INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1871/2020/23–5–994–1002
DOI: 10.17957/IJAB/15.1380
http://www.fspublishers.org

Full Length Article

In Vitro Evaluation of Moringa Whole Seed Cake as a Feed Ingredient to Abate Methane Emission from Ruminants

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Received 05 December 2019; Accepted 25 December 2019; Published 03 March 2020

Abstract

This study aimed to evaluate effect of replacing soybean meal with Moringa Seed Cake (MSC) on degradation of dry matter (DM) and fiber as well as methane production using *in vitro* fermentation technique. Basal diet consisted of Egyptian clover hay and concentrate (1:1). MSC was used to replace 0, 5, 7.5, 10 and 12.5% of soybean meal on total DM basis. Dry matter, neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined before and after 48 h of fermentation. Hemicellulose (hemi), cellulose and degradation of DM, NDF, ADF and hemi (dDM, dNDF, dADF, dhemi) in incubated samples were calculated. Total gas production (TGP) and methane production were measured at different intervals. Use of 5% MSC did not reduce DM degradation as compared to control. Total gas production only differed between MSC levels when expressed per gram ADF at 12 and 36 h, while all other measures of TGP (TGP per unit of degraded DM and fiber fractions) and methane did not differ between 5% MSC and control. The 12.5% MSC ration resulted in decreased TGP per unit DM and fiber fractions from 6 to 48 h of incubation compared to control. Total gas production decreased with increasing ADF and NDF and increased with increasing dNDF, but was unaffected by dADF, at any MSC level. The 12.5% MSC significantly reduced methane to a substantially (>80%). The results show that 5% MSC can be used in ruminant diets without negative effects on fiber degradation, rumen fermentation or methane production.

Keywords: Moringa seed cake; Fiber degradation; Gas production; Methane emission

Introduction

Ruminants contribute about 44% of all greenhouse gas emissions from livestock sector in form of enetric methane. Ruminants produce CH_4 as a result of rumen fermentation especially from roughages which yield more CH_4 per unit of dry matter intake (Grossi *et al.* 2019). Recent scenarios of global warming and climate change compells to devise effective strategies to mediate CH_4 emissions from ruminants through dietary interventions by changing roughage to concentrate ratios (Elghandour *et al.* 2016; Haque 2018) or using feed additives (Elghandour *et al.* 2017; Gomaa *et al.* 2018; Haque 2018). Many studies have focused to evaluate non-conventional feed ingredients beside manipulation of roughage to concentrate ratios in an effort to reduce ruminal methanogensis while increasing nutrient digestion and utilization.

Non-traditional oilseed plants such as Moringa, (*Moringa oleifera* Lam.), is a potential source of ruminant feed that does not directly compete with human food. Moringa seeds and leaves are low cost alternatives of costly imported feed ingredients. Moringa trees possess excellent vegetative propagation potential as it grows quickly to 4 m in the first year and 15 m in later development stages of maturity. Seed oil is used throughout the tropics for food, cosmetics, alternative medicine, as a biodiesel source and as a natural coagulant (Bhutada *et al*. 2016). Moringa tree can produce up to 50 to 70 kg of fruits (pods)/year with an average yield of 12 to 13 ton of seeds/ha (Bridgemohan *et al*. 2014; Ferreira *et al*. 2014). The trees can grow individually in smallholdings or in orchards for commercial harvest of the oilseeds (Ferreira *et al*. 2014). Moringa seed cake is a byproduct after oil extraction and can be used as a protein supplement, as it has a high protein residue (61.4 to 70.3% CP on DM basis) after oil extraction (Makkar *et al*. 2007). Oil extraction can be performed mechanically by pressure or using enzymes and/or solvents. However, solvent extraction is the most efficient method for industrial separation of the oil from the byproducts (Abdulkarim *et al*. 2005).

To cite this paper: Hansen HH, HM Ebeid, NE El-Bordeny, Faiz-ul Hassan (2020). *In vitro* evaluation of Moringa whole seed cake as a feed ingredient to abate methane emissions in ruminants. *Intl J Agric Biol* 23:994–1002

Many studies have been conducted on different fractions of Moringa seeds like whole seeds (Makkar and Becker 1997), seed extract (Hoffmann *et al*. 2003) and seed meal (Salem and Makkar, 2009) to evaluate its potential as a feed ingredient for ruminants. Moringa seeds contain high levels of sulfur-*containing* amino acids that may be beneficial for fiber producing animals (*e.g*., sheep and goats) especially in case of nutritionally balanced ration (Hoffmann *et al*. 2003; Anjorin *et al.* 2016). It also reported that Moringa seeds contain phytochemicals with potent antimicrobial effects (Suarez *et al*. 2005) and could be used in ruminants for CH_4 abatement (Olivares-Palma *et al*. 2013). Moringa seed cake has shown to decrease degradation of dietary protein in an *in vitro* rumen system, revealing its potential to enhance post ruminal protein supply (Hoffmann *et al*. 2003; Makkar *et al*. 2007). Most of the published research has used Moringa seed meal after removing seed coat or seed extracts. Removal of the seed coat is time consuming and increases the cost of production.

