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Moringa oleifera leaf meal as an environmental friendly protein source for ruminants: Biomethane and carbon dioxide production, and fermentation characteristics

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ABSTRACT

Ruminal fermentation produces methane (CH_4) and carbon dioxide (CO_2) which contribute to global warming. Therefore, several enteric CH_4 and CO_2 mitigation strategies have been explored recently. In this trial the effect of replacing soybean meal, as the sole protein source in a control total mixed ration (TMR) with *Moringa oleifera* leaf meal (MLM) at different levels, on ruminal fermentation characteristics were studied. *M. oleifera* leaf meal replaced (g/100 g DM): 0 (TMR0, control), 10 (TMR10), 20 (TMR20), 30 (TMR30), 40 (TMR40), 50 (TMR50), 60 (TMR60), 70 (TMR70), 80 (TMR80), 90 (TMR90), and 100 (TMR100) of soybean meal in the rations. Rations were incubated for 48 h using rumen inoculums from goats and steers. Some interactions between inoculum × TMR were observed ($P < 0.05$) for gas production (GP) parameters, CH_4 production, and fermentation profile. Moreover, most parameters determined responded differently between animal species. Rations containing MLM decreased the asymptotic GP ($P < 0.01$), while they increased ($P < 0.01$) the rate of GP and lag of GP with both inoculums. Decreased ($P < 0.05$) CH_4 production and increased CO_2 production ($P < 0.05$) were observed when MLM replaced soybean meal. Diets containing MLM decreased ($P < 0.05$) ruminal ammonia-N and total protozoal number, while increasing ($P < 0.05$) total bacterial number with both goat and steer inoculums. Replacing soybean meal with MLM increased ($P < 0.05$) fermentation pH, but decreased ($P < 0.05$) organic matter degradability (OMD) with goat inoculum. Conversely, a declined ($P < 0.05$) in SCFA concentrations, and enhanced ($P < 0.05$) OMD and DM degradability compared with the control diet was observed with diets containing MLM. It is concluded that replacing soybean meal in goat and steer diets negatively affected the nutritive value of diets but decreased CH_4 production. From an environmental standpoint, the replacement of soybean meal with MLM is a potential sustainable strategy to reduce CH_4 production from goats and steers, and thus mitigate greenhouse gas emissions. Goat inoculum was more efficient in reducing CH_4 production than that of steers.

1. Introduction

There is a growing global concern for the environmental impact of greenhouse gases (methane, carbon dioxide and nitrous oxide) emissions by ruminant production systems. Estimates of global production of enteric methane from ruminants are about 80,000 Gg (Ku-Vera et al., 2013). Increasing future feedstuffs demand for livestock and food-feed-fuel competition have environmental and social impacts (Gopalakrishnan et al., 2016; Makkar, 2016). The growing demand of livestock products is accompanied by social, economic and environmental challenges (Makkar, 2016). During ruminal fermentation of feeds, large amounts of greenhouse gases are produced making livestock one of most important greenhouse gases producers. Food and Agriculture Organization (FAO, 2006)

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reported that the livestock sector accounts for about 18% of methane (CH_4) and 9% of carbon dioxide (CO_2) productions. Enteric CH_4, a product of ruminal anaerobic fermentation produced by methanogenic archaea during disposal of metabolic H_2 produced by their metabolic activity, contributes 30–40% of the total CH_4 production from agricultural sources (Moss et al., 2000). Many attempts have been made to mitigate CH_4 emission from ruminants including the inclusion of yeast (Elghandour et al., 2017), organic acids salt (Elghandour et al., 2016), exogenous enzymes (Kholif et al., 2017a), and essential oils (Hernandez et al., 2017), with promising results.

Animal producers face a series of problems; one of them is the availability and high price of concentrates, in particular protein sources, which compel nutritionists to seek for less-expensive alternative protein feeds (Kholif et al., 2015). Tree leaves have been used to ameliorate this problem. *Moringa oleifera* Lam (syns. *Moringa pterygosperm* family *Moringaceae*) is a fodder tree growing almost worldwide and yields a high amount of biomass ranging from 43 to 115 tons per hectare (Safwat et al., 2014), with a high protein content. Kholif et al. (2015) reported the chemical composition as a protein feed containing (/kg DM) 241–277 g crude protein (CP), with about 47% of bypass protein (Becker, 1995), adequate amino acid profile (Sánchez-Machado et al., 2010) and polyphenolics contents as antioxidant (Nouman et al., 2016). *M. oleifera* is a cheaper protein ingredient than most traditional protein feeds such as sesame and soybean meal (Kholif et al., 2015). However, like other fodder trees, *M. oleifera* contains secondary metabolites (Kholif et al., 2015). Plants having bioactive products such as essential oils, saponins, and condensed tannins (Guglielmelli et al., 2011; Calabrò et al., 2011) with antimicrobial properties may be exploited in ruminant production to reduce CH_4 emissions and improve fermentation efficiency. Little information about MLM as a protein source for ruminants is available; however, recent experiments that included MLM as a protein feed are gaining increasing interests, with promising results such as enhanced feed utilization and milk production from goats (Kholif et al., 2015). Unfortunately, they did not study the effect of replacing soybean meal with MLM as a protein source on greenhouse gases production using inoculums from two different livestock species. Therefore, the present study aimed to evaluate the effects of replacing soybean meal at different levels with MLM in diets for ruminants, as a "clean" feed for the environment, on the sustainable mitigation of CH_4 and CO_2 production, ruminal fermentation profile, and CH_4-producing protozoa and bacteria using rumen inoculums from goats and steers.

2. Materials and methods

2.1. Substrate and treatments

M. oleifera leaf meal was prepared as previously described by Kholif et al. (2015). Briefly, *M. oleifera* leaves and small twigs were harvested at 40 d of age. *M. oleifera* was air-dried at 60 °C for 48 h, and then kept for further use. Total tannin concentration of *M. oleifera* leaves was determined according to Makkar (2003), and total phenolic content was determined chromatographically as described by Meier et al. (1988). *M. oleifera* contained 22 g/kg DM total tannins and 48 g/kg DM total phenolic contents. A total mixed ration (TMR) was prepared, as a substrate containing (/kg DM) 400 g alfalfa hay (*Medicago sativa*), 250 g crushed yellow corn, 250 g soybean meal, and 100 g wheat bran, and considered as a control. Rations were balanced for minerals and vitamins contents. In the basal TMR, dried MLM replaced soybean meal at (/100 g DM): 0 g (TMR0, control), 10 g (TMR10), 20 g (TMR20), 30 g (TMR30), 40 g (TMR40), 50 g (TMR50), 60 g (TMR60), 70 g (TMR70), 80 g (TMR80), 90 g (TMR90), and 100 g (TMR100). The chemical composition of ingredients and TMRs used is shown in Table 1.

