Research Article

Received: 23 June 2021 Revised: 9 March 2022 Accepted article published: 13 March 2022 Published online in Wiley Online Library: 26 March 2022

(wileyonlinelibrary.com) DOI 10.1002/jsfa.11864

Prosopis juliflora piperidine alkaloid extract levels in diet for sheep change energy and nitrogen metabolism and affect enteric methane yield

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Abstract

BACKGROUND: Ionophore antibiotics improve the efficiency of energy metabolism, which has driven their use as a feed additive in ruminants for decades. Currently, they have not been approved in many countries, generating a challenge for the immediate search for plant extracts with a similar mode of action on rumen metabolism. This study evaluated the effects of enriched *Prosopis juliflora* (mesquite) piperidine alkaloid extract (MPA) levels as an alternative phytoadditive to sodium monensin (MON) in sheep.

RESULTS: The MPA diet did not differ from MON with regard to nutrient intake. A quadratic effect ($P < 0.05$) was observed for organic matter and neutral detergent fibre digestibility, with respective maximum point at 25.40 and minimum point at 0.95 mg kg^{-1} MPA. The MPA levels linearly decreased ($P < 0.05$) faecal nitrogen loss. MPA did not differ from MON with regard to nutrient digestibility, and MPA levels increased ($P < 0.05$) the proportion of digestible energy and metabolizability from dietary gross energy. The MPA levels linearly decreased ($P < 0.05$) enteric CH_4 production, the yield showing lower ($P < 0.05$) energy loss as CH_4 than MON.

CONCLUSION: The results show that MPA levels of 17.3 and 27.8 mg kg^{-1} are enteric CH_4 inhibitors and enhance energy and protein utilization, indicating a promising alternative to MON for ruminants.

Keywords: energy balance; mesquite; methane; monensin; nitrogen balance; plant extract

INTRODUCTION

Improvement in bioeconomic efficiency and environmental impact in the livestock industry is promoted by using ruminal fermentation-modulating additives; thus polyether ionophores increase feed efficiency by as much as 10%.[1] Ionophores are lipid soluble and are able to transport cations across cell membranes, acting against bacteria whose cytoplasmic membrane is exposed or is covered in a thinner cell wall.[2] Its classification as an antibiotic and its long-term application could result in increased control in future years. However, the current demand for production with long-term sustainability and food safety, natural compound extracts from plants, bacteriocins, propolis and others has been researched to modify rumen fermentation with anti-methanogenic potential.[3-8]

Piperidines are phytochemicals belonging to the group of heterocyclic alkaloids, which have marked lipophilicity and polarity, contributing to their incorporation into exposed cell membranes, and which can greatly affect the functioning of various ion channels.[9] The blocking action of calcium transport can affect both bacteria and methanogenic archaea.[9-11]

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The qualitative and quantitative nature of the bioactive piperidine alkaloid profile in *Prosopis juliflora* (Sw.) D.C. (mesquite) can vary significantly depending on geographical location and annuality.[12] Nevertheless, analyses on the alkaloid fraction from mesquite pods collected in Senhor do Bonfim, Brumado, and Manoel Vitorino, located in different geographical regions of the Bahia state, led to identification of two majority alkaloids: juliprosinine and juliprosopine (juliflorine).[3,13,14]

Doses of 130, 260 and 390 mg m^{-3} enriched mesquite piperidine alkaloid extract (MPA), obtained as chlorinated salts from basic chloroform extract (BCE), were used in *in vitro* ruminal fermentation by Pereira *et al*.,[6] who observed an increase in propionate concentration, lower methane yield and unaffected degradability of the dry matter from wheat bran compared with sodium monensin at 110 mg m^{-3}. MPA doses ranging from 2.3 to 31.5 mg kg^{-1} dry matter, providing intraruminal concentrations of approximately 230–3150 mg m^{-3} per day, enhanced the energy and protein utilization and increased the performance of crossbred Santa Inês growing male lambs.[14,15] Additionally, the lambs did not show any clinical signs or histological lesions (unpublished data), as characterized by Silva *et al*.[16] and Figueiredo *et al*.[17]

In view of the MPA potential, this study was performed based on the hypothesis that increasing doses of MPA reduce enteric methane production and yield without negative effects on energy and protein utilization in Dorper lambs. Therefore, the objective of this study was to evaluate the effects of MPA levels and compare them to a diet with MON on the intake, digestibility, nitrogen balance, gas exchange, enteric methane production, and yield and energy use in lambs.

MATERIAL AND METHODS

The study was performed at the Bioenergetics Laboratory of the Brazilian Agricultural Research Corporation (Embrapa), Coronel Pacheco, Minas Gerais, Brazil. All animal care and handling procedures were approved by the Embrapa Dairy Cattle Animal Care and Use Committee (Juiz de Fora, Minas Gerais, Brazil; Protocol CEUA-EGL 8762160316).

