Research Article

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Protection of sunflower seed and sunflower meal protein with malic acid and heat: effects on *in vitro* ruminal fermentation and methane production

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Abstract

BACKGROUND: Combined malic acid–heat treatments of protein supplements have been shown to reduce ruminal protein degradation, but there is no information on their possible influence on ruminal fermentation and methane emissions. This study aimed to investigate the effects of the treatment of sunflower meal (SM) and sunflower seed (SS) with malic acid and subsequent drying at 150°C for 1 (MAL1) or 3 h (MAL3) on *in vitro* rumen fermentation and methane emission using ruminal fluid from sheep as inoculum.

RESULTS: Compared with untreated samples, the MAL3 treatment reduced ($P < 0.05$) the dry matter effective degradability (DMED) by 78% and 46% for SS and SM, respectively, indicating heat damage. The MAL1 treatment reduced the DMED of SS by 22%, but did not affect ($P > 0.05$) total volatile fatty acid production for any feed. This treatment also increased ($P < 0.05$) the propionate proportion (by 17.7% and 15.6% for SS and SM, respectively) and decreased ($P < 0.05$) methane production (by 15.5% and 11.3%, respectively) and ammonia-N concentrations (by 26.5% and 14.5%, respectively).

CONCLUSION: The MAL1 treatment was effective in reducing both ammonia-N concentrations and methane emissions without depressing SS and SM fermentation, but more research is needed to formulate environmentally cleaner diets for ruminants.

Keywords: protein protection; malic acid; heat; methane; sunflower meal; sunflower seed

INTRODUCTION

Reducing environmental impacts of animal production is currently an important goal of nutritionists. In this context, ruminants have a low efficiency of N use compared with non-ruminants and about 70–75% of the N ingested is excreted in manure,[1] this inefficiency being attributable primarily to an inefficient use of N in the rumen, although post-absorptive N utilisation is also inefficient. A reduction in the ruminal protein degradation of high-quality proteins can increase protein utilisation and reduce N environmental emissions. The treatment of high-protein feeds with heat and/or acid solutions has proven to be useful for protecting proteins from microbial degradation in the rumen, but the efficacy of these treatments varies with different factors, such as the type and concentration of acid and the heat intensity and duration.[2] Previous *in vitro* and *in situ* studies have demonstrated the efficacy of a combined treatment with malic acid and heat to increase the protein value of sunflower meal (SM) and spring peas.[3–5]

Malic acid is converted to propionate in the rumen via fumarate and therefore can acts as an electron sink for hydrogen and decrease the hydrogen availability for methanogenesis. In fact, malic acid and its sodium salts have been shown to stimulate propionate formation and inhibit methanogenesis in some *in vitro* studies,[6–8] although no clear reductions of methane emissions were found in others.[9,10] In addition, the 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 resulting in less methane production.[11,12] Based on these results, our hypothesis was that the combined treatment with malic acid and heat for protein protection may not only reduce protein degradability, but may also improve the efficiency of ruminal fermentation by increasing propionate production and reducing methane emissions. The aim of this study was therefore to investigate the effects of treating sunflower seeds (SS) and SM with malic acid at high temperature on their *in vitro* fermentation and methane emissions. These two protein feeds were selected because they are extensively used in

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practical feeding of ruminants and differ in their protein degradability, which is usually higher in SS than in SM.[13]

MATERIALS AND METHODS

Animals and feeding

Four adult rumen-fistulated sheep (64.9 ± 2.04 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 (Cl, Na, P, Ca, Mg, I, K, Zn, Mn, S, Fe, Co, Mo, Se and vitamins A, D_3, E and niacin; Capriovi Produccion, Díaz del Prado Nutrición y Salud Animal, Talavera de la Reina, Spain). Sheep were fed a 2:1 mixed diet of lucerne hay and a commercial concentrate at energy maintenance level[14] distributed in two equal meals. The diet contained 913, 168, 426 and 269 g of organic matter (OM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) per kg of dry matter (DM), respectively. Sheep management and rumen contents sampling were carried out in accordance with the Spanish guidelines for experimental animal protection[15] in line with the European regulations. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Polytechnic University of Madrid.