2.2. In vitro fermentation and biogases production

Rumen inoculums were collected from two ruminally cannulated Holstein steers (450 ± 20 kg LW), and two cannulated Creole goats (50 ± 2 kg LW), housed in individual pens and fed *ad libitum* on a diet consisting of oat hay and concentrate (PURINA®, Toluca, Mexico) at 60:40 ratio. Animals were fed twice daily at 08:00 and 16:00 h, and managed under the conditions stipulated in the Official Mexican Standard of technical specifications for the production, care and use of laboratory animals (NOM-062-ZOO-1999). Rumen contents were placed in a plastic thermo preheated at 39 °C. They were flushed with CO_2, mixed and strained through four layers of cheesecloth into a flask with O_2-free headspace, and maintained at a constant temperature of 39 °C and continuous CO_2 flow.

Before the incubation process, incubation media was prepared according to Goering and Van Soest (1970), mixed in a volumetric flask using a platen and magnetic stirrer at 39 °C to maintain the temperature and homogenize the solution. Then, the ruminal inoculum and the reducing solution were added at 1:4 vol/vol, respectively.

Samples (0.5 g) of the substrate were weighed into 120 mL serum bottles, and 50 mL of previously prepared rumen liquor and the buffer solution were added. Bottles were maintained under a constant CO_2 flow for 30 s, capped with neoprene plugs and sealed with aluminum rings. The vials were placed in an incubator (Riossa®, F-51 D, Mexico State, Mexico) at 39 °C for 48 h. Moreover, three bottles as blanks (rumen fluid only) were incubated for 48 h. Three incubation runs were performed in three weeks.

The total gas production (GP) readings were taken after 2, 4, 6, 8, 10, 12, 24 and 48 h of incubation. A water displacement apparatus was used according to Fedorak and Hrudey (1983). The vials were punctured with a 16-gauge needle placed at the end of the hose. The GP (mL) was measured by the displacement of water in the burette.

After 48 h incubation, 5 mL of gas was taken and stored in the vials containing saturated saline solution prepared with 400 g of NaCl in 1 L of distilled water, and the pH was adjusted at 2, and 5 mL of 20% methyl orange was added as indicator for CH_4 and CO_2 concentrations determination. The saturated saline solution was previously prepared and stored in 60 mL serological vials, leaving no space; and neoprene plugs were placed and sealed with aluminum rings, and stored away from light. For the CH_4 and CO_2 determination from vials, a 10 μL sample of the gas phase was taken and injected into a Perkin-Elmer, Claurus 500 gas chromatograph (Mexico City, Mexico) with a flame ionization detection, and helium as the carrier gas. A thermal conductivity detector was used; the oven, column and FID (A flame ionization detector) temperatures were 80 °C, 170 °C and 130 °C, respectively. Retention times were 0.73 min and 1.05 min for CH_4 and CO_2, respectively.

At the end of 48 h incubation period, the fermentation process was stopped by swirling the bottles in ice for 5 min. The bottles were subsequently uncapped and the pH was measured using a pH meter (Thermo Scientific, Orion Star™ A121, Beverly, MA, USA). The contents of the bottles were filtered in Ankom® Technologies F57 bags (at constant weight). The bottles were rinsed with a hot water 3 times to ensure recovery of all the fermentation residues. The bags were placed in a forced-air oven at 55 °C for 48 h. Dry matter degradation was calculated by considering the initial weight of the substrate and the weight of the residue.

After pH measurement and filtration, 4 mL of the medium were obtained with a syringe and mixed with 1 mL of 25% metaphosphoric acid, shaken slightly and placed under freezing for the

Table 1
Chemical composition[a] of ingredients and total mixed rations (TMR) with different levels of *Moringa oleifera* replacing soybean meal as a protein source.

	DM (g/kg wet material)	OM	CP	EE	NSC	NDF	ADF	ADL	Cellulose	Hemicellulose
Ingredients										
Alfalfa hay	902.0	885.8	189.2	25.3	218.9	452.4	330.1	82.1	248.0	122.3
M. oleifera hay	868.2	891.0	281.1	40.9	224.4	344.6	301.0	77.6	223.4	43.6
Crushed yellow corn	866.0	890.3	90.8	45.2	540.0	214.3	88.8	10.4	78.4	125.5
Soybean meal	889.0	927.9	408.1	21.4	355.7	142.7	96.3	8.8	87.5	46.4
Wheat bran	871.4	852.2	129.7	56.2	204.4	461.9	130.6	38.0	92.6	331.3
Total mixed rations[b]										
TMR0	886.7	894.1	213.4	32.4	331.9	316.4	191.4	41.4	149.9	125.0
TMR10	886.2	893.2	210.2	32.9	328.6	321.4	196.5	43.2	153.3	125.0
TMR20	885.7	892.2	207.0	33.4	325.4	326.5	201.6	44.9	156.7	124.9
TMR30	885.1	891.3	203.9	33.9	322.1	331.5	206.7	46.6	160.1	124.8
TMR40	884.6	890.4	200.7	34.3	318.8	336.6	211.8	48.3	163.5	124.7
TMR50	884.1	889.5	197.5	34.8	315.5	341.6	217.0	50.0	166.9	124.7
TMR60	883.6	888.6	194.3	35.3	312.2	346.7	222.1	51.8	170.3	124.6
TMR70	883.1	887.6	191.2	35.8	308.9	351.7	227.2	53.5	173.7	124.5
TMR80	882.5	886.7	188.0	36.3	305.7	356.8	232.3	55.2	177.1	124.5
TMR90	882.0	885.8	184.8	36.8	302.4	361.8	237.4	56.9	180.5	124.4
TMR100	881.5	884.9	181.6	37.3	299.1	366.9	242.6	58.6	183.9	124.3

[a] ADF, acid detergent fiber; CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fiber; NSC, non-structural carbohydrates; OM, organic matter.
[b] *Moringa oleifera* replaced 0 (TMR0), 10 (TMR10), 20 (TMR20), 30 (TMR30), 40 (TMR40), 50 (TMR50), 60 (TMR60), 70 (TMR70), 80 (TMR80), 90 (TMR90), and 100 (TMR100) g/100 g of soybean meal.

analysis of ammonia-N (NH_3-N) concentration. Other 4 mL of the medium were mixed with 1 mL 10% formaldehyde, shaken slightly and placed in refrigeration for bacterial and protozoal counting.

