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Influence of malic acid-heat treatment for protecting sunflower protein against ruminal degradation on *in vitro* methane production: A comparison with the use of malic acid as an additive

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ABSTRACT

Batch cultures of rumen microorganisms were used to compare the fermentation of sunflower seed (SS) and meal (SM) treated with malic acid and heat (MAH; 150 °C for 1 h) to protect the protein from ruminal degradation with that of the untreated samples supplemented the same amount of malate (as additive), either as free acid (MA) or disodium malate (DSM). As previous studies have shown the influence of donors' diet on *in vitro* fermentation, cultures were inoculated with ruminal fluid from four sheep fed a medium-concentrate diet (MC; 50:50 hay:concentrate) or a high-concentrate diet (HC; 15:85 barley straw:concentrate) in a cross-over experimental design. Cultures were sampled at 6 and 16.5 h of fermentation. In 16.5 h incubations, MAH treatment reduced ($P < 0.001$) CH_4 production and ammonia-N concentrations for both substrates (by 60.3 and 45.3% for SS and by 23.7 and 17.2% for SM substrate, respectively). Whereas the MAH treatment reduced total volatile fatty (VFA) production by 16.5% for SS substrate, no effects were observed for SM substrate ($P = 0.441$). For both substrates, adding the same amount of MA to untreated samples (as a feed additive) resulted in greater CH_4 production and ammonia-N concentrations compared with the MAH treatment. A positive relationship ($P < 0.001$) was observed between the concentrations of NH_3—N and CH_4 production at both incubation times for both substrates. Compared with the untreated feeds, MA and DSM did not affect ($P > 0.05$) CH_4 production for either substrate, but reduced ammonia-N concentrations (13.8%; $P = 0.004$) for SM substrate. Compared with DSM, MA treatment resulted in greater ($P < 0.05$) production of gas, butyrate, isovalerate and valerate and tended ($P < 0.10$) to greater total VFA, and propionate production for SS substrate at 6 h incubation, but most differences disappeared after 16.5 h incubation. For some fermentation parameters, the response to MAH treatment was more pronounced by using fluid from sheep fed the HC diet than that from MC-fed sheep. In conclusion, the MAH treatment was more effective at reducing CH_4 emissions and NH_3—N concentrations than the supplementation of malic acid or disodium malate as a feed additive.

Abbreviations: ADFom, acid detergent fibre; ADIN, nitrogen insoluble in acid detergent solution; ADL, acid detergent lignin; BW, body weight; DM, dry matter; DSM, disodium malate; HC, high-concentrate diet; MA, malic acid; MAH, combined malic acid and heat treatment; MC, medium-concentrate diet; aNDFom, neutral detergent fibre; NDIN, nitrogen insoluble in neutral detergent solution; OMAF, organic matter apparently fermented; SM, sunflower meal; SS, sunflower seed; UNT, untreated substrates; VFA, volatile fatty acids

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1. Introduction

Reducing the degradability of the protein in the rumen can increase the amount of protein digested post-ruminally and reduce N losses to the environment. Recently, Vanegas et al. (2016a) observed the efficacy of a malic acid and heat combined treatment (MAH) to reduce the protein degradation of sunflower seed (SS) and sunflower meal (SM) and to improve the *in vitro* fermentation by lowering CH_4 emissions and increasing propionate production without affecting total volatile fatty acids (VFA) production. However, it is unknown whether the effects were due to the protective effect of the malic acid-heat treatment, to malic acid fermentation itself, or even to a combination of both factors.

Some studies have evidenced the influence of the diet of donor animals on CH_4 production (Martínez et al., 2010) and on the effectiveness of additives to modify *in vitro* fermentation (Kamel et al., 2008; Mateos et al., 2013). As highlighted by Mould et al. (2005), diet characteristics and nutrient intake are major factors affecting both the microbial populations in the rumen and the activity of the ruminal fluid used as inoculum for *in vitro* incubations. The effectiveness of malic acid as feed additive to modify the *in vitro* fermentation has been shown to vary with the incubated substrate (Carro and Ranilla, 2003; Gómez et al., 2005), but to our best knowledge the possible influence of the donors' diet has not yet been assessed; therefore, this study was conducted using ruminal fluid from sheep fed two contrasting diets as inoculum. Our hypothesis was that the effects of the MAH treatment of SS and SM substrates on their *in vitro* fermentation would depend on microbial populations in the inoculum, and therefore on the type of diet fed to the host animal. The main objective of this study was to compare the *in vitro* fermentation of MAH-treated SS and SM substrates with that of the untreated substrates (UNT). In addition, the effects of supplementing SS and SM with either malic acid (MA) or disodium malate (DSM) were compared, as the chemical form of malate supply (free acid *vs*. salts) has been proposed as a factor involved in the variability of the response to malate supplementation observed in different studies (Carro and Ungerfeld, 2015). To our best knowledge the *in vitro* fermentation of the two forms of malate has not yet being compared.

2. Materials and methods

2.1. Substrates, animals and feeding

One sample of SS (oil type) and one sample of semi-dehulled SM were used in the study. Samples of each substrate (500 g) were weighed and sprayed with a solution of 1 *M* malic acid at a rate of 400 ml per kg of substrate, left at room temperature for 1 h and subsequently dried at 150 °C for 1 h, as described by Vanegas et al. (2016a). The chemical composition of the untreated and malic acid-heat treated samples is shown in Table 1.