MPA preparation

Mature pods of *Prosopis juliflora* (SW) D.C. were collected during the dry season from mature trees (10–15 years old). The plantation was established in a private farm located in Brumado (14° 12′ 13″ S, 41° 39′ 55″ W) – a zone classified to have a semi-arid climate, with an average annual rainfall of 642.6 mm. The whole pods were manually harvested in July 2014, sun dried for 3 days and processed in a mill (Wiley mill, AH Thomas, Philadelphia, PA, USA) using a 1 mm mesh screen. The whole pod meal was macerated with 99.5% ethanol over 72 h in a sealed container. The macerate was then percolated and the extracted solution was concentrated in a vacuum evaporator (Rotary Fisatom Evaporator – model 802; São Paulo, Brazil) at −600 mmHg and a controlled temperature of 40 °C to obtain the crude ethanol extract (CEE). The CEE was partitioned using acid–base solution and organic solvents according to the methodology of Ott-Longoni *et al*.[18]

Part of the CEE (100 g) was subsequently solubilized in 1.6 mol L^{-1} acetic acid aqueous solution (AcOH, 200 mL) and the resulting solution was filtered to obtain acidic aqueous solution I (AAS-I). The AAS-I was extracted with chloroform ($CHCl_3$) in two successive 150 mL washes, thereby obtaining acidic aqueous solution II (AAS-II). The AAS-II was alkalized with sodium hydroxide (NaOH) to pH 9.0, and called basic aqueous solution I (BAS-I). The BAS-I was triple-washed with 100 mL $CHCl_3$, obtaining basic chloroform fraction I (BCF-I). The BCF-I was subjected to double washing with sodium chloride solution (NaCl), resulting in basic chloroform fraction II (BCF-II), which was subsequently dehydrated with 5 g sodium sulfate (Na_2SO_4), homogenized and allowed to stand for 2 h.

Next, the BCF-II containing the piperidine alkaloids was filtered using cotton wool to remove sodium sulfate and transferred to a round-bottom flask. The chloroform was evaporated on a rotary evaporator at reduced pressure and temperature of 45 °C to produce the solid basic chloroform extract (BCE) of piperidine alkaloids from *Prosopis juliflora*.[3] The BCE was analysed at the Analytical Instrumentation Centre of the University of São Paulo by high-resolution electron spray ionization mass spectrometry (Amazon Speed ETD, Bruker, Billerica, MA, EUA) and high-performance liquid chromatography (Shimadzu, Kyoto, Japan). The alkaloids found were juliprosopine as the major constituent, and juliprosinine, prosopinine and julifloridine as the minor constituents (Table 1).

Animals, experimental design and diet

Five Dorper lambs – intact males, with an approximate age of 4 months and body weight (BW) at the beginning of the experiment of 25 ± 2.0 kg – were used. The animals were randomly distributed in a 5 × 5 Latin square design. They were numbered, dewormed and allocated individually to 1.0 × 0.8 m (0.8 m^2) metabolic cages provided with individual feeders and drinkers. The experiment lasted 115 days, consisting of five periods of 23 days each (16 days were used for adaptation to the diet and 7 days were used for sample collection).

The feed additives used were sodium monensin (MON; Rumensin, Elanco Animal Health, Indianapolis, IN; 100 g kg^{-1} dry matter (DM)) and enriched mesquite piperidine alkaloid extract from *Prosopis juliflora* (Sw.) D.C. pods (MPA).

Treatments consisted of five diets as follows: MON 2.8 mg kg^{-1} diet DM (positive control), without additive (MPA 0, negative control) and levels of MPA 6.6, 17.3 and 27.8 mg kg^{-1} diet DM. The diet was formulated according to NRC[19] for a hypothetical average daily gain (ADG) of 0.2 kg, consisting of Tifton 85 hay (400 g kg^{-1} DM) and concentrate (600 g kg^{-1} DM) (Table 2). The Tifton 85 hay was chopped to a particle size of 5 cm in a forage grinder. The mixture of concentrate and hay was supplied for ad libitum intake, once a day at 0700 h, to allow a residual feed of 15%. The animals had free access to water, which was supplied in drinking troughs, cleaned daily.

Table 1. Identified piperidine alkaloids in the extract from *Prosopis juliflora* pods

Compound	Molecular formula for compound	Measured mass for $[M + H]^+$	Relative abundance (%) of peak $[M + H]^+$
Juliprosinine	$C_{40}H_{71}N_3O_2$	626.53	38
Juliprosopine	$C_{40}H_{75}N_3O_2$	630.53	15
Prosopinine	$C_{18}H_{35}NO_3$	314.31	100
Julifloridine	$C_{18}H_{37}NO_2$	300.22	100

$[M + H]^+$, molecular ion.

Respiration chamber design and operation

The respiration system adopted for chamber measurements was open circuit, based on Machado *et al.*[20] and adapted for small ruminants individually allocated in metabolic cages. The respiration system consisted of one chamber and one set of flow meter and analysers. The chamber (Intergado Ltda, Contagem, MG, Brazil) had a volume of 6.39 m^3 and was made from aluminium and transparent polyethylene terephthalate glycol (PETG) walls, thus enabling visual contact between animals.