2.3. Total bacteria and protozoa counting

The population of total bacteria was determined after 48 h incubation using a count chamber bacterium Petroff-Hausser (Hausser Scientific®, 3900, Horsham, PA) and a phase contrast microscope (Olympus®, BX51, Mexico City, Mexico) at a magnification of 100×. Exactly, 0.5 mL of the 10% formaldehyde fixed medium sample was taken and diluted in 4.5 mL of distilled water. The bacteria concentration per mL was determined as the average of bacteria observed in each grid, multiplied by the dilution factor and the chamber factor (2×10^7), according to the following formula:

$$\text{Bacterial number/mL} = \mu \times FD1 \times FD2 \times 2^7 .$$

where: μ is the average of bacteria in each grid per treatment, FD1 is the first dilution factor (1.25), and FD2 is the second dilution factor (10).

For the protozoal number determination, 1 mL of the 10% formaldehyde fixed sample was obtained and diluted in 1 mL of distilled water; then 0.5 mL of the mixture was taken with a Pasteur pipette (BRAND, 7712, Wertheim, Germany) which were deposited in a Neubauer chamber (BRAND, 7178-10, Wertheim, Germany) and subsequently observed under contrast-phase microscopy (Carl Zeiss®, Axiostar, Mexico City, Mexico) at 400× magnifications. The count of protozoa was made in eight quadrants (4 of each grid), taking as viable protozoans those that maintained their morphological integrity. Protozoa concentration per mL of culture medium was estimated as the average protozoa observed in each grid, multiplied by the dilution factor and the chamber factor (1×10^4), according to the following formula:

$$\text{Protozoal number} = \mu \times FD1 \times FD2 \times 10^4$$

where: = μ is the average of protozoa in each grid per treatment, FD1 is the first dilution factor (5), and FD2 is the second dilution factor (3).

2.4. Chemical analyses

Samples of diets were analyzed for DM, ash, N and EE according to AOAC (1997), while the neutral detergent fiber (NDF) (Van Soest et al., 1991), and acid detergent fiber (ADF) and lignin (AOAC, 1997; #973.18) analyses were carried out using an ANKOM^{200} Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA) with the use of an alpha amylase and sodium sulfite.

The concentration of ruminal NH_3-N was determined according to Broderick and Kang (1980) methods. Samples of the incubation medium were centrifuged at 3000×*g* for 10 min, and 20 μL of the supernatant was mixed with 1 mL of phenol and 1 mL of hypochlorite, and the mixture was incubated at 39 °C for 30 min, after they were diluted with 5 mL of distilled water. Samples were read on a visible ultraviolet light spectrophotometer (Varian, model Cary 1E, California, USA) at 630 nm. The resulting g/L concentration was divided by 0.8 which was the 25% metaphosphoric acid dilution factor.

2.5. Calculations and statistical analyses

For estimation of GP kinetic, recorded gas volumes (mL/g DM) were fitted using the NLIN procedure of SAS (2002) according to the model of France et al. (2000):

$$y = b \times \left[1 - e^{-c(t-L)}\right] \quad (1)$$

where y is the volume of GP at time t (h); b is the asymptotic GP (mL/g DM); c is the fractional rate of fermentation (/h), and L (h) is the discrete lag time prior to the time any gas was released.

Metabolizable energy (ME, MJ/kg DM) and *in vitro* organic matter digestibility (OMD, g/kg DM) were estimated according to Menke et al. (1979) as:

$$ME = 2.20 + 0.136\ GP(mL/0.5\ g\ DM) + 0.057\ CP(g/kg\ DM) \quad (2)$$

$$OMD = 148.8 + 8.89\ GP + 4.5\ CP(g/kg\ DM) + 0.651\ ash(g/kg\ DM) \quad (3)$$

where GP is net GP in mL from 200 mg of dry sample after 24 h of

Table 2
Biogases production (mL/g DM) of total mixed rations (TMR)[a] containing different levels of *Moringa oleifera* leaf meal in substitution of soybean meal, and incubated with rumen liquors obtained from goats or steers.

Inoculum	TMR	Gas production parameters[b]				CO_2 production at 48 h of incubation			CH_4 production at 48 h of incubation		
		b	*c*	*Lag*	mL gas/g degraded DM	mL CO_2/g incubated DM	Proportional CO_2 production	mL CO_2/g degraded DM	mL CH_4/g incubated DM	Proportional CH_4 production	mL CH_4/g degraded DM
Goat	TMR0	288	0.091	1.39	369	200	70.4	315	61.4	21.6	53.7
	TMR10	291	0.096	1.91	376	235	81.7	321	42.1	14.6	55.1
	TMR20	257	0.108	2.62	336	208	81.5	282	41.5	16.3	54.4
	TMR30	235	0.106	2.77	309	201	86.1	256	30.9	13.2	52.7
	TMR40	267	0.109	2.53	357	212	79.7	291	49.3	18.5	66.3
	TMR50	280	0.116	2.56	381	223	80.0	311	51	18.3	69.5
	TMR60	262	0.106	2.46	343	202	77.5	278	49.7	19.1	65.5
	TMR70	268	0.118	2.48	365	214	80.1	299	48.3	18.1	66
	TMR80	269	0.124	2.54	356	216	80.4	291	49.3	18.3	65.2
	TMR90	260	0.113	2.38	360	205	79.2	289	51	19.7	70.9
	TMR100	264	0.122	2.52	363	206	78.1	292	51.7	19.6	71.2
SEM		4.3	0.0025	0.118	11.5	3.80	0.58	9.50	1.71	0.58	2.97
Linear		0.009	<0.001	<0.001	0.733	0.011	0.026	0.922	0.034	0.037	0.317
Quadratic		0.011	0.003	0.725	0.473	0.037	0.46	0.568	0.114	0.461	0.349
Steers	TMR0	322	0.080	0.95	421	247	78.3	363	67.9	21.5	58.7
	TMR10	341	0.091	1.55	427	272	80.8	358	54.3	16.1	68.9
	TMR20	315	0.109	2.50	390	259	82.8	323	53.5	17.1	66.6
	TMR30	327	0.087	1.13	417	258	80.1	347	53.5	16.6	69.4
	TMR40	335	0.089	1.55	436	264	80.0	362	56.1	17.0	74.2
	TMR50	310	0.107	2.52	393	256	83.2	327	51.6	16.8	65.9
	TMR60	311	0.090	1.58	403	248	80.7	332	54.3	17.7	71.4
	TMR70	331	0.093	0.89	431	260	79.5	346	60.6	18.5	85
	TMR80	322	0.110	2.23	410	256	80.0	329	61.4	19.2	81.3
	TMR90	315	0.098	1.84	417	253	81.2	338	57	18.3	79
	TMR100	314	0.099	1.91	368	250	80.3	296	60.4	19.4	72.7
SEM		6.6	0.0021	0.200	12.7	5.3	0.59	10.9	2.36	0.59	3.18
P value											
Linear		0.381	<0.001	0.003	0.007	0.012	0.532	0.011	0.507	0.532	0.032
Quadratic		0.011	0.652	0.615	0.051	0.800	0.528	0.074	0.016	0.528	0.084
Pooled SEM		5.6	0.0020	0.164	12.1	4.60	0.59	10.2	2.07	0.59	3.08
P value											
Inoculum		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TMR											
Linear		0.007	<0.001	<0.001	0.019	0.076	0.218	0.036	0.068	0.218	0.022
Quadratic		0.004	0.028	0.798	0.049	0.001	0.330	0.079	0.020	0.331	0.054
Inoculum × TMR		0.002	<0.001	<0.001	0.011	<0.001	0.996	0.008	0.623	0.995	0.541

