr; mol/mol)	1.64	1.58	1.73	1.57	0.033	0.009	0.21	0.16
CH ₄ /VFA (ml/mmol)	3.66	3.52	3.87	3.55	0.040	0.001	0.03	0.09
OMAF (mg) ^b	37.1	37.0	38.7	37.5	1.17	0.59	0.39	0.61
24 hr								
Gas (ml)	56.3	55.5	57.7	58.3	3.29	0.98	0.55	0.84
CH ₄ (ml)	5.68	5.53	5.05	5.13	0.125	0.80	0.003	0.39
NH ₃ -N (mg/l)	138	125	177	149	4.7	0.002	<0.001	0.17
Total VFA (mmol)	1.16	1.13	1.02	1.06	0.017	0.95	<0.001	0.09
Individual VFA (mol/100 mol)								
Acetate	54.0	54.3	53.5	54.3	0.64	0.41	0.70	0.66
Propionate	25.8	25.9	24.8	25.2	0.36	0.50	0.05	0.69
Butyrate	14.9	14.7	15.3	14.2	0.43	0.18	0.98	0.34
Isobutyrate	1.55	1.55	1.88	2.02	0.100	0.47	0.003	0.51
Isovalerate	1.85	1.72	2.28	2.07	0.068	0.03	<0.001	0.59
Valerate	1.94	1.90	2.26	2.14	0.069	0.26	0.003	0.59
Ac/Pr (mol/mol)	2.17	2.16	2.20	2.21	0.057	0.98	0.48	0.91
CH ₄ /VFA (ml/mmol)	4.92	4.92	4.96	4.88	0.071	0.54	0.99	0.52
OMAF (mg) ^b	103	99.9	89.8	92.1	1.43	0.81	<0.001	0.10

^a13CON, 13TR, 17CON and 17TR: Diets with two levels of crude protein (130 and 170 g/kg dry matter) and containing either untreated (CON) or treated (TR) sunflower seed and sunflower meal to protect protein against ruminal degradation. Each culture contained 300 mg of diet dry matter.

^bOrganic matter apparently fermented estimated as described by Demeyer (1991).

The ratio CH₄/VFA can be used as an indicator of the efficiency of ruminal fermentation, as CH₄ represents an energy loss and VFA are the main energy source for the host animal and are also precursors for the synthesis of fatty acids and glucose. In agreement with a previous study in which providing protein-N to *in vitro* cultures of ruminal microorganisms containing a rapidly fermented substrate enhanced CH₄/VFA ratio (Vanegas, González, et al. (2017)), CON-diets showed greater CH₄/VFA ratios (*p* = 0.001) than TR diets at 8 hr incubation, thus

indicating a more efficient fermentation in TR diets. In addition, fermentation of TR diets resulted in increased propionate proportion (*p* = 0.001) and reduced isovalerate proportion (*p* = 0.03). Different *in vitro* studies (Carro & Ranilla, 2003; Tejido, Ranilla, García-Martínez, & Carro, 2005) have reported that malate supplementation increased propionate production, which can contribute to a reduction in CH₄ production, as malate acts as an electron acceptor and is converted to propionate by the succinate pathway. In contrast to that observed at 8 hr of incubation,

no effects of treatment ($p = 0.18$ to 0.98) were detected at 24 hr, with the exception of a reduction in both $\text{NH}_3\text{-N}$ concentrations ($p = 0.002$) and molar proportion of isovalerate ($p = 0.03$). As before discussed, these differences would indicate lower protein degradation in TR compared with CON diets. The reduction in $\text{NH}_3\text{-N}$ concentrations for TR diets, relative to those in CON-diets, was 19.2 and 12.6% at 8 and 24 hr, respectively (values averaged across CP levels), which indicates that protein protection was reduced with time, probably as a consequence of the continuous proteolytic activity of ruminal microbes.

There were no differences between high- and low-protein diets at 8 hr incubation, with the exception of greater CH_4/VFA ratios ($p = 0.03$; 3.71 vs. 3.59 ml/mmol) and a trend ($p < 0.10$) to lower gas production (27.4 vs. 31.2 ml) and to greater CH_4 production (1.58 vs. 1.50 ml) and $\text{NH}_3\text{-N}$ concentrations (77.6 vs. 70.2 mg/l) for high-protein diets than for low-protein ones. This would indicate that the substitution of cereal grains (wheat and maize) by SM to reach 170 g CP/kg in high-protein diets resulted in lower fermentation efficiency at 8 hr of incubation. After 24 hr of incubation, both CH_4 and VFA production were lower ($p = 0.003$ and 0.001 respectively) for high compared with low-protein diets, resulting in a lower amount of OMAF for the high-protein diets ($p < 0.001$; 91 vs. 101 mg). Ruminal fermentation of CP generates less CH_4 than carbohydrates (Jentsch et al., 2007), which could help to explain the lower CH_4 production observed for high-protein diets at 24 hr as these diets contained less carbohydrates (698 g/kg) and more CP than low-protein diets (745 g of total carbohydrates/kg; Table 1). The trend to greater CH_4 production observed at 8 hr for high-protein compared with low-protein diets is difficult to explain, but could be related to greater CP degradation in these diets resulting in greater fermentation of carbon skeletons generated from amino acid deamination. This hypothesis is supported by the trend to increased $\text{NH}_3\text{-N}$ concentrations in high-protein diets.

In conclusion, the inclusion of sunflower seeds and sunflower meal treated with a combined malic acid-heat treatment in high-cereal diets resulted in lower in vitro CH_4 and $\text{NH}_3\text{-N}$ production at early stages of fermentation without decreasing the amount of organic matter apparently fermented. The results showed a greater fermentation efficiency of diets including rumen-protected sunflower feeds at 8 hr of incubation, as indicated by the lower CH_4/VFA and acetate/propionate ratios than for diets with unprotected protein. The inclusion of rumen-protected sunflower feeds in the diet to reach CP contents of either 130 or 170 g/kg DM did not affect negatively the in vitro fermentation of diets. If the efficacy of this treatment is confirmed in vivo, it could be a means of reducing both N and CH_4 emissions.

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