Feed samples and *in vitro* incubations

One sample of SS (oil type) and one sample of semi-dehulled SM were used in the study. Five hundred grams of each substrate were weighed and pulverised with a solution of 1 mol L^{-1} malic acid at a rate of 400 mL per kg of feed and left at room temperature for 1 h. Samples were then dried at 150°C for either 1 (MAL1) or 3 h (MAL3). The concentration of malic acid and the duration of the heat treatment were selected from the results of previous studies.[3,4] The chemical composition of the untreated (UNT) and treated samples is shown in Table 1.

Two different incubations were carried out and in each of them there were four replicates per feed and treatment. The first incubation was conducted to assess the gas production kinetics of the samples, whereas the main fermentation parameters were determined in the second incubation. Samples (500 and 300 mg of dry matter in the first and second incubation, respectively) of each substrate were carefully weighed into 120-mL serum bottles. The amount of sample in the second incubation was reduced to avoid high headspace pressures that could have a detrimental effect on ruminal fermentation, as the gas produced was only released at the end of the incubation period. Ruminal contents from each sheep were obtained immediately before the morning feeding and strained through four layers of cheesecloth. The fluid of each sheep was then mixed independently with a buffer solution in a 1:4 ratio (vol/vol) at 39°C under continuous flushing with CO_2. The medium of Goering and Van Soest[16] was modified by replacing the $(NH_4)HCO_3$ with $NaHCO_3$ and excluding the trypticase, and the resulting N-free solution was used as buffer. Bottles were pre-warmed (39°C) prior to the addition of 50 mL of buffered rumen contents under CO_2 flushing. Then, bottles were sealed with rubber stoppers and aluminium caps and incubated at 39°C.

In the first incubation the gas production was measured using a pressure transducer (Delta Ohm DTP704-2BGI; Herter Instruments SL, Barcelona, Spain) and a calibrated syringe at 3, 6, 12, 15, 20, 25, 30, 38, 48, 60, 72, 96, 120 and 144 h and the gas produced was released after each measurement. A total of 24 bottles (one per substrate and inoculum) were used. Additional bottles without substrate (blanks; two per inoculum) were included to correct the gas production values for gas release from endogenous substrates. After measuring the gas produced at 144 h of incubation, the fermentation was stopped by swirling the bottles in iced water; bottles were then opened and their content was transferred to previously weighed filter crucibles (pore size 100–160 μm) and filtered under vacuum. The residue of incubation was washed with 50 mL of hot distilled water and dried at 50°C for 48 h for estimating the apparent disappearance of dry matter after 144 h of incubation (DMD_{144}).

The second incubation analysed the effects of the treatments on the *in vitro* fermentation parameters. The incubation procedure was as before described with the exception that bottles contained 300 mg of substrate DM and were filled with 30 mL of buffered ruminal fluid. Two bottles per substrate and inoculum were incubated for 16.5 h, value corresponding to a mean passage rate from the rumen of 0.06 per h. After 16.5 h, total gas production was measured as described before and a gas sample (10 mL) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of methane. Bottles were then uncapped and the pH was measured immediately with a pH meter (Crison Basic 20; Crisson Instruments, Barcelona, Spain). Three millilitres of content 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 acid (VFA) determination and 2 mL were added to 2 mL 0.5 mol L^{-1} HCl for NH_3-N analysis. Finally, the content of all bottles was filtered through crucibles, oven-dried at 50°C and weighed to determine the disappearance of DM (DMD). Half of the crucibles (one per each feed, treatment and inoculum) were ashed (550°C for 8 h) to estimate OM disappearance (OMD). The residues of the other half of crucibles were analysed for NDF to estimate NDF disappearance (NDFD).

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.[17] The analysis of NDF, ADF and acid detergent lignin (ADL) were carried out according to Van Soest *et al.*[18] using an ANKOM220 Fibre Analyzer unit (ANKOMTechnology Corporation, Fairport, NY, USA). 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. Concentrations of NH_3-N were determined using a spectrophotometer by the method of phenol-hypochlorite as previously described[19] and those of VFA by gas chromatography as described by Carro *et al.*[20] Analysis of methane was carried out following the procedure of Martínez *et al.*[21] using a gas chromatograph (Shimadzu GC 14B; Shimadzu Europa GmbH, Duisburg, Germany) equipped with a flame ionisation detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain).