Water treatment after oil extraction has shown to reduce the amount of anti-nutritional factors in the Moringa cake. Moringa seed cake possesses almost no secondary metabolites such as alkaloids, inhibitors of trypsin and amylase, condensed tannins and lectins but contains cyanogenic glucoside, glucosinolate, saponins and phytate (Makkar *et al*. 1997).

However, MSC is a fibrous byproduct with higher levels of NDF (20 to 28%), ADF (8 to 22%) and ADL (3 to 11%) on dry matter basis (Kakengi *et al*. 2005; Olivares-Palma *et al*. 2013). In this study, we evaluated effect of replacing soybean meal with MSC on *in vitro* dry matter and fiber degradation as well as total gas and methane production.

Materials and Methods

Sample preparation and diet characteristics

Moringa pods were collected from more than 10 trees at the Agricultural Experimental Station, Faculty of Agriculture, Fayoum University. Seeds were identified by the department of Botany, Faculty of Agriculture, Fayoum University. The collected pods were transported to the Animal Nutrition Laboratory at the Animal Production Department, Ain Shams University, Egypt. The seeds (with shells) were sundried for 1 week and ground through a Wiley mill 1 mm screen. Oil was extracted from the ground seeds by petroleum ether at 40 to 60°C in a Soxhelt extractor.

Thereafter, fat-free seed meal was extracted for coagulants according to Makkar and Becker (1997). Forty gram of the meal was suspended in 2,000 mL of distilled water and stirred for 20 min at room temperature. The sample was filtered and dried at 50°C for 24 h to make seed cake.

Five levels of MSC were tested in a standard ration to replace 0, 10, 15, 20 and 25% of the concentrate mixture in the ration. Experimental ration consisted of a concentrate mixture (corn grain, wheat bran, barley, soybean meal and mineral mixture) and Egyptian clover hay. The ration was prepared using 1:1 (w/w) roughage to concentrate ratio, so that MSC comprised 0, 5, 7.5, 10 and 12.5% of the DM of the total ration. The MSC replaced the soybean meal and thereafter proportion of corn grain, barley grain and wheat bran were adjusted to ensure the diets were iso-energetic and iso-nitrogenous. The chemical composition of ingredients and experimental rations are shown in Table 1 and 2. Two hundred grams of each test ration was put into an airtight plastic jar and shipped to the *in vitro* Gas Production Laboratory, Department of Large Animal Science, University of Copenhagen in Denmark, for fermentation studies.

Experimental design

Five levels of MSC as a total mixed ration were incubated using the Ankom^{RF} gas production system (Ankom Technology Instrument and Procedure Manuals 2010). The *in vitro* gas production technique (IVGPT) measures gas from anaerobic fermentation as a measure of microbial activity and degradation of organic matter. Five (for 5, 7.5 and 12.5% MSC) and six (for 0 and 10% MSC) replicates were tested. This was done in 3 incubations, each for 48 h. In each incubation, two bottles containing inoculum but no feed (blanks) were included to establish the baseline fermentation gas production.

Chemical analyses of unfermented feed ingredients

Ration ingredients were analyzed in triplicate for DM and ash according to AOAC (1995). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and sulfuric acid lignin (ADL) contents were analyzed sequentially (Robertson and Soest 1981) using the Ankom^{200} Fiber and Daisy machines and protocols (Ankom Technology Instrument and Procedure Manuals 2010) at the Fiber and Digestion lab of the Department of Veterinary and Animal Sciences, University of Copenhagen. Neutral detergent fiber content was analyzed with 3 additions of 4 mL heat-stable α-amylase and 1:1 g sodium sulfite per gram sample in the neutral detergent solution (Hansen *et al*. 2015). NDF and ADF were expressed inclusive of residual ash and hemi and cellulose calculated from NDF, ADF and ADL values. Non-fiber carbohydrate (NFC) was calculated according to NRC (2001). Crude protein (CP) (Nitrogen x 6.25) and ether extract (EE) contents were determined according to AOAC (1995).

In vitro gas production procedure

Two cannulated Jersey heifers, owned and licensed according to Danish law (authorization nr.2012-15-2934-

Table 1: Chemical composition of experimental ration ingredients (g/kg DM)

Ration ingredients	g/kg DM[1]								
	DM	OM	NDF	ADF	ADL	CP	EE	Ash	NFC[2]
Corn grain	884.5	985.5	184.4	35.9	10.8	82.5	53.15	14.5	665.45
Soybean meal	888.8	932.7	150.6	64.6	8.0	387.6	47.8	67.3	346.7
Barley grain	910.3	974.9	478.8	54.7	14.7	89.3	31.2	25.1	375.6
Wheat bran	893.3	956.0	352.1	98.3	31.6	152.6	37.6	44.0	413.7
Moringa whole seed cake	962.4	961.6	598.7	501.1	282.8	305.6	13.1	38.4	44.2
Clover hay	924.0	867.9	409.4	268.8	58.0	174.1	39.8	132.1	244.6

[1]Average of *n*=3; coefficient of variation < 10 % for all analyses
[2] NFC = non-fiber carbohydrate, NFC = (100− [%NDF + %CP + %fat + %ash]) (NRC 2001)