[a] *Moringa oleifera* replaced 0 (TMR0), 10 (TMR10), 20 (TMR20), 30 (TMR30), 40 (TMR40), 50 (TMR50), 60 (TMR60), 70 (TMR70), 80 (TMR80), 90 (TMR90), and 100 (TMR100) g/100 g of soybean meal.
[b] *b* is the asymptotic gas production (mL/g DM); *c* is the rate of gas production (/h); *Lag* is the initial delay before gas production begins (h).

incubation.

The partitioning factor at 24 h incubation (PF_{24}, a measure of fermentation efficiency) was calculated according to Blümmel et al. (1997). Gas yield (GY_{24}) was calculated as the volume of gas (mL gas/g DM) produced after 24 h of incubation divided by the amount of DMD (g).

Short chain fatty acid concentrations (SCFA) were calculated according to Getachew et al. (2002) as:

$$\text{SCFA (mmol/200 mg DM)} = 0.0222\ \text{GP} - 0.00425 \quad (4)$$

where: GP is the 24 h net GP (mL/200 mg DM).

Microbial biomass production (MCP) was calculated (Blümmel et al., 1997) as:

$$\text{MCP(mg/g DM)} = \text{milligrams DMD} - (\text{milliliter gas} \times 2.2\ \text{mg/mL}) \quad (5)$$

Data of each of the three runs within the same sample of each of the three individual samples of rations were averaged prior to statistical analysis and the mean values of each individual sample were used as the experimental unit. The experimental design was a factorial arrangement with 3 replicates in a randomized complete block design. Data were analyzed using the GLM procedure (SAS, 2002) with the model: $Y_{ijk} = \mu + R_i + M_j + (R \times M)_{ij} + \varepsilon_{ijk}$ where: Y_{ijk} is the observation, μ is the population mean, R_i is the inoculum source effect, M_j is the level of MLM in the ration, $(R \times M)_{ij}$ is the interaction between MLM level and inoculum type, and ε_{ijk} is the residual error. Tukey test (pdiff adjust = tukey; SAS) was used to compare means. Statistical significance was declared at $P < 0.05$.

3. Results

3.1. Chemical composition

Replacing soybean meal with MLM gradually decreased DM, OM, CP, NSC, and hemicellulose, while it gradually increased EE, NDF, ADF, and cellulose contents of the rations (Table 1).

3.2. Biogases production

Inoculum type × TMR interactions were observed ($P < 0.05$) for GP parameters and CH_4 production (Table 2). Gas production parameters, and CH_4 and CO_2 productions differed ($P < 0.001$) between goat and steer rumen liquors. Moreover, replacing soybean