Four adult rumen-fistulated sheep (64.7 ± 2.10 kg body weight) were used as rumen fluid donors for the *in vitro* incubations. Animals were housed in individual pens with free access to water and a mineral-vitamin mixture. Sheep were fed two different mixed diets in a cross-over experimental design with two 15-day periods. The medium-concentrate diet (MC) was composed of 30:20:50 triticale hay:alfalfa hay:commercial concentrate, and the high-concentrate diet (HC) was composed of 15:85 barley straw:commercial concentrate (Table 1). Both concentrates were pelleted and forages were chopped to a length of 4–5 cm. The MC and HC diets were representative of those for medium-lactation animals and fattening ruminants under intensive systems of production, respectively. Both diets were fed daily in two equal meals at a fixed rate of 42 g of dry matter (DM) per kg of body weigh$^{0.75}$. Animal management and rumen contents withdrawal were carried out in accordance with the Spanish guidelines for experimental animal protection (Boletín Oficial del Estado, 2013), and the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Polytechnic University of Madrid.

Table 1
Chemical composition (g/kg dry matter unless otherwise stated) of sunflower seed and sunflower meal either untreated (UNT) or treated with a 1 M solution of malic acid (400 ml/kg substrate) at 150 °C for 1 h (MAH) used as substrates for *in vitro* incubations and composition of mixed diets fed to rumen fluid donor sheep.

	Sunflower seed		Sunflower meal		Donor diets[b]	
Item	UNT	MAH	UNT	MAH	MC	HC
Dry matter (g/kg fresh matter)	977	980	919	969	901	900
Organic matter	969	970	927	932	909	900
Crude protein	179	171	359	337	152	149
Ether extract	467	459	12.9	20.2	31.5	33.7
Neutral detergent fibre (aNDFom)	292	271	490	466	408	364
Acid detergent fibre (ADFom)	159	147	275	261	222	191
Acid detergent lignin (ADL)	53.8	48.7	88.2	84.5	34.4	31.3
NDIN × 6.25[a]	54.3 (0.303)	42.6 (0.249)	89.8 (0.249)	98.3 (0.292)	ND	ND
ADIN × 6.25[a]	8.74 (0.049)	7.77 (0.046)	17.5 (0.049)	16.5 (0.049)	ND	ND

ND: not determined.
[a] NDIN and ADIN: N insoluble in neutral and acid detergent solutions, respectively. Values in brackets are expressed as g/g of total crude protein.
[b] MC: medium-concentrate diet; HC: high-concentrate diet.

2.2. In vitro incubations

All *in vitro* cultures contained 300 mg of substrate DM. Samples (300 mg of DM) of each UNT and MAH samples were carefully weighed into 120-ml serum bottles. Additionally, 283 mg of DM of untreated samples were weighed in bottles which received 1 ml of a solution containing either 17 mg of malic acid (MA treatment) or 22.6 mg of disodium malate (DSM treatment) immediately before incubation. This was done to supply the same amount of malate than in the MAH samples, either in the form of free acid or disodium salt. Bottles from UNT and MAH treatments received 1 ml of distilled water to equalize the liquid volume. In addition, bottles without substrate and receiving 1 ml of distilled water, 1 ml of the MA solution, or 1 ml of the DSM solution were incubated for analysing the fermentation of both additives as the only substrate.

The last day of each experimental period, rumen contents from each donor sheep was obtained immediately before the morning feeding and strained through 4 layers of cheesecloth. Three ml of fluid was added to 3 ml of deproteinising solution (20 g of metaphosphoric acid and 0.6 g of crotonic acid per litre) for volatile fatty acids (VFA) determination, and 2 ml were mixed with 2 ml 0.5 *M* HCl for NH_3–N analysis. The fluid from each sheep was then individually mixed with a culture medium solution in a 1:4 ratio (vol/vol) at 39 °C. The medium of Goering and Van Soest (1970) was modified by replacing the $(NH_4)HCO_3$ with $NaHCO_3$ and excluding the trypticase to obtain a N-free solution. Bottles were inoculated with 30 ml of the mixture before being capped and incubated at 39 °C. All these procedures were conducted under CO_2 flushing. In each experimental period, two bottles were incubated for each inoculum (two inocula from MC-fed sheep and two inocula from HC-fed sheep), substrate (SS and SM), and experimental treatment (UNT, MAH, MA and DSM). A total of 64 bottles with substrate (4 inocula x 2 substrates x 4 treatments x 2 bottles/treatment) and 24 bottles without substrate (4 inocula x 3 treatments (distilled water, malic acid or disodium malate) x 2 bottles/treatment) were incubated in each experimental period.

After 6 h of incubation, the gas production was measured in all bottles using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe, and a gas sample (10 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of CH_4. In addition, 1 ml of each bottle content was taken using an insulin syringe, mixed with 20 µl of H_2SO_4 (100 ml per litre) and stored at −20 °C for VFA and NH_3–N analysis. After 16.5 of incubation (corresponding to a mean passage rate from the rumen of 0.06 per h), gas production was measured and a sample was taken for analysis of CH_4 as described previously. Bottles were opened, the pH was measured immediately (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona. Spain) and the bottles were placed in iced water to slow down fermentation before taken samples of the content for VFA and NH_3–N analyses as previously described.

