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RESEARCH ARTICLE

Effects of *Moringa oleifera* leaf extract on ruminal methane and carbon dioxide production and fermentation kinetics in a steer model

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Abstract

Ruminal fermentation produces greenhouse gases involved in global warming. Therefore, the effect of nutrient combinations on methane, carbon dioxide, and biogas production as well as ruminal fermentation kinetics was evaluated in in vitro studies. In total mixed rations, dietary corn grain was partially replaced by two levels of soybean hulls (a highly reusable residue), and a *Moringa oleifera* extract (a natural extract) at three concentration levels was added. Higher levels of both soybean hulls and *M. oleifera* extract delayed the initiation of methane production and resulted in a lower methane and carbon dioxide production. Thus, total biogas production was also lower. Replacement of corn grain by soybean hulls tended to lower methane production rates and asymptotic carbon dioxide production, and a delay in biogas and methane formation was observed. Asymptotic biogas and carbon dioxide production, however, were increased. The presence of *M. oleifera* extract tended to delay methane formation and to decrease methane production rate as well as asymptotic methane production. Higher *M. oleifera* extract levels decreased asymptotic biogas production with the control and the highest soybean hull levels. In the presence of *M. oleifera* extract, asymptotic carbon dioxide production was shown to be quadratically increased with the control and lowest soybean hull levels, but quadratically decreased with the highest soybean hull level. With the exception of fermentation pH, the interaction of substrate type and *M. oleifera* extract level was shown to have an effect on all fermentation parameters. Most fermentation parameters were shown to be higher when replacing corn grain by soybean hulls, including fermentation pH. Thus, the conclusion could be drawn that corn grain replacement by soybean hulls (an agricultural residue) in the presence of *M. oleifera* extract (a sparing leaf product) could ameliorate greenhouse gas emissions and improve digestion.

Keywords Biogas · *Moringa oleifera* extract · Ruminal fermentation · Soybean hulls

Introduction

Sustainable ruminant husbandry requires diminished impacts on natural vegetation (using residual agricultural products), improved animal well-being (improving digestion when free-range husbandry in the wilderness is restrained), and regulation of the abundance of rumen fermentation gases (methane and carbon dioxide) emitted to the atmosphere. By simultaneously meeting these requirements, animal nutritionists and microbiologists may sustainably improve animal performance, i.e., increasing milk yield or body weight gain for a given amount of feed.

About 18 and 9% of the global methane and carbon dioxide emissions were attributed to the livestock industry (FAO-Food and Agriculture Organization of the United Nations 2006). Methane and carbon dioxide production during fermentation in ruminants were reported to cause a loss in dietary energy of 2 to 12% (Johnson and Johnson 1995). Thus, an improvement of animal performance could be expected by reducing methane production.

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Modification of ruminant feeds is seen as one option to reduce greenhouse gas emission in ruminant production (Kholif et al. 2015). Especially, at least, a partial replacement of traditional feed components by agricultural by-products is gaining interest because of shortages and high prices in grain and protein feeds.

Soybean hulls are an unconventional feed constituent which has been successfully evaluated as a low-cost substitute in ruminant diets in vitro and in vivo (Costa et al. 2012; Elghandour et al. 2016). Replacing corn grain with soybean hulls in ruminant diets was shown to exhibit a good fermentation characteristic and resulted in a decrease in methane and carbon dioxide production (Elghandour et al. 2016). A complete substitution of corn grain is impossible, because of the fibrous nature and the low-energy density of soybean hulls. Furthermore, feed additives such as phytogenic extracts were already reported to have a positive effect on feed utilization and animal performance in ruminants (Cedillo et al. 2014; Kholif et al. 2015) and might, therefore, also decrease greenhouse gas production. Leaves and their extracts contain biologically active compounds such as tannins, saponins, and phenolics capable of affecting fermentation in a ruminal in vitro model (Salem et al. 2014).

However, information about the impact of simultaneously replacing feed constituents by agricultural by-products and supplementing feeds with phytogenic extracts on ruminal fermentation, animal performance, and the production of greenhouse gases is scarce. Nonlinear responses are often due to dose-dependent effects and interactions, so that multiple fermentation kinetic parameters need to be compared in statistical models. Accordingly, ruminal gas, carbon dioxide, and methane production in a ruminal in vitro model after replacing different amounts of corn grain in a steer diet by soybean hulls in the absence and presence of different amounts of an extract of *Moringa oleifera* leaves were assessed. Furthermore, fermentation kinetics and the nutritional values of the different diets were evaluated.

Materials and methods

Production of the phytogenic extract

Freshly cut leaves randomly collected from young and mature *M. oleifera* trees in the state of Veracruz (Mexico) were crushed, and 1 g of the crushed leaves was immediately immersed in 8 mL of water. Extraction was performed in closed jars for 72 h at 28 °C, followed by a second extraction at 39 °C for 1 h. Thereafter, the extracts were immediately clarified by filtration through a gauze and stored at 4 °C until further use.

Substrate preparation

Substrates for in vitro fermentation were obtained by replacing 75 (soybean hull (SH)75) and 150 (SH150) g/kg DM corn grain by the same amount of soybean hulls (Table 1). A control substrate without substitution was also included in the study. All three substrates were evaluated in absence (0 mL/g substrate DM) or presence (0.6, 1.2, 1.8 mL/g substrate DM) of an extract obtained from *Moringa oleifera* leaves.