Table 2: Ingredients and chemical composition of rations with different levels of defatted, coagulant extracted whole Moringa seed cake (MSC)

% MSC in ration	Moringa whole seed cake rations				
	0 MSC	5% MSC	7.5% MSC	10% MSC	12.5% MSC
Ingredients, % of DM					
Clover hay	50.0	50.0	50.0	50.0	50.0
Moringa whole seed cake	0	5	7.5	10	12.5
Corn	19.3	17	11	4.5	14.9
Barley grain	5.8	6	12	17.8	5.5
Soyabean meal	11.5	7.8	6	4	2.3
Wheat bran	12.2	13	12.3	12.5	13.6
Di- Ca-P	0.15	0.15	0.15	0.15	0.15
Minerals &Vitamins	0.15	0.15	0.15	0.15	0.15
NaCl	0.4	0.4	0.4	0.4	0.4
Limestone	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100
Ration chemical composition, g/kg					
Dry matter	908.1	912.0	915.3	918.7	917.5
Organic matter	904.6	905.1	905.0	904.8	906.1
Neutral detergent fiber	328.3	352.2	379.7	408.1	384.7
Acid detergent fiber	163.9	186.7	198.5	210.7	220.3
Acid detergent lignin	36.7	50.6	57.5	64.7	71.2
Crude protein	171.3	171.8	171.8	171.8	172.1
Ether Extract	42.1	40.1	38.0	35.8	37.4
Ash	95.4	94.9	95.0	95.2	93.9
Non-fiber Carbohydrate[1]	362.9	341.0	315.5	288.1	311.9
Hemicelluose	16.44	16.55	18.12	197.4	164.4
Cellulose	127.2	136.1	141.0	146.0	149.1
DE, MJ/kg[2]	11.55	11.56	11.58	11.58	11.58

[1]Non-fiber carbohydrate = (100 − (%NDF + %CP + %fat + %ash)) (NRC 2001)
[2]DE = GE × 0.76 (NRC, 2001). GE = (Crude protein × 4) + (Carbohydrates × 4) + (Fats × 9); Carbohydrates = OM – (CP + EE) (Maynard *et al*. 1979)

00648), and maintained by the University Department of Experimental Animals, were used to obtain rumen fluid. The fistulated heifers were fed a maintenance level diet consisting of grass silage (1.5 kg DM/d; 11.3 MJ/kg DM; 7.5% CP), supplemented with daytime grazing on a ryegrass pasture (13 MJ/kg DM; 18% CP) for 2 weeks before the experiment. A 500 mg sample from each MSC ration was weighed into 100 mL Duran® bottles fitted with an automatic wireless *in vitro* gas production module (Ankom Technology, Macedon, NY, U.S.A.) with pressure sensors (pressure range: from −69 to +3,447 kPa; resolution: 0.27 kPa; accuracy: ± 0.1% of measured value). Each module sends measurements via a receiving base station to an attached computer. Gas tight bags (FlexFoil, 1 L; SKC Ltd., Dorset, U.K.) were flushed with CO_2 and emptied with vacuum suction. The flushed bag was attached to the module for collection of gas produced in the headspace of the bottles. The accumulated gas was automatically released (250 milliseconds vent opening) when the pressure inside the units reached 34.47 kPa above the ambient pressure. The absolute pressure was recorded every 10 min to calculate cumulative pressure.

A buffer solution was prepared before addition of rumen fluid as reported by Menke and Steingass (1998) and flushed with CO_2 for 3 h at 39.5°C. Sodium hydroxide and sodium sulfide were added to the buffer shortly before addition of rumen fluid. The rumen fluid, including particulate matter, was collected before morning feeding and transported to lab in preheated thermos bottles. Rumen fluid was filtered through two layers of cheesecloth to eliminate large feed particles. Moreover, particulate material was squeezed to collect microbes attached to feed particles. A 2:1 buffer to rumen fluid ratio was used. Ninety mL of this inoculum was added to each bottle after which the headspace of each bottle was flushed with CO_2, closed with the module head, and incubated in a Thermoshaker at 39.5°C for 48 h. The initial pH of the inoculum was 6.85 + 0.03. After 48 h of incubation, the IVGPT units were put into an ice bath. The residual material in each bottle was filtered into a prepared ANKOM F57 filter bag (Ankom Technology, Macedon, NY, USA).

Dry matter degradability

Degraded DM (dDM) was calculated as the difference between the sample DM content and residual DM after incubation. Residual DM of the sample was corrected for microbial DM after filtration of 2 bottles to which only the rumen fluid and buffer media was added (blank units). NDF and ADF of the residuals after fermentation were determined with the same methods as used for analysis of feed ingredients. Amount of degraded NDF (dNDF), ADF (dADF) and hemicellulose (dhemi) was calculated as the difference between the calculated contents in the sample before and measured contents after incubation. All variables (DM, NDF, ADF, hemi, mL gas, and mL methane) were reported in proportion to sample DM or to sample contents of NDF, ADF, hemi, dDM, dNDF, dADF and dhemi.