2.3. Chemical analyses

Dry matter (ID 934.01), ash (ID 942.05) and N (ID 984.13) contents were determined according to the Association of Official Analytical Chemists (1999). The analysis of neutral detergent fibre (aNDFom), acid detergent fibre (ADFom) and acid detergent lignin (ADL) were carried out according to Van Soest et al. (1991) using an ANKOM220 Fibre Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulphite and heat-stable amylase were used in the sequential analysis of aNDFom, ADFom and ADL, and they were expressed exclusive of residual ash. Concentrations of NH_3–N were determined by the method of phenol-hypochlorite as previously detailed (Weatherburn, 1967). Analyses of VFA and CH_4 were conducted by gas chromatography as described by Carro et al. (1992) and Martínez et al. (2010), respectively.

2.4. Calculations and statistical analyses

The amounts of VFA produced in each bottle were calculated by subtracting the amount present initially in the incubation medium from that determined at each incubation time. The amount of OM apparently fermented (OMAF) in each culture was estimated from VFA production as described by Demeyer (1991). Values measured of the two bottles incubated for each inoculum, substrate, experimental treatment and sampling time were averaged before statistical analysis to get four replicates per treatment.

Data from each incubation time were analysed independently for each substrate (SS and SM) according to a 2 × 4 factorial model (2 donors' diets x 4 experimental treatments) using the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC, USA) and following the model: $Yijk = \mu + ci + Dj + Tk + Pl + DTjk + eijk$,

where Yijk = the dependent variable; μ = the overall mean; ci = the random effect of sheep (i = 1 to 4); Dj = the fixed effect of donors' diet (j = 1 to 2); Tk = the fixed effect of experimental treatment (k = 1 to 4); Pl = the fixed effect of experimental period (l = 1 to 2); DTjk = the interaction of D and T; and eijk = the residual error. Substrate treatment x period, donors' diet x period and substrate treatment x donors' diet x period interactions were not significant ($P > 0.20$) for any parameter and therefore were not included in the model and their variance was included in the error term. Treatment means were compared using the following four preplanned non orthogonal contrasts, C1: UNT versus MAH; C2: MAH versus MA; C3: UNT versus MA and DSM; C4: MA versus DSM. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered to be a trend. Relationships between CH_4 production and NH_3–N concentrations were investigated by linear regression using the PROC REG of SAS (SAS Inst. Inc., Cary, NC, USA).

Table 2
Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower seed in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 6 h (n = 4).[a]

	Treatment				Donor diet			P[b]					
Item	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4	Donor Diet	Treatment x Donor diet
Gas (ml)	54.2	44.8	61.9	51.8	56.0	50.4	1.72	< 0.001	< 0.001	0.090	< 0.001	< 0.001	0.851
CH_4 (ml)	8.73	4.14	8.55	8.33	7.13	7.75	0.508	< 0.001	< 0.001	0.554	0.653	0.115	0.818
NH_3−N (mg/l)	192	96.4	191	180	170	160	7.6	< 0.001	< 0.001	0.348	0.186	0.082	0.923
Volatile fatty acids (VFA; μmol)													
Total VFA	623	385	715	670	613	544	25.4	< 0.001	< 0.001	0.005	0.088	< 0.001	0.382
Acetate (Ac)	367	213	384	372	373	294	15.2	< 0.001	< 0.001	0.436	0.447	< 0.001	0.822
Propionate (Pr)[c]	132	115	205	195	172	151	5.3	< 0.001	< 0.001	< 0.001	0.066	< 0.001	0.006
Butyrate	81.9	38.7	83.9	67.3	73.3	62.6	7.32	< 0.001	< 0.001	0.328	0.034	0.054	0.621
Isobutyrate	13.5	6.26	13.8	12.1	11.7	11.2	1.09	< 0.001	< 0.001	0.547	0.128	0.557	0.828
Isovalerate	18.5	8.20	18.6	15.8	14.4	16.1	1.22	< 0.001	< 0.001	0.235	0.031	0.065	0.677
Valerate	9.53	4.11	10.3	8.10	7.91	8.13	0.693	< 0.001	< 0.001	0.616	0.004	0.651	0.142
Ac/Pr (mol/mol)	2.76	1.84	1.86	1.89	2.18	1.99	0.113	< 0.001	0.781	< 0.001	0.738	0.007	0.714
CH_4/VFA (ml/μmol)[c]	0.014	0.012	0.012	0.013	0.011	0.015	0.0008	0.002	0.330	0.021	0.472	< 0.001	0.035
OMAF (mg)[d]	53.7	32.8	61.3	56.8	56.1	46.2	2.47	< 0.001	< 0.001	0.022	0.086	< 0.001	0.432

[a] UNT: untreated; MAH: malic acid-heat treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet.
[b] Non orthogonal contrasts "for treatment effects": C1:UNT *vs* MAH; C2:MAH *vs* MA; C3:UNT *vs* MA and DSM; C4:MA *vs* DSM.
[c] Propionate production values for UNT, MAH, MA and DSM were 140, 138, 206 and 206 μmol for MC-diet and 124, 92.7, 204 and 184 μmol for HC-diet, respectively. CH_4/VFA values for UNT, MAH, MA and DSM were 0.012, 0.008, 0.012 and 0.012 ml/μmol for MC-diet and 0.017, 0.015, 0.013 and 0.014 ml/μmol for HC-diet, respectively.
[d] OMAF: organic matter apparently fermented estimated from volatile fatty acids production according to Demeyer (1991).