One separate stream of ambient air was drawn through 75 mm diameter polyvinyl chloride (PVC) pipes from outside the shed and was connected to the chamber's fresh-air inlet in the front ceiling. Inside the chamber a fresh-air inlet presented a valve and a T-connection fitted with two horizontal PVC tubes (50 mm diameter × 1.34 m) punctured with 1 cm holes to avoid laminar flow. A mass flow meter continuously pulled air from the chamber, and a slight negative pressure inside the chamber was ensured. Air from the chamber and ambient air were analysed for one set of gas analysis over the measurement period, with the cycle time set to 10 min.

The chamber was fitted with an air outlet with a filter box (CSL-849-100HC, Solberg Manufacturing Inc., Itasca, IL, USA) in the rear section of the ceiling, and the air was continuously drawn out through a 75 mm PVC pipe, which was reduced to 51 mm diameter flexible polyurethane hose next to the analysis room, and then connected directly to a mass flow meter (Flow Kit model FK 430, Sable International Systems, Las Vegas, NV, USA) and a sealed rotary pump having a capacity range from 75 to 430 L min^{-1}. FC-10 oxygen, CA-10 carbon dioxide and MA-10 CH_4 analysers were used (Sable International Systems) to evaluate O_2, CO_2 and CH_4 concentrations.

Table 2. Ingredients and chemical composition of the experimental diet

Item	DM (g kg^{-1})		
Tifton 85 hay	400		
Corn	450		
Soybean meal	130		
Urea	05		
Mineral salt[a]	15		
Chemical composition	**Concentrate**	**Forage**	**Total diet**
DM (g kg^{-1} NM)	905	837	878
OM (g kg^{-1} DM)	859	779	827
CP (g kg^{-1} DM)	212	68	154
EE (g kg^{-1} DM)	33	19	27
NFC (g kg^{-1} DM)	614	97	407
NDF (g kg^{-1} DM)	95	748	356
GE (MJ kg^{-1} DM)	18.4	18.0	18.2

NM, natural matter; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NFC, non-fibrous carbohydrates; NDF, neutral detergent fibre corrected for ash and protein; GE, gross energy.
[a] 120 g Ca; 87 g P; 147 g Na; 18 g S; 590 mg Cu; 40 mg Co; 20 mg Cr; 1800 mg Fe; 80 mg I; 1300 mg Mn; 15 mg Se; 3800 mg Zn; 300.00 mg Mo; 870 mg F (max.); P solubility in citric acid at 2% (min.) - 95.00%.

Nutrient intake, total tract digestibility and nitrogen balance

During each experimental period, on days 17–23, concentrates, Tifton 85 hay and residual feed samples were taken. The intake of each animal was measured from the 17th to 23th day of each experimental period, calculated as the difference between the supplied feed (concentrate, Tifton 85 hay) and the residual feed. All samples were placed in plastic bags and frozen (−20 °C) for later analysis.

The samples of hay, concentrate (Table 2) and residual feed were collected daily over 5 days and pooled per animal and period for chemical analysis. During each experimental period, on days 17–21, apparent total digestibility of nutrients was obtained by total collection of faeces. The lambs were housed in metabolism cages and had faecal bags attached to them to ensure separate collection of urine and faeces. Total output of urine and faeces was measured every 24 h.

The faeces samples were weighed, dried in a forced-ventilation oven (55 °C) for 72 h and ground through a 1 mm screen (Wiley mill, AH Thomas). The N content was analysed in feed (NI), in faeces (FN) and in urine (UN) to evaluate nitrogen (N) balance. Digested N (DN) was calculated as the difference between NI and FN. Retained N (RN) was calculated by the difference between DN and UN output.

The samples were analysed for DM[21] (method 930.15), ash[21] (method 924.05), total nitrogen[21] (method 984.13), ether extract with petroleum ether[21] (method 920.39), non-fibre carbohydrate (NFC)[22] and neutral detergent fibre (NDF)[23] content with heat-stable amylase and without sodium sulfite, and corrected for residual ash and protein. Gross energy was determined using an adiabatic calorimeter (IKA-C5000, IKA Works, Staufen, Germany).

Gas exchange, methane production and yield, and energy use

The lambs were moved to a respiration chamber following the digestibility trial. They were housed in the open circuit respiration chambers for two 22 h periods and subjected to the same feeding regime as described above. The animals were weighed before and after entering the chamber. Daily O_2 consumption and CO_2 and CH_4 production were measured over 22 h with correction for the CO_2 and CH_4 recovery levels in the chamber and extrapolated for 24 h.

The lambs were kept attached to the faecal bags during the CH_4 measurement. Representative samples of feed, leftovers, urine and faeces were collected for gross energy analysis to assess energy partitioning. The CH_4 production (L d^{-1}) was converted to energy loss using the conversion factor 9.45 kcal L^{-1}. Heat production (HP, kcal day^{-1}) was calculated according to Brouwer:[24]

$$HP(\text{kcal d}^{-1}) = (3.866\times VO_2) + (1.200\times VCO_2) - (0.518\times VCH_4) - (1.431\times UN)$$

<NI>where VO_2 is volume of oxygen, VCH_4 is volume of methane, VCO_2 is volume of carbon dioxide (CO_2) (all in L d^{-1}) and UN is total urine nitrogen.