Estimation of total gas and methane production

The pressure of the accumulated gas was converted into volume (mL) per gram DM sample at standard pressure and temperature (STP). The average baseline gas measured in the blank units was subtracted to calculate blank corrected gas production. Values at 0, 6, 12, 24, 36 and 48 h were used for statistical comparisons. Methane content in the gas-tight bags was measured directly after the end of incubation by gas chromatography (GC) system (Agilent 7820A GC, Agilent Technologies, Santa Clara, C.A., U.S.A.). The GC was equipped with a HPPLOT Q column (30 m × 0.53 mm × 40 µmm), with H_2 as the carrier. Column flow was 5 mL/min and the TCD detector was set to 250°C with a reference and make up flow of 10 mL/min. A 250 μL gas sample was taken from each gas bag and manually injected into the GC machine. Run time was 3 min at an isothermal oven temperature of 50°C. Calibration curves were calculated from standards containing 1, 2.5, 5, 10, 15 and 25% CH_4 in nitrogen (Mikrolab A/S, Aarhus, Denmark). Total methane produced was calculated from TGP and content of methane determined by GC.

Statistical analysis

All data were analyzed using either the linear models LM (Pinheiro *et al*. 2015) or mixed model LMER procedures (Bates *et al*. 2015) in the R software (R Core Team 2013). In LMER model, level of MSC in ration (α) was considered as fixed effect while repeated incubations (β) as random effect. No significant interaction was found between repeated incubations and blank corrected gas production, and the model was therefore reduced to exclude interactions. Tukey HSD contrasts were used to compare treatment means. Differences were deemed significant when $P < 0.05$. The final model tested was;

$$Y_{ijk} = \mu + \alpha_i + \beta j + \varepsilon_{ijk,}$$

Where Y_{ijk} is the blank corrected gas production at a given time, or degraded DM, NDF, ADF or hemi using the given i^{th} level of α in j^{th} run; μ is the overall mean, α_i is the fixed effect of the level (Moringa whole seed cake in the DM= 0, 5, 7.5, 10 and 12.5%); β_j is the random effect of fermentation run, and ε_{ijk} is the residual error term. The error terms and random-effects variables were assumed to have a normal distribution with mean zero and variance ε^2_{ijk} (residual error). Model validation was carried out using visual inspection of residuals and Cook's distances.

Results

Feed ingredients and ration composition

Chemical composition of feed ingredients used in this experiment is presented in Table 1. The residue, after oil extraction followed by water treatment of unshelled ground MSC showed 21% less CP than soybean meal. The cell wall contents of MSC were greater than in any of the other nutrients. As expected, extracted MSC contained very limited lipid contents. The chemical compositions of the 5 rations were calculated to be iso-energetic on a digestible energy (DE) basis (11.578 to 11.593 MJ/kg DM) and iso-nitrogenous (17.13 to 17.21% CP) on a DM basis (Table 2). The rations contained 90 to 91% organic matter. The contents of NDF, ADF, hemi, cellulose and ADL of the ration increased while NFC decreased when MSC contents were increased.

Dry matter degradability

Significant difference was found in the amount of degraded DM, dNDF, dADF and dhemi (g/kg DM) when comparing the 0 to 12.5% MSC (Table 3). Degradation of DM in 5% MSC ration was not significantly different from 0% MSC, while 7.5 and 10% MSC significantly decreased dDM by 8 and 11% in comparison to the 0% MSC (control). Same trend was observed for dNDF and dADF with a decrease of 12 and 26% for 10% MSC and 17 and 24% for 12.5% MSC compared to control. Amount of degraded hemicellulose was only significantly different in 12.5% MSC when compared to control.

Degradation of DM and NDF (Y_{dDM}, Y_{dNDF}) were described by linear equations, within the limits of the samples, as a function of increasing MSC (X_{MSC}) in the ration, (Fig. 1A and B). Decrease in degradation of DM and NDF was equivalent to approximately 8 g/kg feed for each percent increase in MSC in the feed on DM basis. This linear relationship explained more than 62% of the sample variation ($P < 0.05$). The prediction interval showed that use of 5% MSC resulted in between 702 and 777 g/kg dDM and between 646 and 576 g/kg dNDF, 95% of the time.

a) $Y_{dDM} = 728.6 - 7.8\ X_{MSC}$; R-squared = 0.6295; $P < 0.0001$;

b) $Y_{dNDF} = 595.3 - 8.0\ X_{MSC}$; R-squared = 0.6513; $P < 0.0001$.

As expected, dDM decreased (1.7 g/kg) for each gram increase in ADF in the ration (Fig. 2). This increase in ADF

Table 3: Average degradation values of dry matter (dDM), neutral detergent fiber (dNDF), acid detergent fiber (dADF), and hemicellulose (dhemi) (g/kg DM) after 48 h fermentation of Moringa whole seed cake rations (MSC)

Items	Moringa whole seed cake rations				
% MSC in ration	0 (*n* = 6)	5% (*n* = 5)	7.5% (*n* = 5)	10% (*n* = 6)	12.5% (*n* = 5)
dDM	729.7^{a}	688.9^{ab}	667.7^{b}	650.8^{bc}	632.8^{c}
dNDF	591.0^{a}	560.3^{ab}	538.1^{b}	518.6^{bc}	487.6^{c}
dADF	423.7^{a}	398.9^{ab}	347.1^{bc}	312.8^{c}	321.6^{c}
dhemi	757.9^{a}	742.3^{abc}	747.5^{ab}	738.3^{abc}	710.0^{cd}

$^{a, b, c, d}$Means within a row with different superscripts differ ($P < 0.05$)

reflects the increasing MSC level.