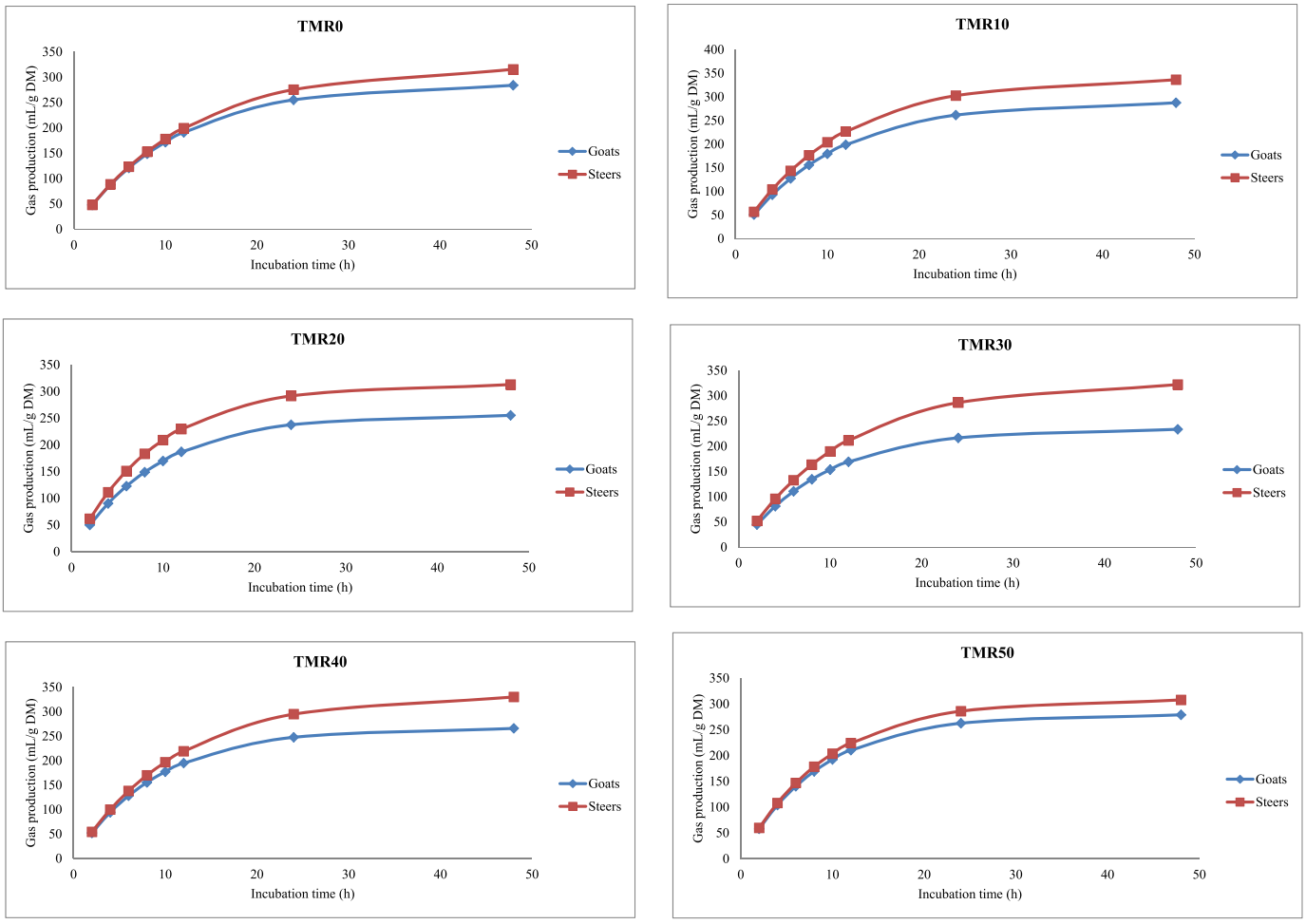

Fig. 1. *In vitro* gas production (mL/g DM) profiles of total mixed rations (TMR)[1] containing different levels of *Moringa oleifera* leaf meal replacing soybean meal, and incubated either with rumen liquors from goats or steers. *Moringa oleifera* soybean meal at (/100 g DM): 0 g (TMR0, control), 10 g (TMR10), 20 g (TMR20), 30 g (TMR30), 40 g (TMR40), 50 g (TMR50), 60 g (TMR60), 70 g (TMR70), 80 g (TMR80), 90 g (TMR90), and 100 g (TMR100).

meal with MLM affected GP parameters and CH_4 production.

With goat rumen inoculum, replacing soybean meal with MLM decreased the asymptotic GP (linear and quadratic effects; $P < 0.01$), but increased the rate of GP (linear and quadratic effects; $P < 0.01$) and lag of GP (linear effect; $P < 0.001$). On the other hand, excluding TMR10 and TMR40 treatments, MLM-containing rations quadratically decreased ($P = 0.011$) the asymptotic GP, and linearly increased ($P \leq 0.003$) the rate of GP and the lag time of GP (Fig. 1). However, a very strong relationship ($r^2 = 0.99$) between gas production of the total mixed rations containing *Moringa oleifera* leaf meal replacing soybean meal using goats and steers was observed (Fig. 2).

With rumen inoculum from goats, the inclusion of MLM decreased (linear and quadratic effects, $P < 0.05$) CH_4 production and the proportions of CH_4 production (linear effect, $P < 0.05$), while it increased (linear effect, $P < 0.05$) CO_2 production (Table 2). With steer inoculum, rations containing MLM quadratically decreased ($P = 0.016$) CH_4 production without affecting its proportion, but linearly increased ($P = 0.012$) CO_2 production (ml/g degraded DM).

3.3. Fermentation characteristics

Inoculum × TMR interactions were observed ($P < 0.05$) for SCFA, NH_3-N, OMD, ME, PF24, GY24 and MCP (Table 3). All fermentation parameters differed between steers and goats. The ration effect on these parameters was quadratic ($P < 0.01$).

With the goats' rumen liquor, MLM increased fermentation pH (6.42–6.46, linear effect, $P = 0.037$) and total ruminal bacteria (9.5×10^5 to 13.5×10^5, quadratic effect, $P = 0.045$), but decreased ruminal NH_3-N (linear and quadratic effects, $P < 0.05$), OMD (quadratic effect, $P = 0.033$), and total protozoa number (5.12×10^5 to 2.88×10^5, $P = 0.015$). The use of steer inoculum decreased SCFA concentrations (quadratic effect, $P = 0.005$), NH_3-N (linear and quadratic effects, $P < 0.001$), and total protozoa number (5.77×10^5 to 1.95×10^5, quadratic effect, $P = 0.027$) were observed when soybean meal was replaced with MLM. However, MLM containing rations increased DMD (linear effect, $P = 0.002$), OMD (quadratic effect, $P = 0.002$), ME (quadratic effect, $P = 0.002$), MCP (quadratic effect, $P = 0.005$), and total number of bacteria (linear effect, $P = 0.021$).

4. Discussion

4.1. Chemical composition

The observed changes in the chemical composition when MLM replaced soybean meal were expected. MLM contains a high fiber