Calculations and statistical analyses

In order to estimate the fermentation kinetic parameters in the first incubation, gas production data were fitted to the exponential model: $\text{gas} = A\{1 - \exp[-c(t - \text{lag})]\}$, where A is the asymptotic gas production, c is the fractional rate of gas production, lag is the initial delay in the onset of gas production and t is the time of gas measurement. The parameters A, c and lag were estimated by an iterative least squares procedure using the NLIN procedures of SAS (version 9.2; SAS Institute, Cary, NC, USA). The half time of gas production ($T_{1/2}$) was the time (h) when half of the asymptotic gas volume (A; mL) was produced and was calculated as $T_{1/2} = [(\ln 2/c) + \text{lag}]$. The average gas production rate

Table 1. Chemical composition (g kg^{-1} dry matter unless otherwise stated) of sunflower seed and sunflower meal either untreated (UNT) or treated with a 1 mol L^{-1} solution of malic acid (400 mL kg^{-1} feed) at 150°C for 1 h (MAL1) or 3 h (MAL3)

	Sunflower seed			Sunflower meal		
Item	UNT	MAL1	MAL3	UNT	MAL1	MAL3
Dry matter (g kg^{-1} fresh matter)	977	980	971	919	969	998
Organic matter	969	970	969	927	932	930
Crude protein	179	171	172	359	337	343
Ether extract	467	459	318	12.9	20.2	15.9
Neutral detergent fibre	292	271	533	490	466	633
Acid detergent fibre	159	147	319	275	261	275
Acid detergent lignin	53.8	48.7	186	88.2	84.5	110
NDIN × 6.25	54.3 (0.303)	42.6 (0.249)	144 (0.834)	89.8 (0.249)	98.3 (0.292)	246 (0.718)
ADIN × 6.25	8.74 (0.049)	7.77 (0.046)	47.2 (0.274)	17.5 (0.049)	16.5 (0.049)	46.8 (0.137)

NDIN and ADIN: insoluble N in neutral and acid detergent solutions, respectively. Values in brackets are expressed as g g^{-1} of total crude protein.

(AGPR; mL gas h^{-1}) was defined as the average gas production rate between the start of the incubation and $T_{1/2}$, and was calculated as AGPR = $Ac/[2(\ln 2 + c \times \text{lag})]$. Finally, the DM effective degradability (DMED) was estimated assuming a rumen particulate outflow (K_p) of 0.06 per h according to the equation: $\text{DMED} = [(\text{DMD}_{144} \times c)/(c + K_p)]e^{(-c \times \text{lag})}$.

In the 16.5-h incubation, the amounts of VFA produced in each bottle were calculated by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation. The volume of gas produced was corrected for temperature (273K; 0°C) and pressure (1 atm) and the amount of methane was calculated by multiplying gas produced by the methane concentration in the analysed sample. The amount of OM apparently fermented (OMAF) in each culture was estimated from acetate, propionate and butyrate production as described by Demeyer.[22] Values measured of the two bottles incubated for each inoculum, feed and experimental treatment were averaged before statistical analysis (four values per feed and experimental treatment).

Accumulative gas production data were analysed independently for SS and SM as a mixed model with repeated measures using the PROC MIXED of SAS. The effects of the treatment, time and the interaction treatment × time were considered fixed and that of the inoculum was random. The rest of the data were analysed independently for each feed as a mixed model using the PROC MIXED of SAS and considering the effect of the treatment fixed and that of the inoculum as random. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered to be a trend. Comparison of means was performed by the Tukey test.