$Y_{dDM} = 1007.9 - 1.70\ X_{ADF}$; R-squared = 0.6295; $P < 0.0001$.

Total gas and methane production

Total accumulated gas decreased with increasing MSC levels (Table 4), in agreement with the result of decreasing DM degradation. Total gas production per gram dDM (TGP/dDM) ranged from 325 to 371 mL/g dDM at 48 h, but was not significantly different between rations at any of the extracted times (data not shown). With 12.5% MSC in the diet, TGP per gram DM (TGP/DM) was significantly less (between 19 and 20%) than the control and 5% MSC at 24, 36 and 48 h. However, the 7.5 and 10% MSC levels showed intermediate values for TGP/DM which were not statistically different from control or other MSC levels. The TGP/DM was more rapid during early stages of fermentation (average of 6.6 mL/g DM·h from 0 to 24 h) for all treatments than later stages (average of 1.8 mL/g DM·h from 24 to 48 h).

TGP per unit of dietary fiber (NDF and ADF) was significantly less for 12.5% MSC levels than for 0 MSC (Table 5). The use of 10 and 12.5% MSC in the diet consistently resulted in significantly less TGP per gram NDF (TGP/NDF) from 6 h and TGP per gram dNDF (TGP/dNDF) after 24 h compared to the 0 MSC. Interestingly, a numerical depression in TGP/dNDF was seen when using 10% MSC as compared to 12.5% MSC. In 10% MSC, the TGP/dNDF was significantly different from control at 6 h, but was similar to 12.5% MSC. There were no significant differences in TGP per gram dADF (TGP/dADF) between MSC levels at any time (data not shown). This lack of significance supports the evidence that TGP is determined by the degradation of the less digestible fiber components. Total gas production per gram hemi (TGP/hemi) or TGP per gram dhemi (TGP/dhemi) was always significantly less in 10% MSC as compared to control.

Total methane production at 48 h expressed as mL per gram of sample DM and fiber fractions (CH_4/DM, CH_4/dDM, CH_4/NDF, CH_4/dNDF, CH_4/ADF, CH_4/dADF, CH_4/hemi and CH_4/dhemi), decreased significantly with inclusion of 12.5% MSC (Table 6). However, amount of methane produced with 5 and 7.5% MSC was not significantly different from control on DM basis or for any of the fiber fractions or their degraded portions. Use of 10% MSC resulted in significantly less quantity of methane per g NDF and ADF compared to control (0% MSC).

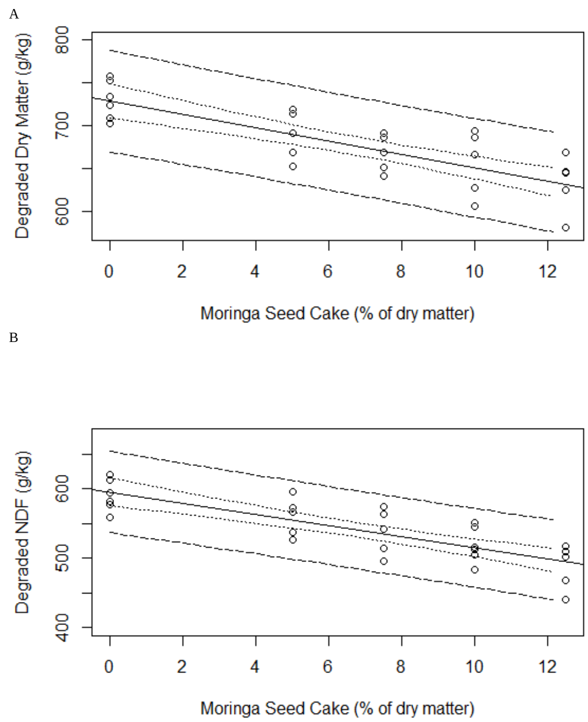

Fig. 1: Linear effect (solid line) of increasing levels of Moringa whole seed cake from 0 to 12.5% of ration DM on: (**A**) dry matter degradation value (dDM, g/kg) and (**B**) degradation value of neutral detergent fiber (dNDF, g/kg). 95% confidence and prediction intervals shown by dotted and dashed lines respectively

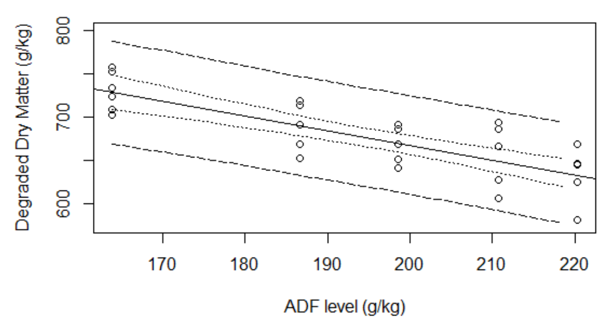

Fig. 2: Linear effect (solid line) of increasing levels of acid detergent fiber (ADF, %), in Moringa whole seed cake rations on dry matter degradation value (% dDM). 95% confidence and prediction intervals shown by dotted and dashed lines respectively