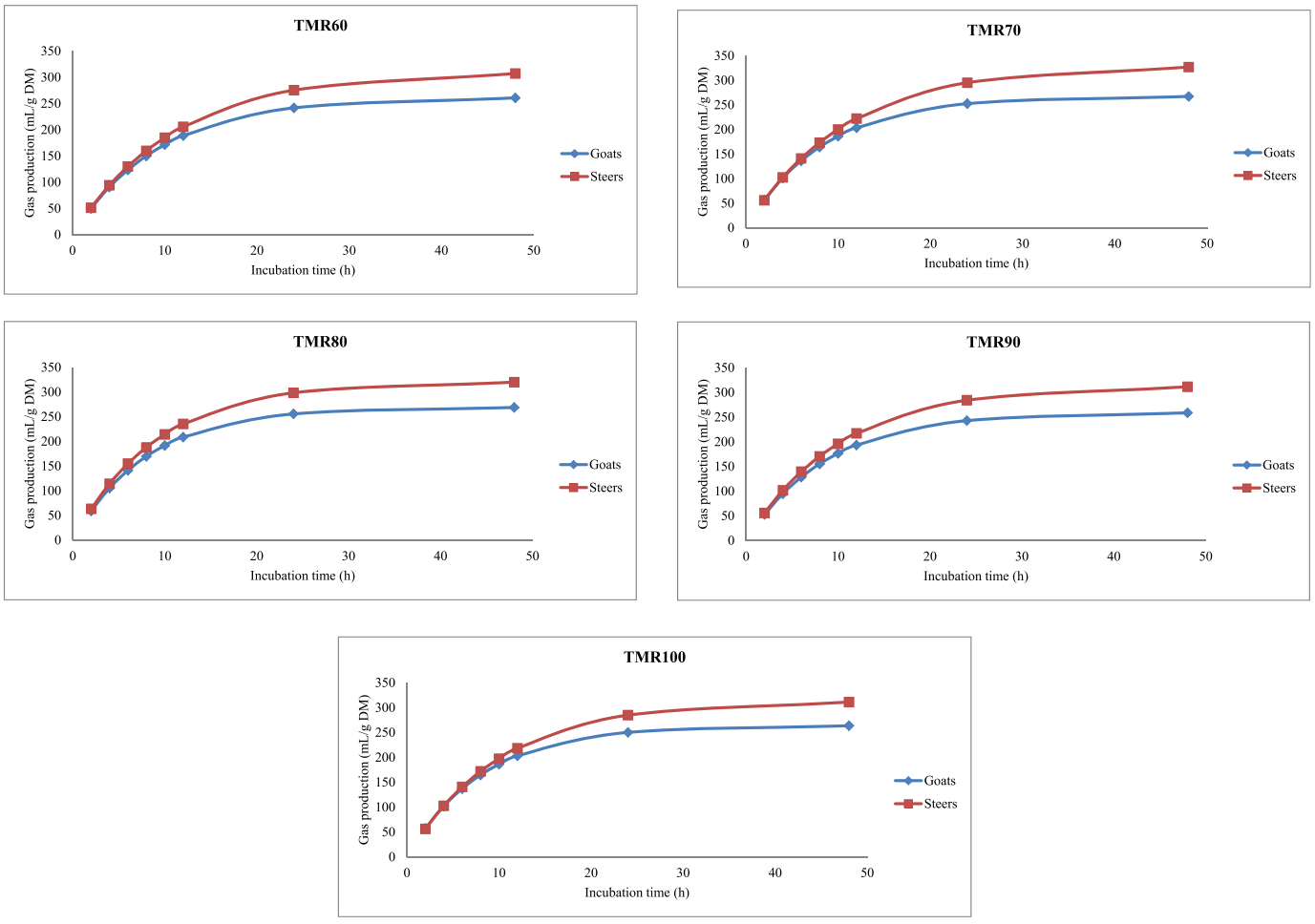

Fig. 1. (*continued*).

fractions and less crude protein compared with soybean meal. In the present experiment, MLM contained 281 g CP which represents about 69% of that contained in the soybean meal (408 g CP/kg MS). On the other hand, MLM contained about 345 g NDF versus 143 g for soybean meal. The chemical composition was expected to affect the fermentation pattern of each ration, as explained later. The protein content of MLM is comparable to sesame meal (26%), however, less than soybean meal (approximately 40–44% CP), cottonseed meal (approximately 40% CP) and sunflower seed cake (approximately 35% CP), which are the most common sources of protein in ruminant nutrition. Kholif et al. (2015) replaced sesame meal in goat diets with fresh MLM at 0, 50, 75, and 100%, and found that the CP content of diets did not change significantly; however, the NDF content of these diets increased. This is a result of almost equally CP content and high NDF content of MLM relative to sesame meal.

4.2. Gas production

The observed interactions between the type of inoculum and MLM level for most measured parameters of total gas production (GP) and fermentation reveal that the response to replacing soybean meal with MLM differed between the inoculum provided by goats and steers. These differences may be supported by the significant different response between steers and goat inoculums, and possibly explain differences in the ruminal microbial population and the digesting capacity of the two ruminant species (Fig. 2). This is an important indicator of the importance of using rumen fluid from different ruminant species to inoculate substrates *in vitro* for incubation cultures, in order to evaluate feed nutritive value. Aderinboye et al. (2016) reported different fermentation parameters with inoculums from cows, sheep and goats. Higher asymptotic GP of steer inoculum suggests that the inoculum supported maximum GP than goat inoculum. It appears that goat inoculum supported greater fermentation but delayed attachment and adaptation of ruminal microbes to the diets than the steer inoculum, based on its higher gas production rate and lag time.

Replacing soybean meal with MLM negatively affected asymptotic GP, lag time of GP and cumulative GP, but positively affected rate of GP. Soliva et al. (2005) observed that complete replacing of soybean meal and rapeseed meal with MLM decreased *in vitro* total GP. The decreased GP with increasing lag time of GP of MLM diets relative to the control soybean meal diet. This is a direct result of increased fiber concentration in the MLM-containing rations, which might have affected fermentability and also possibly delayed microbial attachment and adaptation. Kholif et al. (2017b) stated that increased fiber portion in TMR decreased asymptotic and cumulative GP but increased rate and lag time of GP. In another experiment where a relatively more fibrous and lower protein ingredient (corn silage) was used to replace a less fibrous and higher protein feed (concentrate mixture) in different maize silage: concentrate ratio diets, Elghandour et al. (2015) observed decreased GP and increased the lag time with increasing corn silage ratio in the TMR. Moreover, the reduced CP concentration in MLM

rations can also partially explain the decreased GP, in consonance with previous observations (Elghandour et al., 2017) where increased CP content of a ration enhances GP. It was expected that the observed increased bacterial number with MLM would increase GP, but this did not occur. The reasons for these observations are unknown.

The presence of plant secondary metabolites in MLM can be another reason for the negatively affected ruminal fermentation. Generally, the secondary metabolites at high doses have a great antimicrobial activity against ruminal bacteria, protozoa and fungi (Bodas et al., 2012). The antimicrobial effect depends on plant species, the chemical composition of plants, and the dose fed to animal (Bodas et al., 2012). Therefore, the secondary metabolites sometimes can stimulate rumen microbial activity (Benchaar et al., 2008), and this may explain the greater GP and shorter lag time in the case of TMR10 and TMR40 rations for steers inoculum. Ruminal microflora can tolerate and degrade low and moderate concentrations of secondary metabolites such as phenolic compounds (Varel et al., 1991) and tannins (Frutos et al., 2004), and utilize them as energy sources. Inoculum × TMR interactions for asymptotic and cumulative GP, and rate and lag time of GP indicate the importance of choosing the inoculum for incubating ration in which soybean meal is replaced with MLM. For maximum asymptotic and cumulative GP, and shorter lag time, incubation of TMR10 (10% replacement of soybean meal with MLM) and TMR0 (soybean meal as a sole protein source), respectively, the steer inoculum should be encouraged. However, incubation of TMR80 with goat inoculum is required to improve rate of GP.

Many reports have shown that goats have a higher ability to tolerate high levels of tannins compared with other ruminant species (Frutos et al., 2004; Yisehak et al., 2016). In the present experiment, ruminal microflora from steers showed better response compared with that of goats, which is not in line with Frutos et al. (2004) and Yisehak et al. (2016). This may be due to the previous feed fed to the goats and steers before starting the experiment. Ruminal microbial population depends mainly on the type of diet fed; therefore, based on the fact that both steers and goats in the present experiment were maintained on the same diet, microbial species were not expected to vary (Mould et al., 2005).