Energy balance (retained energy) was calculated by the difference using the following equations:

$$DEI = GEI - FE$$

<NI>where DEI is digestible energy intake (kcal d^{-1}), GEI is gross energy intake (kcal d^{-1}) and FE is faecal energy (kcal d^{-1});

$$MEI = DEI - UE - CH_4E$$

<NI>where MEI is metabolizable energy intake (kcal d^{-1}), DEI is digestible energy intake (kcal d^{-1}), UE is urinary energy (kcal d^{-1}) and CH_4E is energy loss as methane (kcal day^{-1});

$$EB \text{ or } RE = MEI - HP$$

<NI>where EB is energy balance (kcal d^{-1}), MEI is metabolizable energy intake (kcal d^{-1}) and HP is heat production (kcal d^{-1}).

After calculation, GEI, DEI, MEI, HP and RE were expressed as kJ kg^{-1} $BW^{0.75}$ by conversion. The FE, UE, CH_4E, HP and EB were also expressed as a percentage of GEI. The DE and ME contents of the experimental diet (MJ d^{-1}) were calculated by DEI and MEI divided by DMI, respectively. The metabolizability (q_m) of the total diet was calculated as MEI divided by GEI.[25]

Statistical analysis

The data were analysed as a 5 × 5 Latin square design using PROC GLM of SAS (SAS Institute, Cary, NC, USA), considering the period and animals as random effect.

The mathematical model used was

$$y_{ij(k)} = \mu + PER_i + ANI_j + \tau_{(k)} + \varepsilon_{ij(k)}; i,j,k = 1,\ldots,r$$

<NI>where $y_{ij(k)}$ is observation $ij(k)$, μ is the overall mean, PER_i is the effect of period i, ANI_j is the effect of animal j, $\tau(k)$ is the fixed effect of treatment k, $\varepsilon_{ij(k)}$ = random error with mean 0 and variance σ_2, and r is the number of treatments, period and animals.

MON was compared to the MPA levels by contrast (MON *vs.* MPA levels: 6.6, 17.3, 27.8 mg kg^{-1} DM). The effects of increasing MPA levels (0, 6.6, 17.3, 27.8 mg kg^{-1} DM) were evaluated by polynomial contrasts testing linear (L) and quadratic (Q) effects. The contrast coefficients were defined by SAS IML. Significance was declared at $P < 0.05$ and tendency at $P < 0.10$.

The regression mathematical model used was

$$Y_i = \beta_0 + \beta_1 x_i + \beta_2 x_{2i} + \varepsilon_i; i = 1,\ldots,n$$

<NI>where Y_i is observation i of dependent variable y, x_i = observation i of independent variable x, β_0, β_1, β_2 = regression parameters, and ε_i = random error.

RESULTS

Nutrient intake, total tract digestibility and nitrogen balance

It was observed that MPA levels did not affect ($P > 0.05$) the nutrient intake by lambs (Table 3). The diets with MPA showed similar intake and digestibility for most nutritional components ($P > 0.05$)

Table 3. Nutrient intake and coefficients of apparent digestibility of nutritional components by lambs fed with monensin (MON) or with levels of mesquite piperidine alkaloids (MPA)

	MPA (mg kg^{-1} DM)						*P*-value		
Item	MON	0	6.6	17.3	27.8	SEM	MON *vs.* MPA	L	Q
Intake (g d^{-1})									
OM	1082	1176	1104	1147	1125	34.96	0.30	0.53	0.67
DM	1151	1253	1171	1221	1163	36.95	0.46	0.58	0.73
CP	174	198	188	194	188	5.24	0.051	0.15	0.25
EE	40	34	28	31	34	1.01	0.094	0.14	0.23
NDF	397	437	400	415	421	12.39	0.32	0.93	0.62
NFC	483	510	490	513	487	15.16	0.52	0.44	0.99
Intake per day (g kg^{-1} BW)									
CP	4.63	4.96	4.77	4.94	4.72	0.08	0.24	0.38	0.77
Metabolizable energy intake per day									
MJ	13.7	14.3	13.7	14.4	14.9	0.12	0.34	0.25	0.42
MJ kg^{-1} BW	0.33	0.33	0.33	0.38	0.38	0.001	0.49	0.11	0.71
MJ kg^{-1} $BW^{0.75}$	0.88	0.88	0.84	0.92	0.92	0.003	0.41	0.11	0.59
Apparent digestibility (g kg^{-1})									
OMD	737	704	711	736	734	0.60	0.44	0.68	0.023
DMD	730	701	689	727	724	0.60	0.30	0.96	0.92
CPD	733	724	718	765	746	0.74	0.49	0.11	0.050
NDFD	571	535	520	553	568	1.25	0.20	0.45	0.034
NFCD	878	846	863	866	873	0.42	0.28	0.83	0.090
EED	677	660	644	657	716	1.40	0.90	0.63	0.60