3. Results

3.1. Influence of malic acid-heat treatment

As shown in Table 1, the MAH treatment caused only minor changes in chemical composition of both SS and SM substrates, and did not increase the concentration of acid detergent insoluble N in any substrate. The effects of experimental treatments on *in vitro* fermentation at 6 and 16.5 h of incubation are shown in Tables 2 and 3 for SS substrate and in Tables 4 and 5 for SM substrate, respectively. Compared with UNT, the MAH treatment of SS substrate decreased ($P < 0.001$) the production of gas, CH_4, total and individual VFA, NH_3−N concentrations and the amount of OMAF at both incubation times, excepting propionate production at 16.5 h (Tables 2 and 3). In contrast, MAH treatment of SM substrate did not affect ($P > 0.05$) either the production of gas, total VFA, acetate and butyrate or the amount of OMAF at any incubation time (Tables 4 and 5). Concentrations of NH_3−N, production of minor

Table 3
Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower seed in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h (n = 4).[a]

	Treatment				Donor diet			P[b]					
Item	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4	Donor Diet	Treatment x Donor diet
Gas (ml)	94.7	71.8	105.6	92.3	87.3	94.9	5.12	< 0.001	< 0.001	0.349	0.017	0.049	0.928
CH_4 (ml)	17.8	7.08	17.3	17.3	15.0	14.8	1.11	< 0.001	< 0.001	0.577	0.966	0.835	0.968
NH_3−N (mg/l)	364	199	373	351	295	348	16.9	< 0.001	< 0.001	0.874	0.196	< 0.001	0.651
Volatile fatty acids (VFA; μmol)													
Total VFA	1246	1048	1323	1280	1325	1119	43.5	< 0.001	< 0.001	0.200	0.236	< 0.001	0.633
Acetate (Ac)	734	596	744	708	753	639	30.4	< 0.001	< 0.001	0.760	0.242	< 0.001	0.941
Propionate (Pr)	258	259	325	321	299	282	11.3	0.923	< 0.001	< 0.001	0.736	0.051	0.131
Butyrate	157	129	161	159	182	116	5.2	< 0.001	< 0.001	0.594	0.629	< 0.001	0.523
Isobutyrate	25.8	18.5	26.8	25.2	25.5	22.7	1.32	< 0.001	< 0.001	0.902	0.276	0.009	0.775
Isovalerate	27.0	17.3	25.3	26.1	25.2	22.4	2.53	< 0.001	< 0.001	0.176	0.808	0.006	0.628
Valerate	44.3	26.0	40.6	41.3	39.6	36.5	1.38	< 0.001	< 0.001	0.143	0.778	0.101	0.422
Ac/Pr (mol/mol)	2.94	2.30	2.33	2.22	2.53	2.36	0.114	< 0.001	0.803	< 0.001	0.341	0.044	0.737
CH_4/VFA (ml/mol)	0.015	0.007	0.013	0.014	0.011	0.013	0.0010	< 0.001	< 0.001	0.253	0.704	0.015	0.857
OMAF (mg)[c]	106	90.3	113	107	115	93.4	3.51	< 0.001	< 0.001	0.178	0.151	< 0.001	0.630

[a] UNT: untreated; MAH: malic acid-heat treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet.
[b] Non orthogonal contrasts "for treatment effects": C1:UNT *vs* MAH; C2:MAH *vs* MA; C3:UNT *vs* MA and DSM; C4:MA *vs* DSM.
[c] OMAF: organic matter apparently fermented estimated from volatile fatty acids production according to Demeyer (1991).

Table 4
Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower meal in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 6 h (n = 4).[a]

	Treatment				Donor diet			P[b]					
Item	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4	Donor Diet	Treatment x Donor diet
Gas (ml)	62.4400	66.9	65.6	59.6	68.3	58.9	2.72	0.117	0.660	0.926	0.038	< 0.001	0.953
CH_4 (ml)	11.1250	9.1	10.6	10.2	11.4	9.4	0.70	0.020	0.220	0.349	0.641	0.004	0.316
NH_3–N (mg/l)	158	133	136	150	157	132	3.7	< 0.001	0.398	< 0.001	0.002	< 0.001	0.780
Volatile fatty acids (VFA; µmol)													
Total VFA	724	758	781	789	847	679	29.9	0.216	0.408	0.016	0.756	< 0.001	0.935
Acetate (Ac)	479	473	474	480	541	412	15.4	0.666	0.941	0.851	0.755	< 0.001	0.602
Propionate (Pr)	142	198	214	211	202	181	6.2	< 0.001	0.019	< 0.001	0.608	< 0.001	0.988
Butyrate	68.3	61.9	65.1	66.0	72.8	57.9	7.57	0.407	0.682	0.675	0.902	0.012	0.862
Isobutyrate	6.85	5.56	6.09	6.74	6.78	5.84	0.839	0.004	0.199	0.215	0.115	0.003	0.810
Isovalerate	12.2	9.74	10.5	12.1	11.7	10.5	1.23	0.008	0.360	0.224	0.085	0.049	0.763
Valerate	14.5	9.96	11.3	13.8	13.0	11.8	0.395	0.002	0.291	0.089	0.056	0.184	0.244
Ac/Pr (mol/mol)	3.36	2.37	2.20	2.27	2.75	2.34	0.059	< 0.001	0.009	< 0.001	0.256	< 0.001	0.391
CH_4/VFA (ml/µmol)	0.015	0.012	0.014	0.013	0.013	0.014	0.00140	0.035	0.179	0.052	0.082	0.394	0.209
OMAF (mg)[c]	61.4	64.4	66.2	66.6	72.0	57.4	2.53	0.259	0.470	0.034	0.880	< 0.001	0.977

[a] UNT: untreated; MAH: malic acid-heat treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet.
[b] Non orthogonal contrasts "for treatment effects": C1:UNT *vs* MAH; C2:MAH *vs* MA; C3:UNT *vs* MA and DSM; C4:MA *vs* DSM.
[c] OMAF: organic matter apparently fermented estimated from volatile fatty acids production according to Demeyer (1991).