4.3. Biogases production

Reducing CH_4 production from livestock is always desirable from the environmental point of view. The decreased CH_4 production with MLM-containing rations may be related to the secondary metabolites such as tannins and saponins present in MLM, or the high proportion of α-linolenic acid (Machmüller et al., 2000) in MLM (Soliva et al., 2005). A decreased CH_4 production was observed by Soliva et al. (2005) using MLM diets compared with soybean meal diets. The antimicrobial and protozoal effects of secondary metabolites such as tannins can be a direct reason for the decline in CH_4 production (Bodas et al., 2012). Moreover, the adverse effect of secondary metabolites on cellulolytic bacteria (Patra and Saxena, 2009) can cause a reduction in CO_2 and H_2 formation, which are required for methanogenesis, as a result of decreased SCFA production, in particular acetate (Goel and Makkar, 2012), causing a reduction in CH_4 production. Moreover, Jayanegara et al. (2011) reported mitigating effects of phenolic compounds on CH_4 production. Goel and Makkar (2012) reported up to 50% reduction of CH_4 production in response to tannins and phenolic compounds. In the current study, CH_4 production was reduced by 8% and 10% in goats and steers, respectively, fed MLM diets compared to the soybean control diet. Greater gas and CH_4 productions from cattle inoculum indicates cattle contribute more to biogas emission than goats. Inoculum × TMR interaction for CH_4 production showed that CH_4 emission can be abated when soybean bean was completely replaced with MLM (TMR100) using goat inoculum.

4.4. Ruminal microflora

Increased ruminal bacterial number with MLM diets in both goat and steer was not expected based on the antimicrobial properties of secondary metabolites in MLM (Bodas et al., 2012). However, the reports of Varel et al. (1991) and Frutos et al. (2004) on the ability of ruminal microflora to degrade and utilize secondary metabolites as energy sources can explain the increased ruminal bacterial number. The increased bacterial populations seem to be a consequence of the observed inhibition of ruminal protozoa in both ruminant species (Newbold et al., 1997; Goel et al., 2008), as ruminal protozoa is the main predators of bacteria in the rumen (Mathieu et al., 1996). Ruminal bacteria population was greatest for TMR30 in goat and TMR100 in steer, while protozoa population was lowest for TMR100 and TMR20 in goat and steer, respectively. Higher bacterial and protozoa population in goat inoculum than in steer inoculum confirms the assertion that goats have a higher threshold for taninniferous diets compared to other ruminant species.

However, the increased bacterial number with MLM rations did not result in a greater GP, ruminal DM degradability and SCFA production of goats. This may be due to the fact that not all bacteria species are affected in the same way. For example, tannins and saponins are particularly able to inhibit Gram-positive bacteria more than Gram negative bacteria (Bodas et al., 2012). Thus, the increased bacterial number might be due to increase in other species and not cellulolytic bacteria.

The decreased protozoal number when soybean meal was

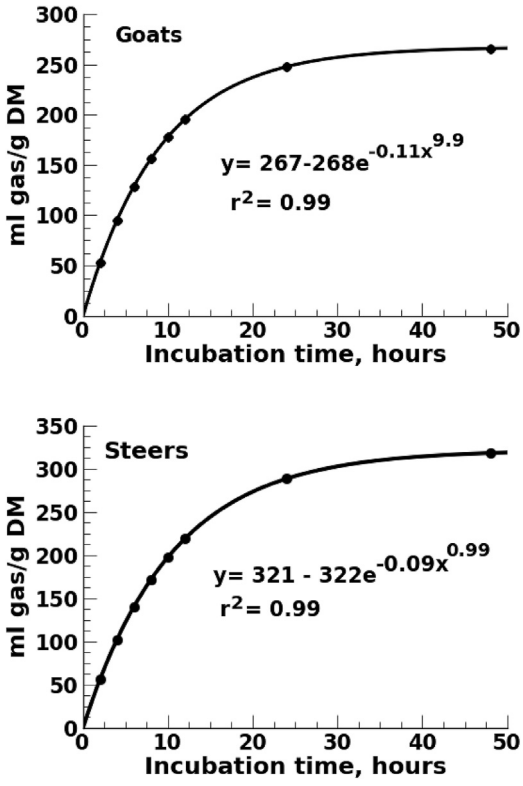

Fig. 2. Relationship (r^2) between *in vitro* gas production using goats and steers of total mixed rations (TMR)[1] containing different levels of *Moringa oleifera* leaf meal replacing soybean meal.

Table 3
Fermentation characteristics[a] of total mixed rations (TMR)[b] containing different levels of *Moringa oleifera* leaf meal replacing soybean meal, and incubated with rumen liquors obtained from goats or steers.

Inoculum	TMR	pH	SCFA	NH_3-N	DMD	OMD	ME	PF_{24}	GY_{24}	MCP	Total bacteria × 10^8	Total protozoa × 10^5
Goat	TMR0	6.42	5.65	69.6	771	702	10.3	5.15	194	753	9.50	5.12
	TMR10	6.44	5.78	65.3	765	712	10.5	5.13	195	765	10.3	4.27
	TMR20	6.47	5.25	58.6	763	665	9.80	5.23	191	720	13.1	3.42
	TMR30	6.45	4.78	58.8	756	629	9.20	5.35	187	681	13.5	3.96
	TMR40	6.44	5.48	60.2	745	679	10.0	5.18	193	739	11.3	3.27
	TMR50	6.46	5.81	58.4	734	708	10.5	5.12	195	767	11.1	3.44
	TMR60	6.48	5.34	53.9	759	668	9.9	5.21	192	728	11.4	3.62
	TMR70	6.44	5.59	56.6	734	685	10.1	5.16	194	748	13.2	4.10
	TMR80	6.46	5.65	49.0	755	689	10.2	5.15	194	754	11.0	3.93
	TMR90	6.40	5.37	51.0	720	667	9.80	5.21	192	730	11.3	3.92
	TMR100	6.44	5.53	52.0	727	681	10.0	5.18	193	744	11.4	2.88
SEM		0.005	0.093	1.37	15.7	7.50	0.11	0.018	0.70	7.80	0.810	0.203
Linear		0.037	0.399	<0.001	0.062	0.059	0.074	0.308	0.412	0.403	0.733	0.812
Quadratic		0.087	0.104	0.014	0.399	0.033	0.338	0.111	0.105	0.100	0.045	0.015
Steers	TMR0	6.40	6.09	65.9	750	738	10.9	5.07	197	791	9.38	5.77
	TMR10	6.43	6.70	63.4	789	785	11.6	4.98	201	842	10.53	3.21
	TMR20	6.44	6.45	61.0	803	761	11.3	5.01	199	821	10.63	1.95
	TMR30	6.40	6.33	56.9	774	752	11.1	5.04	199	811	11.55	3.60
	TMR40	6.44	6.53	61.2	758	763	11.3	5.01	200	827	12.12	3.70
	TMR50	6.46	6.33	53.2	783	750	11.1	5.04	199	811	9.98	3.07
	TMR60	6.39	6.09	57.6	763	728	10.8	5.07	197	791	10.25	3.38
	TMR70	6.42	6.53	61.1	761	760	11.3	5.00	200	828	10.00	2.77
	TMR80	6.40	6.60	61.4	782	765	11.3	5.00	200	834	12.40	3.74
	TMR90	6.38	6.29	53.9	748	740	11.0	5.04	198	807	12.07	3.39
	TMR100	6.41	6.30	56.3	846	742	11.0	5.04	198	809	13.22	4.62
SEM		0.010	0.130	1.92	19.1	10.4	0.16	0.019	0.70	10.9	0.31	1.11
Linear		0.351	0.258	<0.001	0.002	0.771	0.673	0.190	0.229	0.263	0.021	0.410
Quadratic		0.067	0.005	<0.001	0.704	0.002	0.002	0.600	0.441	0.005	0.769	0.027
Pooled SEM		0.008	0.113	1.67	17.5	9.10	0.14	0.019	0.70	9.50	1.963	0.710
P value												
Inoculum		<0.001	<0.001	0.0009	0.0003	<0.001	<0.001	<0.001	<0.001	<0.001	0.748	0.606
TMR												
Linear		0.066	0.660	<0.001	0.146	0.363	0.466	0.790	0.726	0.664	0.023	0.047
Quadratic		0.015	0.001	<0.001	0.805	0.001	0.001	0.002	0.001	0.009	0.477	0.818
Inoculum × TMR		0.700	0.001	<0.001	0.059	0.002	0.001	<0.001	<0.001	0.001	0.941	0.518