Table 4: Gas produced (mL)[1] per gram DM during *in-vitro* fermentation of rations with increasing levels of Moringa whole seed cake (MSC)

% MSC in ration/ Time, h	Moringa whole seed cake rations				
	0 (*n* = 6)	5% (*n* = 5)	7.5% (*n* = 5)	10% (*n* = 6)	12.5% (*n* = 5)
6	139.1^{a}	139.5^{a}	138.1^{a}	132.2^{a}	126.7^{a}
12	183.3^{a}	181.7^{ab}	177.3^{ab}	166.2^{ab}	154.8^{b}
24	234.3^{a}	231.3^{a}	225.3^{ab}	207.9^{ab}	188.5^{b}
36	252.7^{a}	249.1^{a}	241.2^{ab}	225.2^{ab}	201.6^{b}
48	257.1^{a}	255.3^{a}	247.3^{ab}	229.2^{ab}	205.9^{b}

$^{a, b, c}$ Means within a row with different superscripts differ ($P < 0.05$)
[1]Means of gas produced per gram sample DM at standard temperature and pressure

Table 5: Gas produced (mL)[1] per gram DM of fiber fraction during *in-vitro* fermentation of rations with increasing levels of Moringa whole seed cake (MSC)

Item		Moringa whole seed cake rations				
		% MSC in ration				
	Time, h	0 (*n* = 6)	5% (*n* = 5)	7.5% (*n* = 5)	10% (*n* = 6)	12.5% (*n* = 5)
Total gas production per gram NDF	6	423.8^{a}	396.0^{ab}	363.6^{bc}	324.0^{d}	329.4^{cd}
	12	558.4^{a}	515.86^{ab}	467.0^{bc}	407.1^{c}	402.4^{c}
	24	713.68^{a}	656.92^{ab}	593.3^{bc}	509.4^{c}	490.0^{c}
	36	769.8^{a}	707.28^{ab}	635.3^{bc}	551.7^{c}	523.9^{c}
	48	783.1^{a}	724.78^{a}	651.3^{bc}	561.6^{c}	535.3^{c}
Total gas production per gram degraded NDF	6	718.6^{a}	709.0^{ab}	679.3^{ab}	626.8^{b}	678.0^{ab}
	12	947.0^{a}	923.2^{ab}	872.0^{ab}	787.1^{b}	826.4^{ab}
	24	1210.4^{a}	1174.6^{ab}	1105.7^{ab}	985.0^{b}	1005.6^{ab}
	36	1305.7^{a}	1264.4^{ab}	1183.0^{ab}	1067.1^{bc}	1075.0^{bc}
	48	1328.3^{a}	1295.4^{ab}	1211.9^{abc}	1086.3^{c}	1098.3^{c}
Total gas production per gram ADF	6	848.9^{a}	747.2^{b}	695.6^{b}	627.6^{c}	575.3^{c}
	12	1118.5^{a}	973.2^{b}	893.3^{bc}	788.7^{cd}	702.7^{d}
	24	1429.3^{a}	1239.3^{ab}	1134.9^{bc}	986.5^{cd}	855.7^{d}
	36	1541.9^{a}	1334.3^{b}	1215.3^{bc}	1068.6^{cd}	914.9^{d}
	48	1568.6^{a}	1367.3^{ab}	1245.8^{bc}	1087.8^{cd}	934.8^{d}
Total gas production per gram hemicellulose	6	846.3^{a}	842.8^{ad}	762.0^{b}	669.8^{c}	770.9^{bd}
	12	1115.1^{a}	1097.7^{ab}	978.6^{ab}	841.7^{d}	941.7^{bd}
	24	1425.0^{a}	1397.9^{ab}	1243.2^{abc}	1053.0^{c}	1146.6^{c}
	36	1537.2^{a}	1505.1^{ab}	1331.3^{abc}	$1,140.6^{c}$	$1,226.0^{c}$
	48	1563.9^{a}	1542.3^{ab}	1364.8^{abc}	1161.1^{c}	1252.7^{c}
Total gas production per gram degraded hemicellulose	6	1118.4^{a}	1137.7^{a}	1021.7^{ab}	910.1^{c}	1087.7^{a}
	12	1474.0^{a}	1482.0^{a}	1311.5^{ab}	1143.1^{b}	1327.4^{a}
	24	1884.0^{a}	1886.8^{ab}	1665.6^{ac}	1430.2^{c}	1616.4^{abc}
	36	2032.4^{a}	2031.1^{ab}	1782.4^{ac}	1549.4^{c}	1728.0^{abc}
	48	2067.5^{a}	2080.9^{ab}	1826.8^{ac}	1577.2^{c}	1765.2^{abc}

^{a,b,c} Means within a row with different superscripts differ ($P < 0.05$)
[1]Means of gas produced at standard temperature and pressure

Discussion

The objective of this study was to evaluate effect of replacing soybean meal with MSC on dry matter and fiber degradation in ruminants. Moreover, we also studied effect of inclusion of MSC in ration on total gas and methane production. The MSC used in this research was whole seed MSC (seed kernel and seed coat after removal from the pod) which was solvent extracted (with ether) and treated with water. Information about comparable MSC composition is limited as most of the studies used seed meal or seed extract.