RESULTS AND DISCUSSION

Effects of treatments on chemical composition of samples

The chemical composition of raw SS and SM samples was within the range reported in the literature,[13,23] and the MAL1 treatment caused only minor changes in chemical composition of both protein feeds (Table 1). The slight decreases observed in CP, NDF, ADF and ADL content in the MAL1-treated compared with those in the untreated samples may be due to a dilution effect associated with adding malic acid in the protective treatment. The determination of ADIN has been proposed as a parameter to determine the extent of heat damage, as over-heating may lead to the formation of indigestible compounds via the Maillard reactions between sugar aldehyde groups and free amino groups.[2,24] The fact that MAL1 treatment did not increase the concentration of ADIN in SS and SM, either expressed as g kg^{-1} DM or as % of CP content, would indicate that this treatment was not severe enough to produce SS or SM protein damage. In contrast, the MAL3 treatment produced marked changes in the chemical composition of both feeds. The amount of NDF, ADF and ADL was 1.8, 2.0 and 3.5 times greater in MAL3 than in the raw sample for SS, respectively, and 1.3, 1.0 and 1.2 times for SM. In addition, both NDIN and ADIN content increased markedly as a consequence of MAL3 treatment, with values 2.7 and 5.4 times greater than those in the untreated SS sample, respectively, and 2.7 times greater in SM for both NDIN and ADIN. Despite these changes in NDIN and ADIN fractions, total CP content was not markedly affected by MAL3 treatment. Similar results have been reported for other rich-protein feeds,[25–27] indicating that heat treatment did not produce total N loss. The actual effects of acid and heat treatment on protein are influenced mainly by acid concentration, pH, heating time, temperature and moisture,[2,4,25] although differences in susceptibility of different feeds have been reported to be large.[28,29] Due to the impact of heat in feeds previously processed, such as the SM, the increases in NDF and ADF observed after heat treatment are usually greater in raw samples than in samples previously heated, which is in agreement with the higher increases observed in SS compared with SM in the present study. In addition, the large fat content of SS may have produced a 'frying effect' that increased these reactions. The increases in fibre content observed in some studies have been greater for NDF than for ADF,[25,28] which is in agreement with our results. The MAL3 treatment produced a marked decrease in the ether extract content of SS, which was partly attributed to physical losses, as fat residues were observed in the drying trays when removing the sample after its treatment, although some fat combustion cannot be discarded.

Effects of treatments on gas production kinetics

The gas production was chosen to test the effects of the treatments because is a simple technique to evaluate substrate degradation, as the amount of gas produced is assumed to be directly proportional to substrate degradation.[29] The effects of the protective treatments on gas production values and the parameters of gas production kinetics are shown in Fig. 1 and Table 2, respectively. For both feeds, gas production values for MAL3-treated samples were lower ($P < 0.05$) than those for untreated and MAL1 samples