In vitro fermentation

A 1:1 (*w*/*w* DM) mixture of alfalfa hay and a commercial feed concentrate was fed ad libitum to two cannulated Holstein steers with a body weight of 450 ± 20 kg according to NRC (2001). After collecting the rumen contents from both steers, they were thoroughly mixed and flushed with carbon dioxide. Any particles were removed by filtration through a cheesecloth (four layers). For incubation, 10 mL of the filtrate diluted 1:5 (*v*/*v*) with a buffer solution described by Goering and van Soest (1970) was added to 0.5 g of the substrate. Furthermore, blanks without substrate were included in the study. Fermentation was performed for 72 h at 39 °C in closed flasks under agitation and terminated by putting the incubation flasks on ice. Thereafter, the pH values of the fermentation broths were measured. The fermentation broths were filtered

Table 1 Composition of experimental diets

	Control	SH75	SH150
Ingredients (g/kg DM)			
Soybean hulls	250	250	250
Oats straw	249	248	248
Wheat bran	30	30	30
Steam rolled barley	120	110	120
Steam rolled corn	250	175	100
Soybean hulls	0	75	150
Corn gluten feed	30	30	20
Molasses	70	80	80
Minerals/vitamins[1]	1	2	2
Chemical composition (g/kg DM)			
Organic matter (OM)	964	940	957
Ether extract (EE)	24	22	23
Acid detergent fiber (ADF)	121	130	122
Neutral detergent fiber (NDF)	356	428	340
Nonstructural carbohydrates	455	371	481
Crude protein	130	119	113

[1] 12×10^3 IU/g vitamin A, 2.5×10^3 IU/g vitamin D_3, 15 IU/g vitamin E, 2 mg/g vitamin K, 2.25 mg/g vitamin B_1, 7.5 mg/g vitamin B_2, 3.5 mg/g vitamin B_6, 20 μg/g vitamin B_{12}, 12.5 mg/g pantothenic acid, 1.5 mg/g folic acid, 125 μg/g biotin, 45 mg/g niacin, 50 mg/g Fe, 50 mg/g Zn, 110 mg/g Mn, 12 mg/g Cu, 300 μg/g I, 200 μg/g Se, 200 mg/g Co

using a glass frit under vacuum in order to obtain the nonfermented residues. The dry residues obtained by drying the filtrates overnight at 65 °C were used for quantifying the fermented substrate. All fermentations were performed in triplicate.

Methane, carbon dioxide, and biogas determination

Biogas production was determined using an Extech Instruments Pressure Transducer (Waltham, USA) according to Theodorou et al. (1994). Carbon dioxide and methane production were quantified with a Tetra3 Gas Analyzer (CROWCON, Abingdon, UK). Fitting the recorded gas volumes using the SAS (2002) NLIN procedure (France et al. 2000) was used to estimate methane, carbon dioxide, and biogas production kinetics.

Substrate analysis

Substrate composition was determined either according to the AOAC (1997) methods (dry matter, ash, nitrogen, ether extract, lignin, acid detergent fiber) or according to van Soest et al. (1991) (neutral detergent fiber).

Calculations

In vitro metabolizable energy (ME) and organic matter digestibility (OMD) were calculated as described by Menke et al. (1979). Short-chain fatty acid concentrations (SCFA) were obtained as described by Getachew et al. (2002), and the microbial biomass produced (MCP) (mg/g DM) as described by Blümmel et al. (1997). The partitioning factor (PF_{24}), the ratio of in vitro dry matter degradability (mg) and biogas production (mL) after 24 h of fermentation, was used as a measure for fermentation efficiency. Gas yield (GY_{24}) represents the gas volume (mL gas/g DM) obtained after a fermentation time of 24 h per dry matter digestibility (DMD, g).

Statistical analyses

Trends in in vitro biogas production and ruminal fermentation parameters were assessed in a factorial design with SAS (2002) PROC GLM as:

$$Y_{ijk} = \mu + R_i + D_j + (R \times D)_{ij} + (E_{ijk})$$

where μ represents the general mean, R_i the type i substrate, D_j the level of *M. oleifera* extract j, $(R \times D)_{ij}$ the interaction of substrate type and *M. oleifera* extract level, and E_{ijk} the experimental error. Responses of a certain substrate to increasing levels of the *M. oleifera* extract were examined using linear and quadratic polynomial contrasts. $P < 0.05$ was used as a threshold of significance.

Results

Significant interaction at higher levels of SH and *M. oleifera* extract delayed the initiation of methane production by almost a factor of 2 (Table 2). In addition, methane production was observed to be lower (Table 2). At higher levels of soybean hulls and *M. oleifera* extract, asymptotic biogas and methane production were found to be lower, while asymptotic carbon dioxide production was still in a quite low range (Figs. 1, 2, and 3). Furthermore, higher soybean hull and *M. oleifera* extract levels resulted in a lower methane production during the entire fermentation (Fig. 1).

Significant trends identified after replacing corn grain by soybean hulls were lower asymptotic methane production ($P < 0.024$, linear effect) (Table 2), a shorter lag time for methane production ($P < 0.001$, quadratic effect), a longer lag time for biogas production ($P < 0.004$), and lower carbon dioxide production rates ($P < 0.001$, quadratic effect). However, both the asymptotic production of biogas and carbon dioxide ($P < 0.001$) were found to be higher.

The addition of *M. oleifera* extract tended to lower asymptotic methane production ($P < 0.001$, quadratic effect) and resulted in a prolonged lag time for the production of methane and a lower production rate of methane ($P < 0.008$, linear effect). From *M. oleifera* extract levels of 1.2 mL/g DM, a decrease in asymptotic biogas production with the control and the SH150 substrate was found. It is worth mentioning that in the presence of the *M. oleifera* extract, asymptotic carbon dioxide production with the control and the SH75 substrate was observed to be quadratically increased ($P < 0.007$), whereas carbon dioxide production of the SH150 substrate was found to be quadratically decreased ($P < 0.007$).