SEM, standard error of the mean; L, linear; Q, quadratic; OM, organic matter; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre corrected for ash and protein; NFC, non-fibrous carbohydrates; MJ, megajoule; BW, body weight; $BW^{0.75}$, metabolic weight; OMD, organic matter digestibility; DMD, dry matter digestibility; CPD, crude protein digestibility; NDFD, neutral detergent fibre corrected for ash and protein digestibility; NFCD, non-fibrous carbohydrate digestibility; EED, ether extract digestibility.

compared to MON diet, with crude protein (CP) intake tending ($P < 0.10$) to be higher and ether extract (EE) intake lower for diets with MPA. For CP intake corrected for BW, MPA and MON did not differ ($P > 0.10$). The MPA levels did not affect ($P > 0.05$) the metabolizable energy intake (ME). However, there was a tendency of quadratic variation ($P < 0.10$) for the crude protein digestibility (CPD) and non-fibre carbohydrate digestibility (NFCD) with respective maximum points at MPA 22.0 and 27.0 mg kg^{-1} (Table 3). Organic matter digestibility (OMD) also showed quadratic variation ($P < 0.05$) with MPA levels. Neutral detergent fibre digestibility (NDFD) varied ($P < 0.05$) with MPA levels, showing a minimum point at 0.95 mg kg^{-1} MPA.

The nitrogen balance variables were similar between MON and MPA in the diets (Table 4). Levels of MPA did not affect the nitrogen intake (NI, g d^{-1}), but linearly decreased ($P < 0.05$) the faecal nitrogen excretion (FN, g d^{-1}) and showed a tendency to reduce ($P < 0.10$) the urine nitrogen excretion (UN). Digested nitrogen (DN) and retained nitrogen (RN) were not affected by MPA levels. However, the percentage of DN relative to ingested nitrogen (DN % of NI) tended ($P < 0.10$) to increase, while RN and NI ratio (RN % of NI) showed a linear increase ($P < 0.05$) with levels of MPA in the diets.

Gas exchange, enteric CH_4 production and yield, and energy use

There was no difference ($P > 0.05$) in O_2 consumption, and CO_2 production (L kg^{-1} $BW^{0.75}$) tended to increase ($P < 0.10$) in lambs fed MON diet compared to MPA levels (Table 5). CO_2 production linearly decreased ($P < 0.05$) with levels of MPA. The respiratory quotient (RQ) tended to be higher ($P < 0.10$) with MON compared to MPA in the diets and also decreasing ($P < 0.10$) with MPA levels. Heat production (HP) was similar between experimental diets. The methane yield (L kg^{-1} $BW^{0.75}$) linearly decreased ($P < 0.05$) with MPA levels, with a lower average ($P < 0.05$) compared to MON (Table 5).

Diets with MPA showed a trend towards lower ($P < 0.10$) methane production (g d^{-1}) and the daily CH_4 yield (g kg^{-1} BW) was lower ($P < 0.05$) for lambs fed MPA than MON. The MPA levels

Table 4. Nitrogen balance in lambs fed with monensin (MON) or with levels of mesquite piperidine alkaloids (MPA)

	MPA (mg kg^{-1} DM)						*P*-value		
Item	MON	0	6.6	17.3	27.8	SEM	MON *vs.* MPA	L	Q
(g d^{-1})									
NI	28.9	32.0	29.8	31.3	30.3	1.13	0.15	0.47	0.64
FN	7.22	8.52	8.24	7.11	7.40	0.27	0.42	0.020	0.23
UN	8.06	10.8	8.52	9.59	8.21	0.53	0.40	0.076	0.67
DN	21.3	23.2	21.6	24.0	22.7	0.95	0.15	0.71	0.84
RN	13.7	12.7	13.0	14.6	14.7	0.75	0.75	0.16	0.76
(% of NI)									
RN/NI	47.6	39.8	42.7	45.9	48.7	1.51	0.61	0.047	0.84
DN/NI	73.3	72.4	71.8	76.5	74.6	0.74	0.49	0.062	0.32

SEM, standard error of the mean; L, linear; Q, quadratic; NI, nitrogen intake; FN, faecal nitrogen; UN, urine nitrogen; DN, digested nitrogen; RN, retained nitrogen; RN/NI, ratio of retained nitrogen to ingested nitrogen; DN/NI, ratio of digested nitrogen to ingested nitrogen.

Table 5. Gas exchange, respiratory quotient, heat production and methane production by lambs fed with monensin (MON) or with levels of mesquite piperidine alkaloids (MPA)

	MPA (mg kg^{-1} DM)						*P*-value		
Item	MON	0	6.6	17.3	27.8	SEM	MON *vs.* MPA	L	Q
Gas exchange (L kg^{-1} $BW^{0.75}$)									
O_2	32.3	33.1	32.1	32.6	32.4	0.50	0.94	0.81	0.94
CO_2	38.4	38.5	37.2	36.8	37.1	0.47	0.061	0.048	0.39
CO_2 production/O_2 consumption									
RQ	1.19	1.17	1.17	1.13	1.15	0.01	0.064	0.058	0.28
Heat production (kJ kg^{-1} $BW^{0.75}$)									
HP	169.0	172.3	166.8	168.6	168.1	2.36	0.64	0.55	0.91
CH_4 yield (L kg^{-1} $BW^{0.75}$)									
CH_4	2.40	2.31	2.27	2.15	2.02	0.06	0.002	<0.001	0.57

SEM, standard error of the mean; L, linear; Q, quadratic; $BW^{0.75}$, metabolic body weight; RQ, respiratory coefficient.