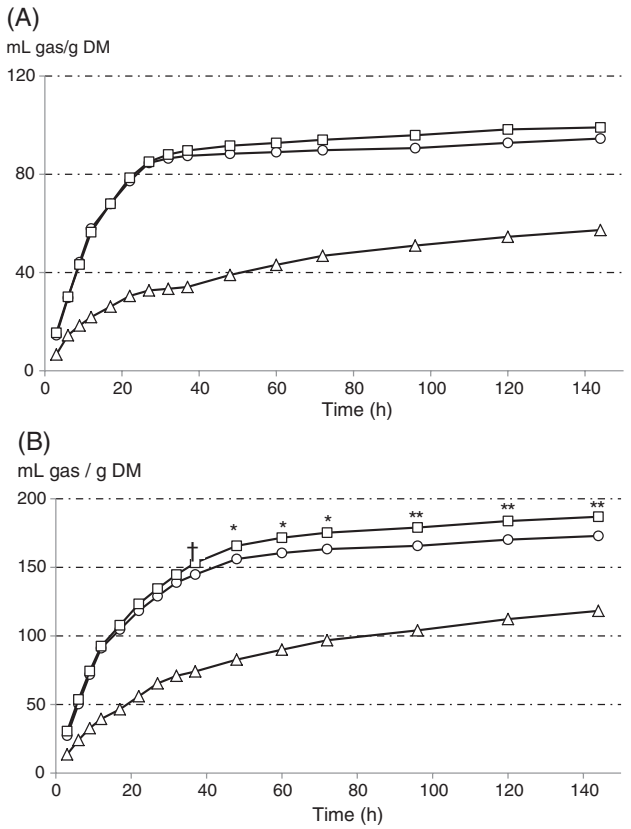

Figure 1. Gas production kinetics of sunflower seed (A) and sunflower meal (B) samples untreated (UNT; circles) and treated with malic acid at 150°C for 1 h (MAL1; squares) or 3 h (MAL3; triangles). Values of SEM and *P* of the ANOVA were 3.63 and $P < 0.001$ for SS and 3.16 and $P < 0.001$ for SM, respectively. For both samples MAL3 had lower ($P < 0.05$) gas production than UNT and MAL1 at all incubation times. Differences between UNT and MAL1 are indicated by †$P < 0.10$, *$P < 0.05$ and **$P < 0.01$.

at all measured times, which indicates a reduced fermentation of MAL3 samples. These results are in accordance with the greater content of NDF and ADL observed in MAL3-treated SS and SM samples compared with the untreated and MAL1 samples. In agreement with the results of gas production, the values of *A*, *c*, and AGPR were 0.58, 0.38 and 0.24 of those for untreated SS and 0.67, 0.53 and 0.36 of those for untreated SM, respectively ($P < 0.05$). Moreover, the DMED of MAL3 samples was decreased ($P < 0.05$) by 78% and 46% compared with the values for untreated SS and SM, respectively. All these results indicate that the MAL3 treatment reduced the amount of substrate available to the microbes for degradation, possibly by the formation of indigestible lignin-like polymers and phenolic compounds that are formed by heating and drying at high temperatures.[30]

There were no differences ($P > 0.05$) in the amount of gas produced between untreated and MAL1 samples at any time for SS, but the fermentation of MAL1-treated SM produced greater amounts of gas than that of the untreated SM from the 38 h until the end of the incubation ($P < 0.10$ at 38 h; $P < 0.05$ at 48, 60 and 72 h; $P < 0.01$ at 96, 120 and 144 h). These results indicate a different response to MAL1 treatment for the two tested feeds, which is in agreement with the differences observed for other protein-rich feeds treated with heat.[26,27] The greater gas production of MAL1-treated SM might be due to the direct fermentation of the added malic acid, as malic acid is rapidly fermented by rumen microorganisms and amounts similar to that used in the present study were fermented *in vitro* within the first 12 h of incubation.[31] However, the fact that the increase in gas production for MAL1 treatment was first noticed at 38 h of incubation would indicate that other mechanisms are involved in the observed response. In addition, a hypothetical rapid fermentation of malic acid would had been decreased the lag time for MAL1 treatment, but no differences ($P > 0.05$) between treatments were observed in this parameter for any feed. For both SS and SM, there were no differences ($P > 0.05$) between untreated and MAL1-treated samples in any gas production kinetics parameter (*A*, *c*, AGPR and $T_{1/2}$), indicating that this treatment did not cause negative effects on substrate degradation. The MAL1 treatment of SS decreased ($P < 0.05$) DMED from 0.325 to 0.259, associated to numerically reductions in the rate of gas production (0.0896 and 0.0779 h^{-1} for untreated and MAL1 samples, respectively), but no effects ($P > 0.05$) were observed for SM (0.334 and 0.322 for untreated and MAL1 samples, respectively).

Effects of treatments on *in vitro* fermentation

As shown in Table 3 and Table 4, the results of the 16.5 h incubation confirmed the negative effects of MAL3 treatment on substrate degradability, as it reduced ($P < 0.05$) the production of gas and VFA by 28% and 42% for SS and by 27% and 38% for SM, respectively. The MAL3 treatment also reduced ($P < 0.05$) the methane emission by 35% and 44% for SS and SM, respectively. The amount of malic acid incubated with the feed per bottle (120 µmol) had a potential decrease in methane production of 30 µmol according to the stoichiometric equation $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$, which is much lower than the reduction of 81 and 142 µmol of methane per bottle observed for MAL3 treatment for SS and SM, respectively. Therefore, this reduction was attributed to decreased fermentable substrate rather than to a direct effect on methanogenesis, as indicated by the lower ($P < 0.05$) amount of OMAF and the lower values of DMD and OMD observed for MAL3-treated samples. The MAL3-treated SM had lower NDFD values than the untreated samples (Table 4), which is consistent with the reduced methane production due to the close relationship between the amount of fibre fermented and that of methane generated.[32] The extremely low values (close to 0; values not shown) of NDFD of SS for all treatments were attributed to the high ether extract content of SS, as the free fatty acids resulting from the triglyceride hydrolysis are toxic for the fibrolytic bacteria.[33]