The interaction of soybean hulls and *M. oleifera* extract levels ($P < 0.001$) resulted in a lower methane production during the entire fermentation and in a lower proportional methane production after 6 h of fermentation (Table 3). When corn grain was replaced by soybean hulls, a decrease in methane production after 48 h of fermentation ($P < 0.011$, quadratic effect) and a reduced dry matter digestibility ($P < 0.046$, linear effect) were observed. Furthermore, a lower proportional methane production was found during the entire fermentation ($P < 0.05$, linear and quadratic effects). Supplementation with an extract from *M. oleifera* resulted in a lower production of methane per incubated and degraded dry matter as well as in a lower proportional methane production ($P < 0.01$, linear and quadratic effects).

At higher levels of *M. oleifera* extract, carbon dioxide production was observed to be lower after replacing corn grain by soybean hulls when compared with low levels of

Table 2 Kinetics of in vitro rumen gas (BG), methane (CH_4), and carbon dioxide (CO_2) production in dependence of soybean hull (SH) and *Moringa oleifera* extract (mL/g DM) levels

Substrate[1]	*M. oleifera* extract	BG production (mL/g DM)[2]			CH_4 production (mL/g DM)[2]			CO_2 production (mL/g DM)[2]		
		b	*c*	Lag	*B*	*c*	Lag	*b*	*c*	Lag
Control	0	281	0.030	1.92	59		12.6	91	0.080	5.72
	0.6	281	0.035	1.97	52	0.020	11.2	113	0.065	5.71
	1.2	270	0.040	1.98	43	0.018	11.3	105	0.070	5.47
	1.8	265	0.036	1.95	46	0.026	11.5	104	0.060	5.75
SH75	0	277	0.033	2.17	46	0.022	15.4	103	0.055	5.80
	0.6	389	0.035	2.84	56	0.024	13.8	151	0.060	5.19
	1.2	359	0.033	3.38	40	0.024	13.7	155	0.055	5.64
	1.8	336	0.033	3.04	40	0.022	13.5	137	0.035	5.92
SH150	0	367	0.033	2.05	46	0.027	29.1	152	0.040	4.86
	0.6	472	0.035	1.78	50	0.034	17.5	203	0.035	5.89
	1.2	339	0.025	2.59	36	0.020	18.4	136	0.040	5.95
	1.8	252	0.028	2.09	46	0.012	19.9	109	0.055	5.87
Pooled SEM		9.0	0.0033	0.307	2.4	0.0023	1.11	7.2	0.0068	0.380
Ration effect										
Linear		< 0.001	0.502	0.004	0.024	0.878	0.026	< 0.001	0.003	0.924
Quadratic		< 0.001	0.051	0.153	0.051	0.859	< 0.001	< 0.001	0.001	0.981
Dose effect										
Linear		0.003	0.902	0.225	0.010	0.008	0.006	0.850	0.158	0.236
Quadratic		0.001	0.868	0.050	0.001	0.252	0.010	0.007	0.865	0.899
Ration × dose		< 0.001	0.214	0.499	0.047	0.001	0.002	0.003	0.078	0.423

SEM, standard error of the mean

[1] Control: no replacement of corn grain by soybean hulls; SH75: replacement of 75 g/kg DM of corn grain by the same amount of soybean hulls; SH150: replacement of 150 g/kg DM of corn grain by the same amount of soybean hulls

[2] *b* represents the asymptotic gas production (mL/g DM) of either rumen gas, methane, or carbon dioxide; *c* the rate of gas production (/h); *Lag* the time (h) elapsed until gas production starts

M. oleifera extract (Table 4). Moreover, at high soybean hull and *M. oleifera* extract levels, carbon dioxide production was only slightly higher than that with the control. Thus, the trend of increasing carbon dioxide production with increasing levels of soybean hulls was significantly slowed down in the presence of high *M. oleifera* extract levels. Lower soybean hull levels, however, increased carbon dioxide production per incubated and degraded dry matter after 24 and 48 h of fermentation as well as proportional carbon dioxide production after 48 h of fermentation, and lower *M. oleifera* extract levels increased carbon dioxide production and proportional carbon dioxide production per incubated and digested dry matter after 48 h of fermentation (Table 4).

With the exception of fermentation pH, the interaction of substrate type and *M. oleifera* extract levels was shown to have an effect on all fermentation parameters (Table 5). By replacing corn grain with soybean hulls, higher fermentation pH values ($P < 0.003$, quadratic effect), higher metabolizable energy ($P < 0.003$, linear effect), higher short-chain fatty acid concentrations ($P < 0.001$, linear effect), higher dry matter digestibility ($P < 0.001$, quadratic effect), higher organic matter digestibility ($P < 0.004$, linear effect), higher production of microbial biomass ($P < 0.003$, linear effect), higher GY_{24} ($P < 0.006$, linear effect), and lower PF_{24} ($P < 0.016$, linear effect) were obtained. Inclusion of *M. oleifera* extract resulted in higher metabolizable energy ($P < 0.03$, linear effect; $P < 0.036$, quadratic effect), higher short-chain fatty acid concentrations ($P < 0.02$, quadratic effect), higher production of microbial biomass ($P < 0.027$, linear effect; $P < 0.023$, quadratic effect), and higher organic matter digestibility ($P < 0.02$, quadratic effect) with the control and the SH75 substrate. It is worth mentioning that the favorable effects of the replacement of corn grain by soybean hulls peaked at the lowest *M. oleifera* extract level (metabolizable energy, organic matter digestibility, and production of microbial biomass), while at the highest *M. oleifera* extract level, fermentation pH was substantially raised.