VFA (isobutyrate, isovalerate and valerate) and acetate/propionate and CH_4/total VFA ratios were lower ($P < 0.05$) in MAH-treated SM substrate compared with the untreated SM substrate (Tables 4 and 5).

3.2. Influence of malic acid and disodium malate as feed additives

Compared with the MAH treatment, the direct supply of MA to cultures with SS as substrate resulted in greater ($P < 0.001$) production of gas, CH_4 and total and individual VFA at both incubation times (Tables 2 and 3). In contrast, there were no differences ($P > 0.05$) between MA and MAH treatments in the production of gas, CH_4 and total and individual VFA (excepting that of propionate which was higher for MA) for SM substrate at 6 h incubation (Table 4). After 16.5 h incubation, the cultures with SM as substrate and receiving MA as additive produced more CH_4 and minor VFA than those with MAH-treated SM substrate (Table 5).

For both substrates, MA and DSM treatments resulted in increased ($P < 0.001$ to 0.016) production of total VFA and propionate and reduced acetate/propionate ratios compared with the untreated substrates at both incubation times, with the exception of total

Table 5
Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower meal in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h (n = 4).[a]

	Treatment				Donor diet			P[b]					
Item	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4	Donor Diet	Treatment x Donor diet
Gas (ml)	109	114	112	106	107	114	5.2	0.383	0.661	0.943	0.321	0.076	0.996
CH_4 (ml)	22.3	16.4	23.3	20.9	20.9	20.6	1.06	< 0.001	< 0.001	0.850	0.035	0.732	0.117
NH_3–N (mg/l)	325	269	280	308	278	314	10.9	< 0.001	0.337	0.004	0.018	< 0.001	0.803
Volatile fatty acids (VFA; µmol)													
Total VFA	1470	1456	1550	1557	1408	1605	25.3	0.441	< 0.001	0.001	0.762	< 0.001	0.899
Acetate (Ac)	903	882	913	927	862	951	22.2	0.358	0.181	0.398	0.550	< 0.001	0.902
Propionate (Pr)	278	315	361	350	303	349	11.0	0.003	< 0.001	< 0.001	0.323	< 0.001	0.920
Butyrate	188	186	186	185	163	206	3.0	0.215	0.501	0.351	0.780	< 0.001	0.939
Isobutyrate	25.8	19.9	24.9	25.1	21.9	26.0	0.99	0.003	0.011	0.600	0.890	0.004	0.460
Isovalerate	28.5	20.4	26.6	27.1	23.4	26.9	2.09	< 0.001	< 0.001	0.061	0.223	< 0.001	0.990
Valerate	45.8	31.8	42.0	43.3	35.3	44.9	1.79	< 0.001	0.001	0.027	0.199	< 0.001	0.915
Ac/Pr (mol/mol)	3.25	2.80	2.55	2.65	2.87	2.76	0.112	< 0.001	0.043	< 0.001	0.391	0.183	0.880
CH_4/VFA (ml/µmol)[c]	0.015	0.011	0.015	0.012	0.014	0.013	0.0008	< 0.001	< 0.001	0.046	0.003	0.241	0.006
OMAF (mg)[d]	126	126	133	133	121	139	1.93	0.995	0.001	< 0.001	0.990	< 0.001	0.897

[a] UNT: untreated; MAH: malic acid-heat treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet.
[b] Non orthogonal contrasts "for treatment effects": C1:UNT *vs* MAH; C2:MAH *vs* MA; C3:UNT *vs* MA and DSM; C4:MA *vs* DSM.
[c] CH_4/VFA values for UNT, MAH, MA and DSM were 0.015, 0.012, 0.014 and 0.013 ml/µmol for MC-diet, and 0.016, 0.008, 0.016 and 0.012 ml/µmol for HC-diet, respectively.
[d] OMAF: organic matter apparently fermented estimated from volatile fatty acids production according to Demeyer (1991).

VFA production at 16.5 h incubation for SS ($P = 0.20$; Table 3). Compared with UNT substrates, neither MA nor DSM treatments reduced CH_4 production ($P > 0.05$). Whereas both additives reduced ($P < 0.01$) NH_3–N concentrations for SM substrate (Tables 4 and 5), no effects were observed for SS substrate (Tables 2 and 3).

Compared with the supply of DSM, adding MA as additive to SS substrate resulted in greater ($P < 0.05$) production of gas and tended ($P < 0.10$) to increase total VFA production and the amount of OMAF (Table 2), but after 16.5 h of incubation, only differences in gas production were detected (Table 3). A different response was observed for SM substrate, as MA treatment resulted in greater gas production ($P < 0.05$) at 6 h (Table 4), greater CH_4 production ($P < 0.05$) at 16.5 h (Table 5) and lower NH_3–N concentrations ($P < 0.05$) at both incubation times (Tables 4 and 5) compared with the supply of DSM.