Table 6. Enteric methane by lambs fed with monensin (MON) or with levels of mesquite piperidine alkaloids (MPA)

	MPA (mg kg^{-1} DM)					*P*-value		
Item	MON	0	6.6	17.3	27.8	SEM	MON *vs.* MPA	L	Q
Daily CH_4 production									
g day^{-1}	23.6	23.9	23.2	21.4	20.4	0.56	0.055	<0.001	0.99
Daily CH_4 yield (g)/*Body weight* (kg)									
g kg^{-1}	0.62	0.58	0.58	0.55	0.51	0.02	<0.001	<0.001	0.35
Daily CH_4 yield (g)/*Nutrient intake* (kg)									
DM	21.9	19.7	19.7	19.9	17.0	0.53	<0.001	<0.001	0.015
OM	26.4	24.0	23.8	24.0	20.5	0.63	<0.001	<0.001	0.024
NDF	61.0	54.9	55.8	57.0	46.6	1.50	<0.001	<0.001	0.001
NFC	53.3	49.1	47.9	48.1	42.7	1.26	<0.001	<0.001	0.14
Daily CH_4 yield (g)/*Digested nutrient* (kg)									
DM	30.0	28.0	28.6	27.4	23.5	0.73	0.002	<0.001	0.016
OM	35.8	34.1	33.5	32.6	27.9	0.88	0.001	<0.001	0.065
NDF	106.9	102.6	107.4	103.1	82.0	2.77	0.026	<0.001	<0.001
NFC	60.8	58.0	55.5	55.5	48.9	1.46	0.001	<0.001	0.22

SEM, standard error of the mean; L, linear; Q, quadratic; DM, dry matter; OM, organic matter; NDF, neutral detergent fibre corrected for ash and protein; NFC, non-fibre carbohydrates.

Table 7. Energy use by lambs fed with monensin (MON) or with levels of mesquite piperidine alkaloids (MPA)

	MPA (mg kg^{-1} DM)						*P*-value		
Item	MON	0	6.6	17.3	27.8	SEM	MON *vs.* MPA	L	Q
Gross energy intake and energy losses (MJ d^{-1})									
GEI	21.4	23.2	22.0	22.4	22.6	0.64	0.23	0.73	0.30
FE	5.98	7.16	6.63	6.33	6.18	0.18	0.20	0.018	0.35
CH_4E	1.41	1.37	1.32	1.32	1.15	0.05	0.069	0.034	0.40
UE	0.29	0.40	0.32	0.35	0.37	0.02	0.33	0.92	0.39
ME	13.7	14.3	13.7	14.4	14.9	0.49	0.34	0.25	0.42
HP	11.2	11.5	11.0	11.1	11.1	0.38	0.65	0.60	0.52
Digestible energy									
MJ d^{-1}	15.4	16.1	15.3	16.0	16.4	0.51	0.43	0.40	0.45
% of GEI	72.0	69.0	69.5	71.5	72.5	0.56	0.51	0.018	0.89
Energy losses (% of GEI)									
FE	28.0	31.0	30.5	28.5	27.5	0.56	0.51	0.018	0.89
CH_4E	6.62	6.00	6.03	6.10	5.10	0.23	0.020	0.049	0.11
UE	1.35	1.72	1.50	1.52	1.65	0.10	0.41	0.90	0.43
HP	52.2	49.5	50.2	49.7	48.9	0.92	0.23	0.74	0.72
Energy use									
ME/DE	0.90	0.89	0.89	0.89	0.91	0.01	0.29	0.046	0.48
ME/GE	0.64	0.61	0.62	0.64	0.66	0.01	0.92	0.010	0.87
EB	2.47	2.82	2.73	3.25	3.80	0.27	0.16	0.097	0.63

SEM, standard error of the mean; L, linear; Q, quadratic; GEI, gross energy intake; FE; energy losses in faeces; CH_4E, energy lost as methane; UE, energy lost in urine; ME, metabolizable energy; HP, energy lost as heat production; MJ, megajoule; ME/DE; ratio of metabolizable energy to digestible energy; ME/GE, metabolizability (q_m); EB, energy balance (MJ d^{-1}).

reduced ($P < 0.05$) the daily methane yield (Table 6). The daily CH_4 yield (g kg^{-1} of ingested and digested dry matter, organic matter, neutral detergent fibre and non-fibre carbohydrates) was lower ($P < 0.05$) for lambs fed MPA than MON. This represents an average decrease of 12% in g kg^{-1} of ingested and digested nutritional component in comparison to MON. The daily CH_4 yield from nutrients ingested and digested linearly decreased ($P < 0.05$) with MPA levels.