Compared with the untreated samples, the MAL3 treatment increased ($P < 0.05$) molar proportion of acetate for both feeds, but effects on other VFA were variable. Treating the SS with MAL3 resulted in lower ($P < 0.05$) proportions of propionate, butyrate and valerate and higher ($P < 0.05$) caproate proportions, whereas the treatment of SM decreased the proportion of isovalerate and valerate without affecting the propionate, butyrate, isobutyrate and caproate proportions. These results indicate again a great variability between feeds in the response to acid–heat treatments.

In agreement with the results of the gas production trial, the MAL1 treatment of SS and SM did not negatively affect the production of gas and total VFA and the amount of OMAF indicating that this treatment did not reduce the substrate fermentability. The average reduction of 36 µmol of methane per bottle observed for MAL1 treatment for both feeds is slightly greater than the potential decrease in methane production of 30 µmol calculated for the amount of malic acid added per bottle. However, in a meta-analysis of malate effects on methanogenesis in ruminal batch cultures, Ungerfeld and Forster[34] observed that methane decrease with

Table 2. Parameters of *in vitro* gas production kinetics and dry matter effective degradability (DMED) of sunflower seed and sunflower meal either untreated (UNT) or treated with a 1 mol L^{-1} solution of malic acid (400 mL kg^{-1} feed) at 150°C for 1 h (MAL1) or 3 h (MAL3)

	Sunflower seed					Sunflower meal				
Item	UNT	MAL1	MAL3	SEM	*P* value	UNT	MAL1	MAL3	SEM	*P* value
A (mL g^{-1} DM)	96.3^{b}	96.5^{b}	55.7^{a}	1.79	<0.001	168^{b}	181^{b}	113^{a}	3.83	<0.001
c (h^{-1})	0.0896^{b}	0.0779^{b}	0.0341^{a}	0.00471	<0.001	0.0577^{b}	0.0535^{b}	0.0307^{a}	0.00180	<0.001
Lag (h)	1.17	1.20	0.25	0.311	0.113	0.11	0.00	0.00	0.053	0.298
AGPR (mL h^{-1})	5.39^{b}	4.75^{b}	1.30^{a}	0.367	<0.001	6.85^{b}	6.93^{b}	2.48^{a}	0.223	<0.001
$T_{1/2}$ (h)	9.08^{a}	10.4^{a}	26.5^{b}	4.14	0.044	12.3^{b}	13.3^{b}	23.0^{a}	0.65	<0.001
DMED (g g^{-1})	0.325^{c}	0.259^{b}	0.073^{a}	0.0100	<0.001	0.334^{b}	0.322^{b}	0.179^{a}	0.0524	<0.001

$^{a-c}$Within each feed, means in the same row with different superscript letters differ ($P < 0.05$).
A is the asymptotic gas production (mL g^{-1} DM); *c* is the fractional rate of gas production (h^{-1}); lag is the initial time delay in the onset of gas production; AGPR is the average gas production rate; $T_{1/2}$ is the time at which *half* of *A* is reached; DMED is the dry matter effective degradability for a rumen particulate outflow of 0.06 h^{-1}.