Results of our study revealed that whole seed cake used in this experiment had a greater NDF, ADF, and ADL contents (Table 2) than those reported by Kakengi *et al.* (2005), who found 27.7; 22.2 and 11.0% for NDF, ADF and ADL, respectively, in pressed whole seeds. However, mechanical pressing and solvent extraction (with petroleum ether) are quite different methods of making seed cake. Olivares-Palma *et al.* (2013) also reported fiber components of pressed Moringa oil seed cake and found 39.6, 42.1, and 25.5% lower levels of NDF, ADF and ADL, respectively, compared to our study.

Despite the higher fiber fraction, the CP content of the MSC found in this experiment is similar (30.6% *vs*. 30.8%) to results obtained by Kakengi *et al.* (2005). Anwar *et al.* (2006) reported similar results of CP (29.6 to 31.3% of DM) in pressed Moringa whole seeds and shelled seeds. However, these CP contents were different from Olivares-Palma *et al.* (2013) and Morais *et al.* (2015) who reported a substantially higher level of CP (58 and 57%) in a commercially available Moringa pressed seed cake (a byproduct of biodiesel production). Makkar *et al.* (2007) reported that Moringa seed hulls contain low CP content (99 g/kg) but very high fiber fraction (NDF, ADF and ADL: 842, 805 and 452g/ kg, respectively). Anwar *et al.* (2006) observed a 30:70 DM ratio between hulls and kernels. The

Table 6: Methane (CH_4) produced (mL)[1] at 48 h per unit sample dry matter (DM) or fiber fraction from *in-vitro* fermentation of Moringa seed cake rations (MSC)

Item	Moringa whole seed cake rations				
	0 (n = 6)	5% (n = 5)	7.5% (n = 5)	10% (n = 6)	12.5% (n = 5)
CH_4 per gram DM	29.7[a]	28.7[a]	26.8[a]	18.6[ab]	7.6[b]
CH_4 per gram degraded DM	41.7[a]	41.4[a]	39.9[a]	28.5[ab]	11.8[b]
CH_4 per gram NDF	90.4[a]	81.4[a]	70.5[a]	45.5[b]	19.8[b]
CH_4 per gram degraded NDF	129.2[a]	113.4[a]	109.6[a]	69.9[ab]	38.3[b]
CH_4 per gram ADF	181.0[a]	153.5[a]	134.9[ab]	88.2[b]	34.6[b]
CH_4 per gram degraded ADF	427.7[a]	379.7[a]	390.5[a]	294.8[ab]	108.1[b]
CH_4 per gram hemicellulose	180.5[a]	173.2[a]	147.8[a]	94.1[ab]	46.3[b]
CH_4 per gram degraded hemicellulose	238.4[a]	234.5[a]	196.3[a]	128.0[a]	64.4[b]

[a, b, c] Means within a row with different superscripts differ ($P < 0.05$)

[1]Means of mL methane produced at standard temperature and pressure

differences between nutrient compositions are probably due to different varieties, plant phenology and/or plant production systems. Higher ADL and reduced protein contents of Moringa seeds in present experiment suggest that seeds were more mature than earlier studies as lignin content increases with increasing seed age (Jung and Allen 1995). Therefore, appropriate seed age should be considered for harvesting keeping in view oil and other dry matter contents while making seed cake.

Inclusion of 5% MSC did not decrease DM or fiber degradation but addition of >5% MSC in ration decreased dDM, dNDF and dADF as compared to control (Table 3, Fig. 2) with a maximum significant loss (13%) of dDM with 12.5% MSC. This negative linear relationship between dDM and MSC level is in complete contrast with findings of Olivares-Palma *et al.* (2013), who replaced a basal diet of dried beard grass *(Brachiaria brizantha)* grass with 10, 20 and 40% MSC in an IVGPT study. They reported a non-significant effect of MSC on methane production but increase in in vitro DM degradability (50 to 51%) with increasing Moringa seed cake was observed. Morais *et al.* (2015), also found a linear increase in degradation from 53 to 60% (Y_{dDM}=51.51 + 0.08$X_{MSC\%}$; R^2 = 0.5; p = 0.0004) when substituting 0, 30, 50 and 70% dried elephant grass *(Pennisetum purpureum)* DM with commercial Moringa seed cake in an IVGPT experiment.

The Moringa seed cake used by Morais *et al.* (2015) had higher CP and EE contents than our study and was used as a substitute in a basal diet consisting of dried grass. The fiber contents of grasses in both studies were greater than Moringa seed cake, which subsequently decreased cumulative fiber contents in experimental ration with increasing substitution. Salem and Makkar (2009) supplemented defatted Moringa seeds (pure kernels without shells) at extremely low levels (0, 0.19, 0.34 and 0.52% of DM intake) together with soybean meal and oat-vetch hay to growing lambs with approximately 1:10 concentrate to roughage DM ratio. They observed a significant increase in the apparent digestibility of NDF per gram organic matter (60 to 65%) but no significant difference in the apparent digestibility of DM, CP and OM with increasing substitution. The NDF contents of the MSC in the present experiment were 5 times higher than MSC used in mentioned study (Salem and Makkar 2009). However, in contrast to our study, they used basal ration consisting of oat vetch hay which contained 59% NDF, which resulted in a higher total fiber contents in the basal diet.