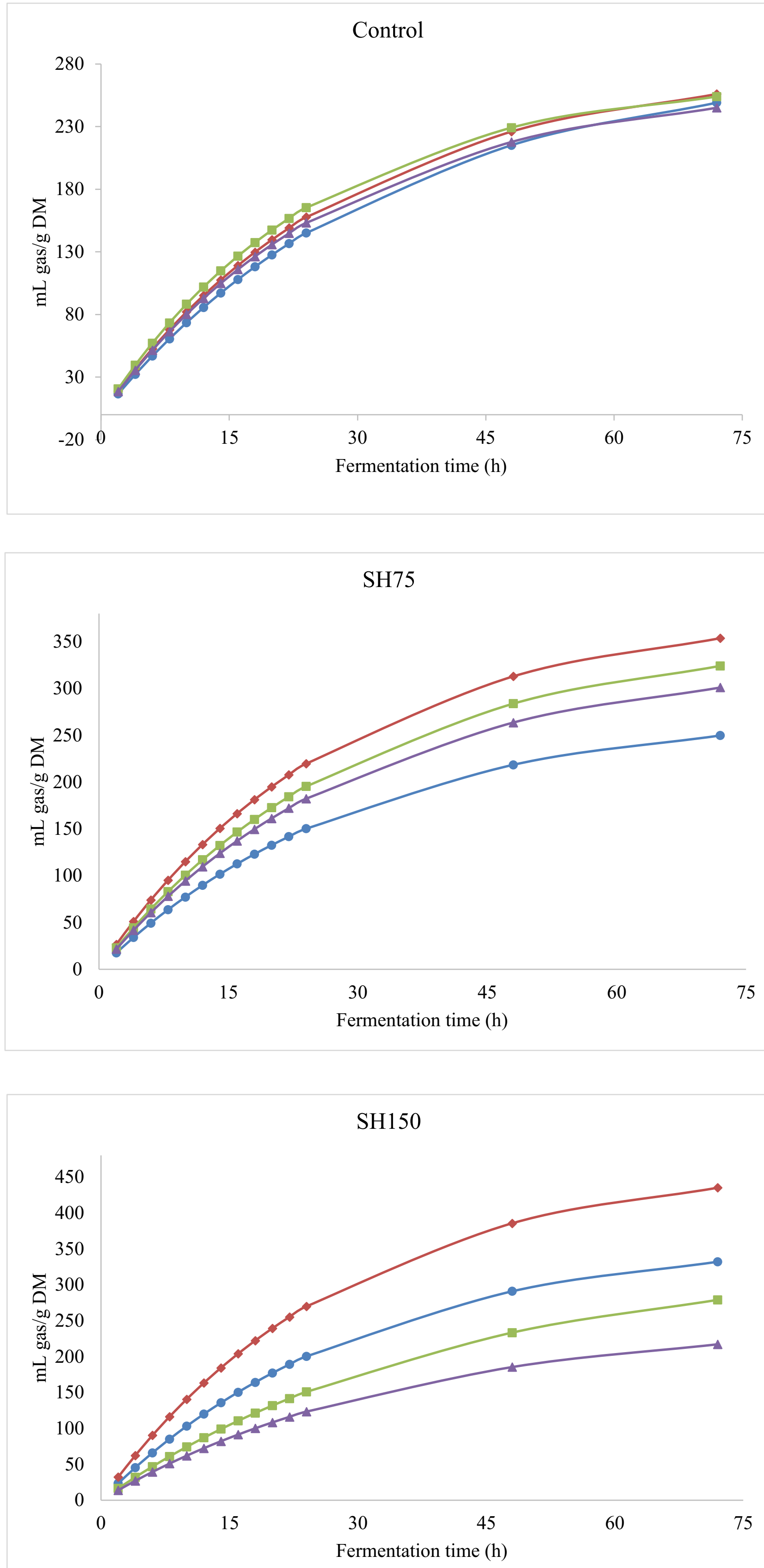

Fig. 1 In vitro rumen gas production (mL/g DM) in dependence of soybean hull and *Moringa oleifera* extract: 0 (circle with solid line), 0.6 (diamond with solid line), 1.2 (square with solid line), and 1.8 (triangle with solid line) mL/g DM. Control: no replacement of corn grain by soybean hulls; SH75: replacement of 75 g/kg DM of corn grain by the same amount of soybean hulls; SH150: replacement of 150 g/kg DM of corn grain by the same amount of soybean hulls