3.3. Influence of donorś diet

The two types of inoculum used in this experiment differed in fermentation characteristics (results not shown). The fluid from MC-fed sheep had greater ($P < 0.05$) pH (6.68 *vs.* 6.03), NH_3–N concentrations (162 *vs.* 129 mg/l) and acetate:propionate ratio (4.72 *vs.* 4.39), but lower propionate proportion (14.0 *vs.* 15.4 mol/100 mol; $P = 0.042$), compared with the HC-inoculum.

The donorś diet influenced most of the *in vitro* measured parameters, and treatment x donors' diet interactions ($P < 0.05$) were observed for propionate production and CH_4/VFA ratio at 6 h of fermentation for SS substrate (Table 2), and for CH_4/VFA ratio at 16.5 h of fermentation for SM substrate (Table 5). For both substrates, the use of MC inoculum resulted in greater ($P < 0.05$) gas and VFA production, NH_3–N concentrations (only a trend for SS; $P = 0.082$) and OMAF at 6 h of incubation compared with the HC inoculum (Tables 2 and 4). In contrast, after 16.5 h incubation the MC-inoculated cultures had lower ($P < 0.05$) VFA production, NH_3–N concentrations and OMAF for SM substrate, and tended ($P = 0.076$) to show lower gas production (Tables 3 and 5) than the HC-inoculated cultures. For SS substrate, the MC-inoculated cultures also had lower ($P < 0.05$) gas production and NH_3–N concentrations at 16.5 h of incubation than those HC-inoculated, but VFA production was greater ($P < 0.001$; Table 3).

4. Discussion

4.1. Influence of malic acid-heat treatment

The slight decreases observed in crude protein, aNDFom, ADFom and ADL content after the MAH treatment were attributed to a dilution effect by the addition of malic acid in the protective treatment (Vanegas et al., 2016a). The MAH treatment did not increase the concentration of acid detergent insoluble N in any substrate, which would indicate that no protein damage was produced (Goering et al., 1972; Pereira et al., 1998).

The lower production of gas and total and individual VFA observed in the cultures containing MAH-treated SS substrate indicates reduced fermentation of MAH-SS substrate compared with the untreated sample. The lack of differences on propionate production was attributed to malic acid fermentation itself, as malic acid is mainly metabolized to propionate in the rumen (Carro and Ungerfeld, 2015). A decrease in protein degradation in the cultures with MAH-treated SS substrate would be supported by the reduced NH_3–N concentrations and molar proportions of isobutyrate and isovalerate, as these minor VFA are generated in the degradation of branched-chain amino acids.

The lack of effects of MAH treatment on gas and total VFA production observed for SM substrate at any incubation time, indicated a different response of SS and SM substrates to this treatment, which is in agreement with the differences observed for other high-protein feeds treated with heat (Vanhatalo et al., 1995; Mustafa et al., 2003). The effects of acid-heat treatments of feeds on their ruminal fermentation are based on protein denaturation and condensation reactions with other compounds that reduce protein degradability. Thus, previous studies (Arroyo et al., 2013; Díaz-Royón et al., 2016) reported that about 85–90% of the reduction in the DM degradation of SM substrate produced by MAH treatment was due to a reduction in protein degradation. The different response to MAH treatment observed for SS and SM substrates may be explained by the higher susceptibility of proteins in an untreated material such as SS, compared with a heat-treated material such as SM (Vanegas et al., 2016a). In addition, the large fat content of SS substrate may have produced a "frying effect" that increased the chemical reactions that contribute to reduce ruminal degradability (Vanegas et al., 2016a). In summary, the results indicate that MAH treatment caused a reduction of SS substrate DM fermentation, whereas for SM substrate the reduction in degradation was possibly limited to proteins.

The MAH treatment caused a reduction in CH_4 production and NH_3–N concentrations for both substrates, but the decreases were more pronounced for SS than for SM. Compared to UNT samples, CH_4 production in MAH-cultures was reduced by 56.4 and 22.6% for SS and SM substrates, respectively (values averaged across incubation times), and NH_3–N concentrations were decreased by 47.6 and 16.5%. As shown in Fig. 1, positive correlations ($P < 0.001$) between NH_3–N concentrations and CH_4 production in the fermentation of UNT and MAH samples were detected for both SS ($r = 0.899$; $n = 32$) and SM ($r = 0.848$; $n = 32$) substrates. The inclusion of data from MA and DSM treatments resulted also in significant correlations ($P < 0.001$) between NH_3–N concentrations and CH_4 production for both SS ($r = 0.891$; $n = 64$) and SM ($r = 0.870$; $n = 64$) substrates. Positive correlations between NH_3–N concentrations and CH_4 production have also been reported in other *in vitro* studies in which fermentations were conducted at different pH (Lana et al., 1998) or using rumen fluid with different protozoa species as inoculum (Ranilla et al., 2007). The relationships between NH_3–N and CH_4 may just reflect the different amounts of organic matter fermented in the cultures, but in the current study there were no differences in the OMAF between UNT and MAH-treated SM samples, and still a highly significant correlation was observed (see Fig. 1). Therefore, it seems that the lower CH_4 production observed for the MAH-treated SM substrate was due to the reduction in protein degradation. Protein degradation usually results in an increase in NH_3–N concentrations and the