Table 3. Effects of the treatment of sunflower seed with a 1 M solution of malic acid (400 mL kg^{-1} feed) at 150°C for 1 h (MAL1) or 3 h (MAL3) on *in vitro* fermentation and dry matter (DMD) and organic matter disappearance (OMD) in batch cultures of sheep rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h ($n = 4$)

	Treatment				
Item	UNT	MAL1	MAL3	SEM	*P* value
Gas (μmol)	1541^{b}	1564^{b}	1113^{a}	54.1	0.002
CH_4 (μmol)	233^{c}	197^{b}	152^{a}	10.4	0.005
Proportion of CH_4 in gas (mol mol^{-1})	0.155	0.127	0.140	0.0074	0.099
NH_3-N (mg L^{-1})	302^{c}	222^{b}	122^{a}	4.64	<0.001
Total volatile fatty acids (μmol)	784^{b}	731^{b}	456^{a}	31.6	<0.001
Molar proportion (mol 100 mol^{-1})					
Acetate (Ac)	59.2^{a}	58.9^{a}	64.7^{b}	0.37	<0.001
Propionate (Pr)	19.5^{b}	23.7^{c}	16.2^{a}	0.55	<0.001
Butyrate	9.93^{b}	8.16^{a}	8.65^{ab}	0.377	0.039
Isobutyrate	4.03	3.70	3.92	0.139	0.295
Isovalerate	4.33	3.35	3.81	0.243	0.084
Valerate	2.53^{c}	2.05^{b}	1.83^{a}	0.062	<0.001
Caproate	0.50^{a}	0.15^{a}	0.89^{b}	0.134	0.023
Ac/Pr (mol mol^{-1})	3.05^{b}	2.53^{a}	4.00^{c}	0.067	0.001
CH_4/VFA (mol mol^{-1})	0.300	0.269	0.333	0.0310	0.049
Organic matter fermented (mg)	62.8^{b}	58.8^{b}	34.2^{a}	2.58	<0.001
DMD (g g^{-1})	0.346^{c}	0.269^{b}	0.123^{a}	0.0130	<0.001
OMD (g g^{-1})	0.355^{c}	0.294^{b}	0.137^{a}	0.0148	<0.001

$^{a-c}$Means in the same row with different superscript letters differ ($P < 0.05$).
Values of organic matter fermented were estimated from volatile fatty acids production according to Demeyer.[22]
Values for neutral detergent fibre degradability were close to 0 for all treatments and are not reported.
UNT, untreated sample.

malic acid has generally been quite lower than expected according to stoichiometry calculations. In fact, the MAL1 treatment decreased methane production by 15.5 and 11.3% for SS and SM, respectively, values greater than the 7–8% decrease in methane formation found by others[7,8,34] in ruminal batch cultures for similar initial concentrations of malic acid or malate salts, which suggest that methane reductions in the present study cannot be exclusively attributed to the fermentation of malate itself. The observed reduction in NDFD of MAL1-treated SM compared with raw SM would support this hypothesis, as the degradation of NDF usually results in high proportions of acetate and butyrate and the formation of both VFA requires the production of CO_2 and hydrogen which are used by the methanogenic archaea to form CH_4.[35]

The lower OMD observed for MAL1-treated SS compared with untreated SS is also in agreement with this hypothesis. In addition, the fermentation of protein has been shown to contribute to methane generation[11,12] and a decrease in protein fermentation in MAL1-treated samples is supported by the lower ($P < 0.05$) ammonia-N concentrations observed for both feeds and by the reduced molar proportions of isobutyrate, isovalerate and valerate, which are generated in the degradation of branched-chain amino acids. The MAL1 treatment also increased ($P < 0.05$) the molar proportions of propionate for both feeds and decreased that of butyrate ($P < 0.05$) for SS. The 0.45 mol/mol recovery of malic acid as propionate observed for the SM agrees well with the average value of 0.48 mol mol^{-1} reported by Ungerfeld and Forster,[34]

Table 4. Effects of the treatment of sunflower meal with a 1 mol L^{-1} solution of malic acid (400 mL kg^{-1} feed) at 150°C for 1 h (MAL1) or 3 h (MAL3) on *in vitro* fermentation and disappearance of dry matter (DMD), organic matter (OMD) and neutral detergent fibre (NDFD) in batch cultures of sheep rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h ($n = 4$)