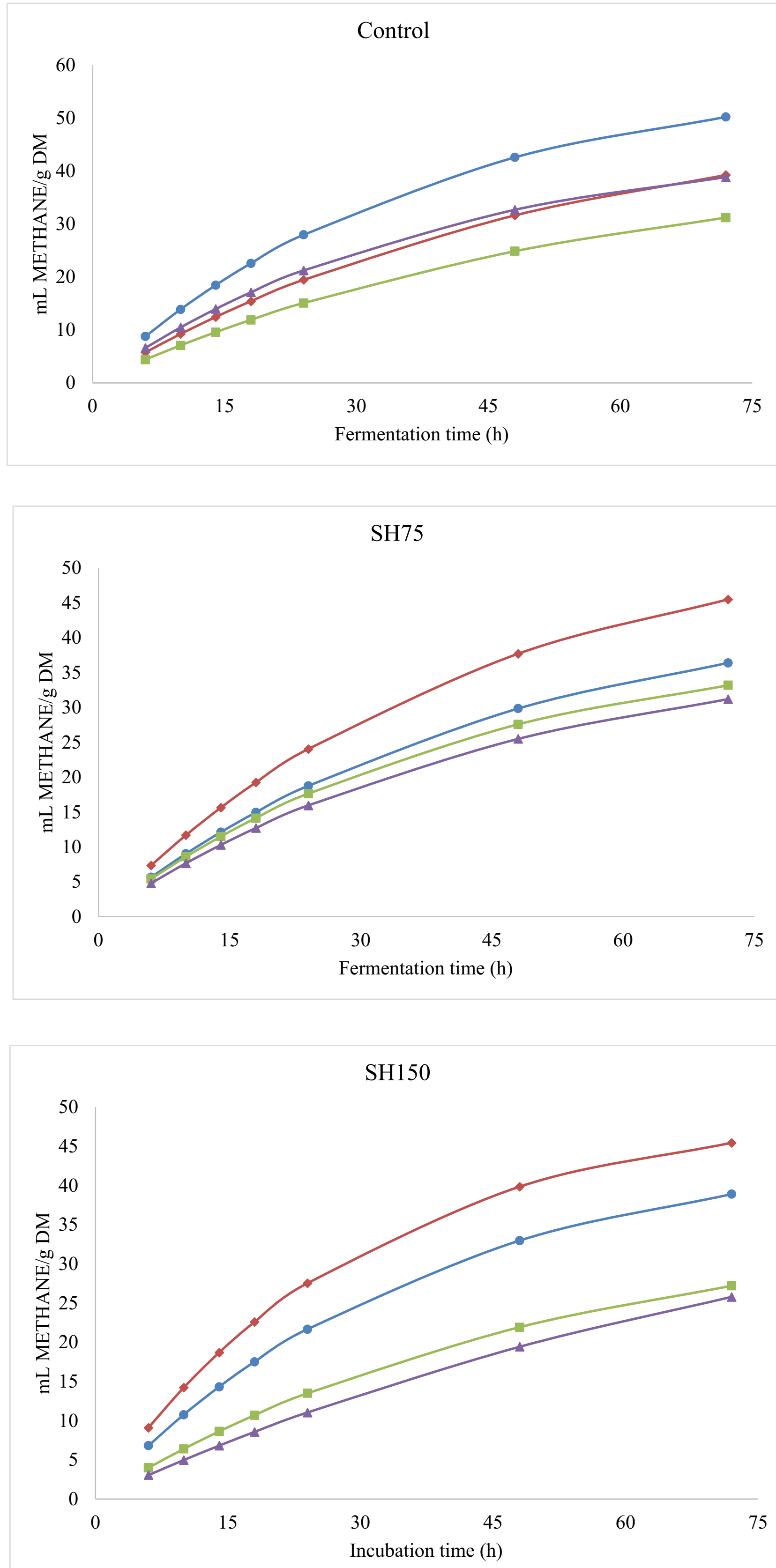

Fig. 2 In vitro methane production (mL/g DM) in dependence of soybean hull and *Moringa oleifera* extract: 0 (circle with solid line), 0.6 (diamond with solid line), 1.2 (square with solid line), and 1.8 (triangle with solid line) mL/g DM. Control: no replacement of corn grain by soybean hulls; SH75: replacement of 75 g/kg DM of corn grain by the same amount of soybean hulls; SH150: replacement of 150 g/kg DM of corn grain by the same amount of soybean hulls