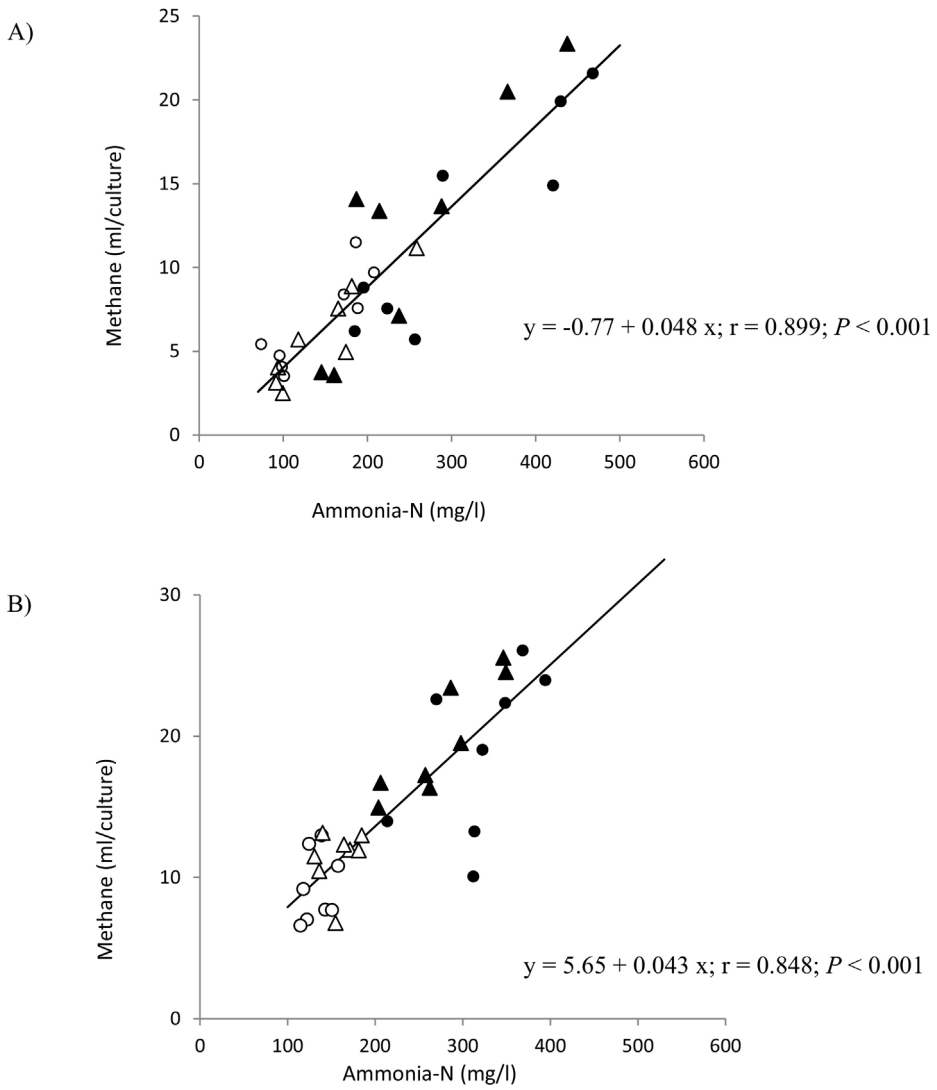

Fig. 1. Relationship between ammonia-N concentrations and methane production in batch cultures containing sunflower seed (Fig. 1A) or sunflower meal (Fig. 1B), either untreated or treated with malic acid and heat, as substrates and incubated for 6 (empty symbols) and 16.5 h (full symbols). Batch cultures were inoculated with ruminal fluid from sheep fed a medium-concentrate diet (triangles) or a high-concentrate diet (circles).

fermentation of the carbon chains resulting from amino acid deamination can contribute to the hydrogen supply to methanogenic archaea, thereby the decrease in protein degradation can result in lower NH_3—N concentrations and less CH_4 production as previously reported (Carro and Miller, 1999; Vanegas et al., 2016b).

4.2. Influence of malic acid and disodium malate as feed additives

The greater production of gas, CH_4 and VFA observed for SS substrate in the cultures with malic acid added (MA treatment) compared with that in MAH-treated cultures is consistent with the depression of SS substrate fermentation produced by MAH treatment and also with the rapid fermentation of MA by rumen microorganisms (Russell and Van Soest, 1984). The lack of differences between MA and MAH treatments in the production of gas, CH_4 and total VFA observed for SM substrate at 6 h of incubation would indicate a similar amount of DM degraded for both treatments, confirming that MAH treatment did not depress the fermentation of SM substrate. The greater propionate production observed for MA was attributed to malic acid fermentation itself (Carro and Ungerfeld, 2015). Nevertheless after 16.5 h incubation, cultures containing MAH-treated SM substrate had lower productions of CH_4 and minor VFA and numerically lower NH_3—N concentrations (Table 5) than those with MA-supplemented SM substrate, showing the greater efficacy of MAH treatment to reduce both CH_4 emissions and protein degradation compared with the use of malic acid as a feed additive.

The increase in the production of total VFA and propionate observed for both substrates at both incubation times is consistent with the results of previous *in vitro* studies on the efficacy of MA and DSM as feed additives (reviewed by Carro and Ungerfeld, 2015).