	Treatment				
Item	UNT	MAL1	MAL3	SEM	*P* value
Gas (μmol)	2173[b]	2240[b]	1578[a]	15.2	<0.001
CH_4 (μmol)	320[c]	284[b]	178[a]	6.81	<0.001
Proportion of CH_4 in gas (mol mol^{-1})	0.148[c]	0.127[b]	0.112[a]	0.00353	0.001
NH_3-N (mg L^{-1})	303[c]	259[b]	147[a]	5.07	<0.001
Total volatile fatty acids (μmol)	1220[b]	1276[b]	756[a]	26.0	<0.001
Molar proportion (mol 100 mol^{-1})					
Acetate (Ac)	62.8[a]	64.3[a]	67.8[b]	0.45	0.004
Propionate (Pr)	18.4[a]	21.8[b]	18.4[a]	0.26	<0.001
Butyrate	8.22	7.48	7.68	0.27	0.199
Isobutyrate	2.83[b]	2.58[a]	2.83[b]	0.059	0.039
Isovalerate	2.73[b]	2.16[a]	1.93[a]	0.074	<0.001
Valerate	1.75[c]	1.53[b]	1.18[a]	0.066	0.003
Caproate	0.32	0.23	0.16	0.070	0.354
Ac/Pr (mol mol^{-1})	3.61[b]	2.96[a]	3.69[b]	0.069	<0.001
CH_4/VFA (mol mol^{-1})	0.262[b]	0.224[a]	0.235[a]	0.0078	0.029
Organic matter fermented (mg)	99.6[b]	104.7[b]	62.4[a]	2.19	<0.001
DMD (g g^{-1})	0.344[b]	0.333[b]	0.171[a]	0.0071	<0.001
OMD (g g^{-1})	0.373[b]	0.357[b]	0.188[a]	0.0188	<0.001
NDFD (g g^{-1})	0.259[c]	0.189[b]	0.043[a]	0.0194	<0.001

[a–c]Means in the same row with different superscript letters differ ($P < 0.05$).
Values of organic matter fermented were estimated from volatile fatty acids production according to Demeyer.[22]
UNT, untreated sample.

but the recovery for SS was much lower (0.17 mol mol^{-1}), indicating differences between feeds. The effects of malic acid and malate salts on VFA profile have been reported to vary with the incubated substrate,[7,8] but an increase in the propionate production has been observed in most *in vitro* studies because malate is an intermediate of the succinate pathway of propionate production in the rumen.[10] The observed increase in propionate production is in agreement with the reduction in the production of methane, as the generation of propionate incorporates reducing equivalents.[10] Due to these changes, the MAL1 treatment reduced ($P < 0.05$) the acetate/propionate ratio for both feeds and decreased the ratio methane/VFA from 0.300 to 0.269 for SS and from 0.262 to 0.224 for SM, differences being only significant ($P < 0.05$) for SM. These results indicate an improvement in the efficiency of ruminal fermentation and could imply a greater energy supply to the host animal.

The lower ($P < 0.05$) DMD and OMD values observed in MAL1-treated SS compared with untreated SS are in accordance with the lower DMED of SS estimated in the gas production trial (22.2, 17.2 and 20.3% of reduction, respectively) and may be due to a reduction in the substrate degradation rate, as previously discussed. Thus, values of c were numerically lower for MAL-1 than for the untreated SS samples, although the differences did not reach the significance level ($P = 0.129$). This slow degradation rate may also affect the ruminal fermentation of the unsaturated fat of this SS. Therefore, studies investigating the effects of this treatment on the biohydrogenation of SS fatty acids and its subsequent intestinal digestibility would be of interest to evaluate the actual increase of its nutritive value. Finally, it should be noticed that the amount of DM and OM digested and incorporated into microbial biomass has not been taken into account in any of the fermentation variables measured, and studies determining the microbial protein synthesis with protein protected feeds are needed.

CONCLUSIONS

The results indicate that the MAL1 treatment is effective at protecting a highly degradable protein rich in sulfur-containing amino acids and tryptophan such as sunflower protein from the ruminal degradation. In addition, this treatment improved the efficiency of sunflower seed and sunflower meal fermentation by decreasing the emission of methane and increasing the proportion of propionate without negatively affecting total VFA production. However, over-protection by excessive heat duration (150°C for 3 h) should be avoided because it reduced the fermentability of both feeds and led to protein damage as indicated by the increased NDIN and ADIN fractions.

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