[a] DMD is dry matter degradability (mg/g DM), GY_{24} is gas yield at 24 h (mL gas/g DMD), MCP is microbial protein production (mg/g DM), ME is metabolizable energy (MJ/kg DM), NH_3-N (g/L) is ammonia-N, OMD is *in vitro* organic matter digestibility (g/kg DM), PF_{24} is partitioning factor at 24 h of incubation (mg DMD/mL gas), pH is ruminal pH, SCFA is short-chain fatty acids (mmol/g DM).
[e] [b]*Moringa olifera* replaced 0 (TMR0), 10 (TMR10), 20 (TMR20), 30 (TMR30), 40 (TMR40), 50 (TMR50), 60 (TMR60), 70 (TMR70), 80 (TMR80), 90 (TMR90), and 100 (TMR100) g/100 g of soybean meal.

replaced with MLM is a result of the marked anti-protozoal activity of secondary metabolites such as saponins, tannins and phenolic compounds (Makkar et al., 1995; Bodas et al., 2012). Bhatta et al. (2009) reported that tannins have a clear defaunating effect, without a clear mode of action. Decreasing ruminal protozoa population is desirable because this will result in lower CH_4 production and higher bacterial numbers.

4.5. Fermentation profile

Increasing ruminal pH in goat nutrition is desirable for better ruminal condition for cellulolytic bacteria activity. Ruminal pH values ranged between 6.42 and 6.48, and fell within the range considered acceptable for fiber digestion (Ørskov and Ryle, 1990). Greatest ruminal pH was observed in TMR60 for goats. Generally, ruminal pH was higher in MLM diets than the control diet possibly due to higher fiber content of MLM (Olafadehan and Adebayo, 2016).

Ruminal NH_3-N concentrations, in the present experiment, ranged from 49.0 to 69.6 g/L, and were above the range required for sufficient microbial protein synthesis (Satter and Slyter, 1974). The decreased ruminal NH_3-N with MLM is a result of the reported low degradability of MLM protein in the rumen (Kholif et al., 2015) due to its tannins and phenolic compounds (Bodas et al., 2012). Tannins in feeds may reduce ruminal protein degradation because tannins bind to dietary protein and protect it from ruminal degradation (Frutos et al., 2004). Besides, secondary metabolites such as saponins and tannins have the ability to decrease ruminal protozoa (Newbold et al., 1997), as we previously showed. Protozoa play a major role in ruminal feed protein degradation (Jouany, 1996). Another probable reason for the decreased NH_3-N is the inhibition of hyper NH_3-producing bacteria activity and their deaminase activity (Newbold et al., 2004).

The increased bacterial numbers with the MLM-containing diets did not result in increased OMD or SCFA production when goat inoculum was used. The negatively affected nutrient degradability in MLM-containing rations may be due to the negative effects of increasing fiber concentration and the declining CP concentration on ruminal fermentation. Frutos et al. (2004) reported that less than 50 mg/g DM is the acceptable level of tannins in feeds without negative effects on digestibility. In the present experiment, tannins concentration was 22 mg/g DM, which is far below the critical level that suppresses ruminal fermentation. Therefore, tannins cannot be the main reason for the decreased degradation, but the increasing fiber concentration in MLM rations could have decreased degradation, as speculated earlier. Elghandour et al. (2015) observed that increasing fiber concentration in a ration reduced nutrient degradability. In steer nutrition, the result of DMD was contrary to the observed result in goat nutrition. Differences in ruminal microflora response to secondary metabolites might be the reason.

Decreased SCFA concentration can be interpreted as a result of declined degradability of MLM-containing ration. Olafadehan and Okunade (2016) attributed decreased ruminal SCFA concentration to reduced digesta degradability in the rumen. Results of decreasing NH_3-N and total SCFA concentrations are an evidence of improved synchronization between dietary energy and protein, which is expected to increase microbial-N production within the rumen (Seo et al., 2013). Soliva et al. (2005) compared the ruminal fermentation of MLM with soybean meal and rapeseed meal, and observed unaffected ruminal pH values and SCFA concentration, and a decreased ruminal NH_3-N concentration with MLM. Higher SCFA, NH_3-N, DMD, OMD ME and MCP in cattle inoculum suggests that the inoculum enhanced ruminal fermentation relative to goat inoculum.

From the present results, future studies in which CH_4 emission abatement due to replacement of soybean meal with MLM in *in vivo* trials, using the three major ruminant species (cattle, sheep and goats), to validate our current results should be conducted.

5. Conclusion

From the nutritional perspective, *M. oleifera* cannot replace soybean meal as a protein source in diets for goats and steers because of the negative effect on ruminal fermentation. However, from an environmental point of view, replacing soybean meal with *M. oleifera* leaf meal reduced CH_4 production, which can be used as a good "cleaner" product for the environment and feedstuff for ruminants to mitigate the environmental contamination by biogases pollution emanated from these animals. More research will be desirable to determine the best levels of replacement on feed utilization and methane production in dairy and beef cattle, wool and meat sheep and dairy goats. Besides, more experiment should be carried out to replace other protein feeds with low protein concentrates such as sesame meal and rapeseed meal with *M. oleifera* at different levels in both *in vitro* and *in vivo* trials.

Conflict of interest

All authors declare that there are no present or potential conflicts of interest between them and other people or organizations that could inappropriately bias their work.

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