The amount of added malate (120 μmol) recovered as propionate was 0.61 and 0.56 mol/mol for MA and DSM, respectively (values averaged across substrates and incubation times), which is slightly higher than the average value of 0.48 mol/mol reported by Ungerfeld and Forster (2011) in a review of *in vitro* studies. These results support previous studies reporting a rapid fermentation of both MA and DSM by rumen microorganisms, as propionate increases were similar at 6 and 16.5 h incubation (73 *vs.* 75 μmol per culture for MA, respectively, and 66 *vs.* 68 μmol for DSM; values averaged across substrates) (Russell and Van Soest, 1984; Callaway and Martin, 1997). The lack of effects of MA and DSM on CH_4 production was consistent with the variable effects of malate supplementation on CH_4 production reported in other *in vitro* studies (Carro and Ranilla, 2003; Gómez et al., 2005). The lower NH_3—N concentrations observed for MA and DSM treatments of SM substrate compared with UNT could be explained by a greater NH_3—N capture by ruminal microorganisms as a consequence of the greater fermentable organic matter supply as malate; however, this effect was not detected for SS substrate. The observed differences between SS and SM substrates in the response to MA and DSM supplementation are in agreement with the inconsistency reported in previous *in vitro* studies with substrates of variable composition (Carro and Ranilla, 2003; Gómez et al., 2005; Tejido et al., 2005).

Compared with the supply of DSM, adding MA to SS substrate resulted in greater production of gas and total VFA production at 6 h incubation, but after 16.5 h of incubation, only differences in gas production were detected. A different response was observed for SM substrate, as MA treatment resulted in lower NH_3—N concentrations and greater CH_4 production at 16.5 h compared with the supply of DSM. These results indicate a different response due to the form of malate supply (free acid or sodium salt), which is in agreement with the controversial effects of malate on rumen methanogenesis reported in the literature (Carro and Ungerfeld, 2015; Ungerfeld and Forster, 2011). However, there were no differences in the VFA and CH_4 production in the batch cultures with MA and DSM as the only substrate (blanks with additives) either at 6 or 16.5 h of incubation (averaged values, 478 and 475 μmol VFA, and 6.73 and 6.73 ml CH_4 for MA and DSM, respectively; results not shown). This indicates that MA and DSM were fermented similarly when they were the only substrate, but differently when they were added to a fermentable substrate (SS or SM).

4.3. Influence of donorś diet

The fermentation characteristics of the two types of inoculum were in accordance with previous studies in sheep fed diets of different composition (Carro et al., 2000; Ramos et al., 2009), and might indicate the existence of different microbial communities in MC and HC ruminal fluids. However, the high buffer capacity of the incubation medium of the buffer-mineral solution prevented a pH drop in the cultures and the pH decrease observed in sheep fed HC could not be reproduced in the cultures, resulting in similar values ($P = 0.324$) of final pH for both donors' diets (6.78 and 6. 74 for MC and HC diets, respectively; values averaged across treatments). The lack of differences in pH might have influenced treatment differences, as cultures did not fully mimic rumen conditions.

The greater gas and VFA production and NH_3—N concentrations observed at 6 h incubation for the MC inoculum would indicate that this inoculum had higher initial degradative activity than the HC ones. However, after 16.5 h incubation the MC-inoculated cultures had lower VFA production and NH_3—N concentrations than those HC-inoculated for SM substrate, indicating increased microbial growth and/or activity in HC-cultures. For SS substrate, the MC-inoculated cultures also had lower NH_3—N concentrations at 16.5 h of incubation, but VFA production still was greater than in HC-inoculated cultures. This might be due to a lower development of bacterial microcolonies as a consequence of a possible greater sensibility of HC microbes to negative effects of SS fat.

The lower CH_4 production observed in the HC-cultures for SM substrate at 6 h of incubation might reflect a lower concentration and/or activity of methanogenic archaea in this inoculum (Wallace et al., 2014), although no differences between inocula were observed for SS substrate. Differences in the fermentation parameters at 6 and 16.5 h incubation are also related to the adaptation of the microbial populations to the incubation conditions in the batch cultures (substrate, temperature, pH, etc.), as observed by others (Mateos et al., 2015).

Treatment x donors' diet interactions were observed for propionate production and CH_4/VFA ratio. Whereas the MAH treatment of SS substrate decreased the propionate production at 6 h of incubation with the HC-inoculum (124 and 92.7 μmol for UNT and MAH, respectively), no changes were observed with the MC-inoculum (140 and 138 μmol for UNT and MAH). Similarly, the decrease in the CH_4/VFA ratio produced by the MAH-treatment of SM substrate at 16.5 h incubation was more pronounced for the HC-inoculum (0.016 and 0.008 ml/μmol for UNT and MAH, respectively) than for the MC-inoculum (0.015 and 0.012 ml/μmol). These results illustrate that the effectiveness of treatments to modify *in vitro* fermentation may depend on the type of diet fed to donor animals, as previously observed for some feed additives (Kamel et al., 2008; Mateos et al., 2013).

5. Conclusion

The results indicate that the combined malic acid-heat treatment was effective to improve *in vitro* fermentation by reducing CH_4 emissions and NH_3—N concentrations. This treatment was more effective than the use of the same amount of malic acid as feed additive, either in the form of free acid or as disodium salt. The positive relationships observed between CH_4 emissions and NH_3—N concentrations suggest that a decrease in protein degradation could result in less CH_4 production, highlighting the importance of avoiding an excess of degradable protein in the diet to reduce both N and CH_4 emissions. The differences in the response to either malic acid or disodium malate supplementation help to explain the controversial results observed in the literature regarding malate effects on *in vitro* fermentation.

Conflict of interest

The authors declare that there are no conflicts of interest.

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