In parameters of energy use, the only difference ($P < 0.05$) between MPA and MON was evidenced for energy loss as methane from gross energy intake (CH_4E % of GEI), where MPA showed a lower ($P < 0.05$) average value than MON (Table 7). This represents a decrease of 13% in gross energy lost as methane. Consistently, there was a linear decrease ($P < 0.05$) for energy lost as methane (MJ d^{-1} and % of GEI) with levels of MPA levels added to diet, and MPA showed a trend ($P < 0.10$) towards lower energy loss as methane than MON.

The faecal energy loss (FE, MJ d^{-1}) and FE expressed as a percentage of GEI linearly decreased ($P < 0.05$) with MPA levels. The digestible energy (DE) as a percentage of GEI showed a linear increase ($P < 0.05$) with MPA levels.

DISCUSSION

Nutrient intake, total tract digestibility and nitrogen balance

Considering that all diets presented the same roughage and concentrate ratio and the intake was not affected, the doses of MPA promoted changes in digestion. The diets with MPA showed similar digestibility compared with the MON diet. However, increased CP, NDF and NFC digestibility at levels higher than 6.6 mg kg^{-1} MPA contributed to variation in OM digestibility, because these constitute the organic fraction of the ration.

The dose dependence of MPA on CP, NDF and NFC digestibility allows us to infer that possibly MPA altered the core microbiome composition in the rumen, in which resistant species were able to grow in the presence of higher doses, similar to the effects of MON.[2,26,27] MON acts by selecting proteolytic and fibrolytic bacteria[2,3,6] and, depending on the concentration, inhibits hyperammonia-producing bacteria.[2,27-29] Possibly, the populations of rumen selected microbes fermented less amino acid and/or were more efficient in nitrogen and energy utilization in the rumen,[2,30-32] because the urine nitrogen excretion tended to reduce and a greater proportion of dietary nitrogen was retained with the use of MPA without differing from the diet with MON.

The high amino acid fermentation rate usually presents low efficiency of dietary protein use due to increased ammonia absorption through the rumen and, as a consequence, increased excretion of nitrogen (N) in urine. The trend towards reduced urinary N excretion and the decrease in faecal N excretion indicated improved N utilization in the gut, proven by the increase in N retention proportional to the ingested N.

The importance of using an additive that reduces the deamination rate of dietary protein in the rumen is the possibility of providing more absorbed amino acid through the intestine for body metabolism. This may contribute to a decrease in the amount of nitrogen excreted in the environment[14] and in the metabolic energy expenditure for urea formation.

Gas exchange, enteric CH_4 production and yield, and energy use

It is possible that O_2 consumption was not affected due to the similarity of dry matter intake by the lambs. The unchanged RQ was expected as the sheep in the current study were fed ad libitum and feed intake was not affected.[33,34] An RQ greater than 1 is usually reported in trials with ruminants, as shown by Nichols *et al.*[35] and Morris and Kononoff.[36] Although the oxidation of lipids, protein and carbohydrates results in an RQ of 0.71, 0.81 and 1.00, our results greater than 1 can be explained by lipid synthesis that results in an RQ greater than 1.[37] Also, the RQ above 1.0 (apparent RQ) has been justified due to the rumen fermentative process and energy obtained from anaerobic metabolism. Additionally, the meta-analysis study of Aubry and Yan[38] showed a physiological range for RQ from 0.7 to 1.2 (average of 1.04), so our results are in agreement with this range for ruminants.

The rapid degradation of carbohydrates by rumen microbes is the major pathway for release of CO_2.[39] Possibly the decrease in CO_2 production by MPA would be a consequence of rumen fermentation modification, since the RQ tended to reduce and the CH_4 production decreased. This linear decreasing trend for RQ may indicate an increase in gluconeogenesis from rumen propionic acid to support protein accretion.[33] Such effects could predominate because MPA doses showed less energy released as CH_4, a tendency for reduced urinary nitrogen excretion, and an increase in the proportion of nitrogen retained relative to nitrogen ingested and in metabolizability.[33,40,41] It can be inferred that MPA could adjust the CO_2 and CH_4 emission in the rumen and improve the energy and protein utilization in sheep.

A positive relationship between CO_2 and CH_4 in the current study agreed with those reported by Zheng *et al.*[34] and Patra and Yu.[42] However, some studies have shown that there was a negative relationship, because CO_2 and H_2 are, in general, the precursors for CH_4 formation in the rumen.[43,44]

The reduced methane production by MON can be a consequence of activity against bacteria providing carbon dioxide, formate, methyl-containing compounds and acetate, or to an increase in bacteria species that compete for hydrogen or a decrease in hydrogen production through the inhibition of protozoa.[2,45,46] Carbon dioxide utilization to produce methane using H_2 as an energy source may be the main process carried out by rumen methanogen.[47-51] Therefore, it seems likely that MPA might be an inhibitor of methanogen because CO_2 production reduced with MPA dose, and CH_4 production was lower for MPA compared to MON. However, CO_2 production did not differ significantly from that of MON.