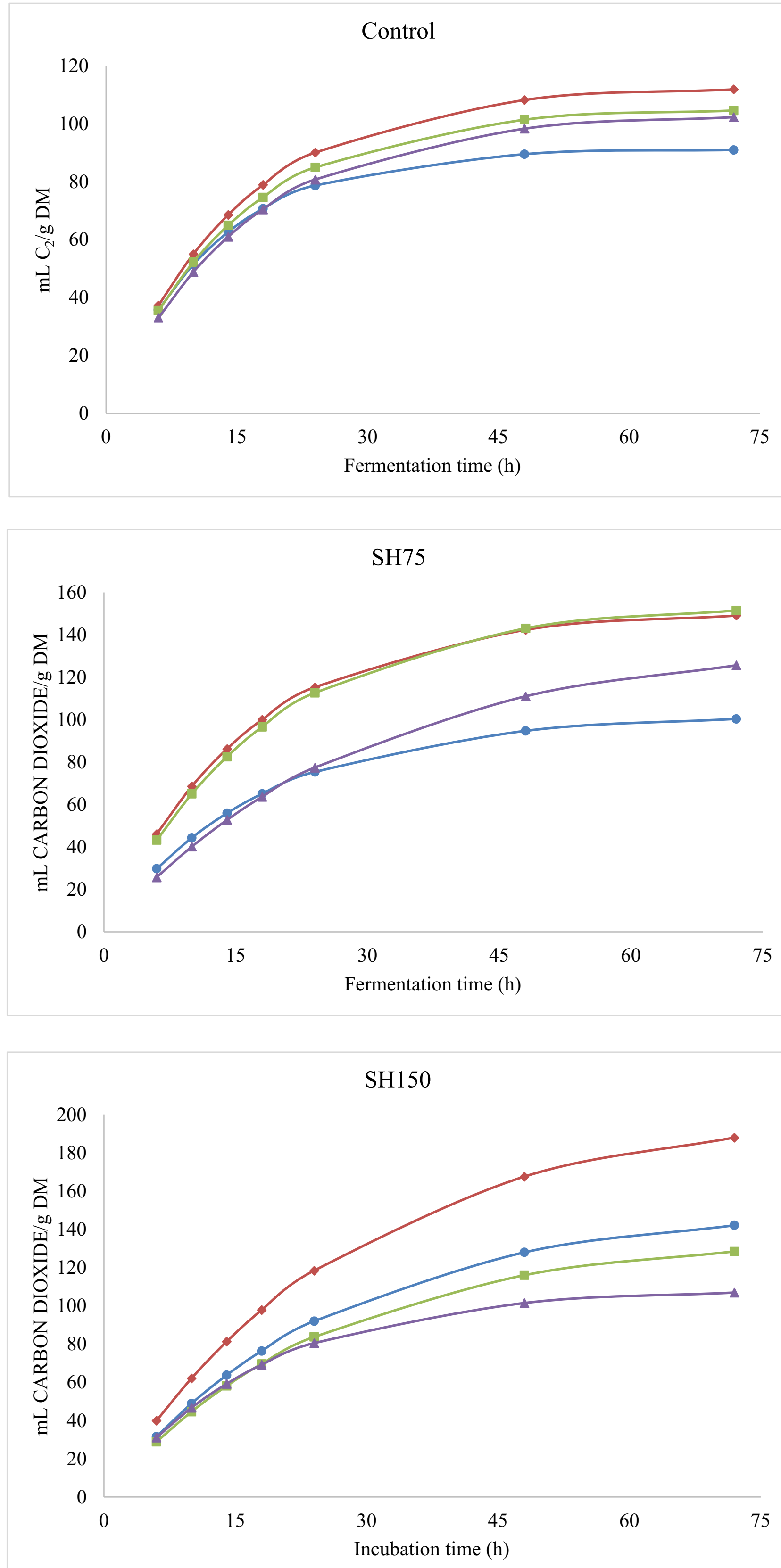

Fig. 3 In vitro carbon dioxide production (mL/g DM) in dependence of soybean hull and *Moringa oleifera* extract: 0 (circle with solid line), 0.6 (diamond with solid line), 1.2 (square with solid line), and 1.8 (triangle with solid line) mL/g DM. Control: no replacement of corn grain by soybean hulls; SH75: replacement of 75 g/kg DM of corn grain by the same amount of soybean hulls; SH150: replacement of 150 g/kg DM of corn grain by the same amount of soybean hulls

Table 3 Proportional in vitro methane (CH_4) production as a percent of total gas production in dependence of soybean hulls and *Moringa oleifera* extract (mL/g DM) levels

Substrate[1]	*M. oleifera* extract	CH_4 production								
		mL/g incubated DM			mL/g degraded DM			Proportional CH_4 production		
		6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Control	0	8.8	27.9	42.5	13.6	43.4	66.1	18.4	19.0	19.7
	0.6	5.7	19.4	31.6	8.9	30.1	49.0	10.4	11.9	13.8
	1.2	4.4	15.1	24.8	6.8	23.2	38.2	7.9	9.2	10.9
	1.8	6.6	21.2	32.7	9.8	31.5	48.4	12.6	13.8	15.1
SH75	0	5.7	18.8	29.8	8.7	28.9	46.0	12.4	13.3	14.3
	0.6	7.3	24.0	37.7	11.3	37.0	58.1	9.0	10.1	11.4
	1.2	5.4	17.6	27.6	8.2	26.9	42.0	8.0	8.7	9.5
	1.8	4.8	16.0	25.5	7.4	24.5	39.1	9.2	9.7	10.2
SH150	0	6.8	21.7	33.0	10.7	33.9	51.6	11.0	11.3	11.7
	0.6	9.1	27.5	39.8	14.1	42.5	61.5	10.0	10.2	10.4
	1.2	4.0	13.5	21.9	6.4	21.5	34.9	8.2	8.7	9.2
	1.8	3.1	11.0	19.4	5.0	18.1	31.9	7.5	8.6	10.0
Pooled SEM		0.51	1.30	1.62	0.82	2.09	2.62	1.29	1.11	0.95
Ration effect										
Linear		0.135	0.072	0.032	0.159	0.091	0.046	0.013	0.002	< 0.001
Quadratic		0.291	0.073	0.011	0.545	0.218	0.058	0.042	0.006	< 0.001
Dose effect										
Linear		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	0.001	0.001
Quadratic		0.003	0.001	< 0.001	0.004	0.001	0.001	0.001	0.001	< 0.001
Ration × dose		0.001	< 0.001	< 0.001	< 0.001	0.001	< 0.001	0.001	0.109	0.103

SEM, standard error of the mean

[1] Control: no replacement of corn grain by soybean hulls; SH75: replacement of 75 g/kg DM of corn grain by the same amount of soybean hulls; SH150: replacement of 150 g/kg DM of corn grain by the same amount of soybean hulls

Discussion

Gas production

Replacement of corn grain by soybean hulls resulted in a delay of biogas production reflecting that the rumen microflora needed some time to adapt their metabolism on the soybean hull-rich substrates. This observation might be due to the higher content of fibrous carbohydrates of soybean hulls compared to corn grain. However, a dose-dependent increase with significant nonlinear interactions with *M. oleifera* extract levels was also obtained when corn grain was replaced by soybean hulls. The higher biogas production indicated that soybean hulls provided significant amounts of nutrients and fermentable material for the microbial community present in the in vitro model system, because the availability of nutrients (Kholif et al. 2017) and rapidly fermentable carbohydrates for rumen microorganisms (Elghandour et al. 2015) was reported to increase biogas production. The higher biogas production may indicate enhanced degradability and fermentability of the substrates, and partial replacement of corn grain by soybean hulls might, therefore, increase feed intake and, consequently, animal productive performance (Khazaal et al. 1996). Elghandour et al. (2016), however, did not find any effect on biogas volumes by the same replacement of corn grain by soybean hulls as used in this study, but higher biogas production rate and an extension of the delay of biogas production with the increase in soybean hulls were observed. The use of different animal species and/or the physiological status of the donors of the rumen fluids is often responsible for the variability in the obtained results.

The increase in asymptotic biogas production in the presence of the *M. oleifera* extract was only observed with the SH75 substrate and with low levels of the extract with the SH150 substrate. This observation might be explained by significant interactions between substrate type and *M. oleifera* extract level and indicates that the *M. oleifera* leaf extract had the most significant effect at lower concentrations of fibrous carbohydrates. The content of secondary metabolites such as tannins, saponins, and phenolics has been suggested to be responsible for the observed effects (Bodas et al. 2012). Low levels of plant secondary metabolites were reported to

Table 4 Proportional in vitro carbon dioxide (CO_2) production as a percent of total gas production in dependence of soybean hull and *Moringa oleifera* extract (mL/g DM) levels

Substrate[1]	*M. oleifera* extract	CO_2 production								
		mL/g incubated DM			mL/g degraded DM			Proportional CO_2 production		
		6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Control	0	36	79	89	55	122	139	75	53	41
	0.6	37	90	108	58	140	168	68	55	47
	1.2	35	85	101	55	131	156	64	52	44
	1.8	33	81	98	49	119	146	63	52	45
SH75	0	30	75	95	46	116	146	64	53	46
	0.6	46	115	142	71	178	219	57	48	43
	1.2	43	113	143	66	172	218	64	55	49
	1.8	26	77	111	40	119	170	49	47	44
SH150	0	32	92	128	49	144	200	50	48	45
	0.6	40	118	168	62	183	259	44	44	44
	1.2	29	84	116	46	133	185	60	54	49
	1.8	31	81	101	51	133	167	74	60	51
Pooled SEM		3.5	6.2	6.4	5.5	10.1	11.0	6.5	3.2	1.2
Ration effect										
Linear		0.722	0.021	0.002	0.705	0.027	0.005	0.081	0.339	0.232
Quadratic		0.200	0.278	0.009	0.421	0.099	0.004	0.162	0.732	0.022
Dose effect										
Linear		0.408	0.634	0.931	0.408	0.650	0.937	0.819	0.529	0.021
Quadratic		0.079	0.012	0.004	0.089	0.017	0.007	0.989	0.492	0.042
Ration × dose		0.102	0.024	0.002	0.119	0.061	0.014	0.076	0.052	0.010

SEM, standard error of the mean

[1] Control: no replacement of corn grain by soybean hulls; SH75: replacement of 75 g/kg DM of corn grain by the same amount of soybean hulls; SH150: replacement of 150 g/kg DM of corn grain by the same amount of soybean hulls

represent an energy source for rumen microorganisms (Hart et al. 2008), whereas higher levels seem to negatively affect rumen microorganisms because of their antimicrobial properties (Bodas et al. 2012). Thus, supplementation of a diet with an extract of *M. oleifera* needs to be optimized in respect to animal performance and environmental protection.

Methane and carbon dioxide production

Lowering methane emission from ruminants is the most important issue in respect to lowering the concentrations of greenhouse gases in the atmosphere. Replacing corn grain by soybean hulls seems to be a way to contribute to this goal. A trend to lower methane production was observed when replacing corn grain by soybean hulls. However, Elghandour et al. (2016) reported recently in a comparable study that methane production was unaffected. This contradictory result might be due to the use of different rumen liquor donors in both studies. Replacing corn grain by soybean hulls certainly changed the types and composition of the carbohydrate fractions of the substrates. Therefore, a reduction in methane production by altering the rumen microbial population seems possible (Johnson and Johnson 1995). In particular, increasing the amount of soluble carbohydrates should have an effect on the production of short-chain fatty acids with the consequence of a decreased production of acetate and an increased production of propionate (Boadi et al. 2004). This limits methanogenesis by a reduced availability of hydrogen (Polyorach et al. 2014) and a reduced population of protozoans (Iqbal et al. 2008), the main ruminal methane producers (Hook et al. 2010).

Methane production was found to be lower in the presence of *M. oleifera* extract. This is in good agreement to the results obtained by Dey et al. (2014) and Soliva et al. (2005) for roughage-based diets. The observed effects were ascribed to the secondary metabolites present in such extracts (Mueller-Harvey 2006; Bodas et al. 2012). Those have already been reported to suppress methane as well as hydrogen production in the rumen. Different mechanisms have been suggested: (a) inhibition of methanogens (Carulla et al. 2005), (b) a reduced fiber digestion (Tiemann et al. 2008), and (c) a reduced protein digestion (Salem et al. 2012).