Conversely, substrate limitation of hydrogenotrophic methanogenesis must always be caused by a lack of the electron donor H_2. A strategy employing inhibitors of methanogenesis to redirect [H] sinks should evaluate possible direct effects of the inhibitors on non-methanogenic rumen microorganisms, so as to avoid affecting processes such as fibre digestion or propionate production. The amount of hydrogen generated in the rumen is directly influenced by the volatile fatty acid (VFA) pattern of fermentation.[52-54]

Methane production corrected for DM, OM and NDF digested and ingested is essential, given that the enteric methane production is directly related to the quantity and quality of the ingested feed.[50] The CH_4 yield relative to ingested and digested DM, OM and NDF showed lower values for MPA diets.

The increased digestion of fibrous fraction by MPA did not generate methane. This is consistent with the results reported by Pereira *et al.*[6] in an *in vitro* assay using wheat bran and Tifton 85 hay with MPA (260 and 390 mg m^{-3}) and MON (110 mg m^{-3}), which showed a decrease of 58% in CH_4 production with MPA compared to MON at 24 h incubation, as well as shorter lag time and higher

gas production from the fibrous fraction for MPA. Santos *et al.*[3] reported that MPA reduced *in vitro* gas production during ruminal fermentation without affecting the degradability of the DM of wheat bran.

Among the evaluated additives, energy lost as heat (HP) represented the main method of energy loss, making up 50% of the gross energy intake (GEI). Loss as HP was followed by the energy loss in faeces (FE; 29% of GEI), energy loss as CH_4 (CH_4E; 5.96% of GEI) and energy loss in urine (UE; 1.51% of GEI). Therefore, the levels of MPA showed decreases of faeces energy loss and of enteric methane as a consequence of improved dietary energy utilization during digestion (DE % of GEI), mainly from NDF and CP.

The energy loss observed in faeces (% of GEI) is close to 27%, as reported by Arndt *et al.*[55] According to Blaxter,[56] an average of 10–70% of lost energy in faeces (% of GEI) is observed in the diets normally offered to ruminants.

Values obtained in the respiration chamber for CH_4 emissions of 2–12% GE have been reported for several diets.[57] In this study, the enteric CH_4 varied from 5% to 6% of GEI, respectively, for the diet with MPA 27.8 mg kg^{-1} and the diet without additive.

The energy loss in urine cannot be higher than 5% of GE[58] and is relatively constant,[59] which is consistent with the values found for UE (1.55% GE). Possibly, MPA acted to improve the synchronization between carbohydrate fermentation and protein degradation in the rumen. The determining factors of nitrogen utilization efficiency in the rumen are the supply of fermentable carbohydrates and the modification of protein degradation rate.[60]

Several actions of MON contribute to increase the energy availability in the animal because it alters rumen microbiota, increasing propionic acid production and reducing loss of methane in the rumen.[2,61] Consistently, Pereira *et al.*[6] reported promising results with MPA during *in vitro* ruminal fermentation, such as higher propionate concentration and lower methane yield with MPA, which depended on its dose, fermentation time and food type, compared to MON, in the medium with rumen fluid.[2,8]

In the present study, the decrease in enteric CH_4 production with MPA levels contributed to increase the metabolizable energy from the digested energy.

The non-difference for EB is probably a result of similarities observed with both additives for energy loss in urine and HP, since it represents a large fraction of the energy balance (EB). Despite the EB not differing, the increasing levels of MPA provided a lower gross energy loss as methane. Thus, this corroborates the hypothesis that MPA has the potential to improve energy use in the rumen.

CONCLUSIONS

Enriched mesquite piperidine alkaloid extract (MPA) ranging from 17.3 to 27.6 mg kg^{-1} of DM in the diet increases the fibre digestion as well as the proportion of digestible energy from the ingested gross energy and metabolizability. MPA ranging from 6.6 to 27.8 mg kg^{-1} reduces the enteric CH_4 production and yield and improves the energy and protein utilization in lambs. The findings point to the potential use of MPA as an alternative additive for ruminants. To achieve a better description of the piperidine alkaloid-rich extract, additional studies on rumen metabolism and microbial diversity are required.

ACKNOWLEDGEMENTS

The results from this study are part of the results of the projects 'PECUS – Rumen Gases' and 'Precision Nutrition', Embrapa (Brazilian Agricultural Research Corporation). The authors gratefully acknowledge the funding support from FAPESB (Bahia State Research Support Foundation), CNPq (National Council for Scientific and Technological Development, Brasília, Brazil), Embrapa and UESB (State University of Southwest of Bahia).

The first author gratefully acknowledges CNPq for the master scholarship; authors A.L.F and D.B.O. acknowledge CAPES for the postdoctoral scholarship and CNPq scholarship holder (313110/2014-0) of the corresponding author. The authors would like to thank Research Support Foundation of the State of Bahia (FAPESB, PET Number 0013/2013) for provision of financial support.

CONFLICT OF INTEREST

There is no conflicts of interest.

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