Table 5 In vitro rumen fermentation profile in dependence of soybean hull and *Moringa oleifera* extract (mL/g DM) levels

Substrate[1]	*M. oleifera* extract	pH	ME	DMD	OMD	SCFA	PF_{24}	MCP	GY_{24}
Control	0	5.82	6.87	611	467	3.20	5.97	515	167
	0.6	5.80	7.23	639	490	3.47	5.87	539	171
	1.2	5.91	7.40	652	504	3.63	5.73	555	174
	1.8	5.96	7.10	666	482	3.40	5.87	532	170
SH75	0	6.33	6.97	659	470	3.30	5.93	525	169
	0.6	6.05	8.87	653	594	4.87	5.37	649	187
	1.2	6.09	8.17	652	551	4.33	5.50	605	182
	1.8	6.20	7.83	649	527	4.03	5.60	582	178
SH150	0	6.40	8.40	634	566	4.43	5.47	614	183
	0.6	6.22	10.27	645	690	5.93	5.10	738	196
	1.2	6.55	7.03	624	478	3.33	5.93	523	169
	1.8	6.35	6.27	610	429	2.73	6.43	476	156
Pooled SEM		0.176	0.342	7.3	22.3	0.272	0.141	23.4	4.0
Ration effect									
Linear		0.028	0.003	0.483	0.004	0.001	0.016	0.003	0.006
Quadratic		0.003	0.048	< 0.001	0.037	0.060	0.962	0.095	0.606
Dose effect									
Linear		0.915	0.030	0.644	0.048	0.026	0.137	0.027	0.144
Quadratic		0.972	0.036	0.890	0.020	0.020	0.133	0.023	0.129
Ration × dose		0.903	< 0.001	0.010	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

DMD, DM degraded substrate (mg/g DM); *ME*, metabolizable energy (MJ/kg DM); *OMD*, in vitro organic matter digestibility (mg/g DM); *SCFA*, short-chain fatty acids; PF_{24}, partitioning factor after a fermentation time of 24 h; GY_{24}, gas yield after a fermentation time of 24 h; *SEM*, standard error of the mean

[1] Control: no replacement of corn grain by soybean hulls; SH75: replacement of 75 g/kg DM of corn grain by the same amount of soybean hulls; SH150: replacement of 150 g/kg DM of corn grain by the same amount of soybean hulls

A tendency to a higher carbon dioxide production was observed when replacing corn grain by soybean hulls. Because biogas is the sum of carbon dioxide, methane, nitrogen, and traces of other gases, the higher final biogas volumes found in this study could be explained by the increase in carbon dioxide production. However, Elghandour et al. (2016) did not observe any effect of corn grain replacement by soybean hulls on carbon dioxide production. A decrease in the production of methane might result in higher carbon dioxide concentrations, because carbon dioxide and hydrogen are in general the precursors for methane formation in the rumen (Sirohi et al. 2010). A further source of carbon dioxide in the rumen is formic acid (Fenchel and Finlay 1995). Several methanogens are capable of directly metabolizing formic acid, and therefore, their inhibition will result in an increased use of formic acid for carbon dioxide formation.

In the presence of *M. oleifera* extract, asymptotic carbon dioxide production was increased with the SH75 substrate, but decreased with the SH150 substrate, illustrating again significant interactions between substrate type and *M. oleifera* level. To the best of our knowledge, no information is available on the effects of *M. oleifera* extracts on carbon dioxide production so far.

Fermentation kinetics

Fermentation pH was determined to be between 5.80 and 6.55 and, therefore, within the range suggested by Ørskov and Ryle (1990) for optimal growth and activity of ruminal microorganisms. Replacing corn grain by soybean hulls resulted in higher fermentation pH values making the environment more suitable for cellulolytic activity, since a positive correlation between fermentation pH and readily fermentable carbohydrates (Walsh et al. 2009) as well as fermentation pH and the production of volatile fatty acids has been reported (Ramos et al. 2009).

Higher metabolizable energy, short-chain fatty acid concentrations, and microbial biomass production when replacing corn grain by soybean hulls were consistent with higher organic matter digestibility and dry matter digestibility (Elghandour et al. 2015). Short-chain fatty acids and gases are in general generated by fermentation of dietary organic matter (Blümmel and Ørskov 1993). In addition, the expected increase in microbial biomass due to the conversion of degraded substrate (Blümmel et al. 1999) was confirmed by a lower PF_{24} with both SH75 and SH150 substrates. The improvement of fermentation parameters, for example fermentability, could be explained by a higher content of easily fermentable carbohydrates of soybean hulls compared to corn grain which are required for ruminal microbial activity. A higher availability of nutrients, particularly nitrogen, for microbial activity could explain the linear increase in organic matter digestibility with increasing levels of soybean hulls (Elghandour et al. 2016). The results obtained in this study are in good agreement to those reported by Elghandour et al. (2016). They reported also improved fermentation kinetic parameters when replacing corn grain by soybean hulls at the same levels as in this study.

Inclusion of an extract derived from *M. oleifera* leaves enhanced microbial biomass production, organic matter digestibility, metabolizable energy, and short-chain fatty acid concentrations with the SH75 and the control substrates compared to the SH150 substrates. This observation is in good agreement with the already drawn conclusion that the effects induced by the *M. oleifera* extract were more pronounced at lower levels of fibrous carbohydrates.

Conclusion

Partial replacement of corn grain with soybean hulls enhanced the nutritional value of the substrates and resulted in a decreased methane and an increased carbon dioxide production. The *M. oleifera* extract positively affected fermentation at lower concentrations of fibrous material. However, an extract derived from *M. oleifera* leaves reduced methane production in the highest level of replacement of corn grain by soybean hulls. The results suggest that simultaneous improvements can be made toward sustainable husbandry using optimal levels of soybean hulls and *M. oleifera* extract in order to reduce methane emissions, enhance the nutritional value of feed, and partially replace a staple crop by an agricultural waste product and a perennial plant. Further experiments with different amounts of soybean hulls and different levels of an extract from *M. oleifera* leaves should focus on in vivo methane and carbon dioxide production.

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