Higher level of nutrients, lower fiber and oil contents of the basal ration in the present study compared to previous studies (Salem and Makker 2009; Olivares-Palma *et al.* 2013; Morais *et al.* 2015) is most probable explanation for the variations observed in dry matter degradation or digestibility. Moreover, fiber contents of the ration in these studies decreased with substitution, while in our study fiber contents increased with increasing MSC level. The decrease in dDM, dNDF and dADF in conjunction with the increase of substitution with MSC in the present experiment was expected due to higher fiber and lignin contents of Moringa seed cake (Table 2). However, presence of anti-microbial compounds, cyanogenic glucoside, glucosinolate and/or cationic polyelectrolyte proteins may also have influenced rumen microbial activity subsequently leading to decrease DM, NDF and ADF degradation, as suggested previously (Makkar and Becker 1997; Makkar *et al*. 2007).

Increasing levels of MSC reduced TGP from 6 h, when expressed as TGP per gDM or fiber (NDF and ADF) and ADL contents (Tables 4 and 5). Specifically, use of 12.5% MSC significantly reduced TGP per gram DM, NDF, ADF and hemi. These results are in line with decreased DM degradation. Increase in MSC level did not decrease TGP/dDM or TGP/dADF supporting the assumption that gas production is determined by the dDM and this in turn is influenced by the ratio of easily digestible to lesser digestible components.

Gas production from the basal diet at initial stage in present study was much higher (5.5 times) than observed with grass based diet used by Olivares-Palma *et al.* (2013) but after 48 h of fermentation, we observed only 1.6 times more gas per gram DM. Similarly, our basal diet produced much more gas after 48 h of fermentation than basal diet used by Morais *et al.* (2015). Both above mentioned studies reported a lower DM degradation than observed in present study. However, effective digestibility calculated from digestion kinetics reported by Olivares-Palma *et al.* (2013) was between 1.1 and 1.4 times greater than the actual degradation observed in our study. This indicates either a very slow degradation rate and/or a very slow rumen fractional disappearance. Despite the increased digestibility, both studies reported decrease in gas production with increasing Moringa seed cake substitution. However, we observed non-significant differences in TGP/dDM with increasing levels of MSC substitution which is in agreement with earlier studies (Olivares-Palma et al., 2013). The substantially different levels of gas produced at the highest level of MSC inclusion (93 *vs*. 364.9 mL/g dDM) suggest variation in rumen microbial activity between the studies. These variations suggest different microbial population diversity and activity in the experiments and warrant further studies to explore effect of MSC on rumen microbial populations to better explain these findings.

The significant reduction in TGP/dNDF, while non-significant difference in TGP/dADF between the lowest and highest levels of MSC suggests that hemi could be a determinant for gas production. Heterogeneous hemi composition and/or hemi interactions with ADL can stimulate specific microbial population growth. This could explain why variable hemi degradation with same TGP /dDM was observed in present study.

Significant reduction in CH_4 per unit of degraded fiber contents (dNDF, CH_4/dADF and CH_4/dhemi) also suggests possible inhibition of methanogens by phytochemicals present in MSC. A reduction in methane production has also been reported by Olivares-Palma *et al*. (2013) who found a significant 50% reduction in methane production per gram dDM with MSC substitution (from 0 to 40%) in diets. Inclusion of 12.5% MSC showed significant reduction in CH_4/DM, CH_4/dDM, CH_4/NDF, CH_4/dNDF, CH_4/ADF, CH_4/dADF, CH_4/hemi and CH_4/dhemi by 74, 72, 78, 70, 81, 75, 74 and 73%, respectively, compared to control in this study. These findings revealed much greater extent of reduction in methane in proportion to the reduction observed in TGP (20%) suggesting greater impact from inhibition of specific methanogens. However, inclusion of 7.5% MSC did not significantly reduce methane production on a DM or fiber content basis, suggesting that methane production due to MSC fiber components is not linear. This may be due to a minimum threshold level of phytochemicals required to inhibit methanogens. A reduction in the degraded DM and fiber fractions should be considered together with reduced CH_4 emission, as methane represents dietary energy that potentially can be used to replace energy not available due to reduced degradation. Total methane produced from fermentation of basal diet (29.7 mL) represented 9% of DE of the experimental rations. With inclusion of 12.5% MSC in the diet, reduction in methane (mL) accounted for about 0.804 MJ or 7% of the total DE of the diet. However, degraded DM, NDF and ADF decreased significantly (13, 17 and 24%, respectively) with increase from 0 to 12.5% MSC in the ration. Therefore, the decrease in methane production will most likely be unable to compensate for the reduced digestibility *in vivo* even with 100% utilization of the lost methane to digestible energy. Previous studies have shown that 16% decrease in methane production through supplementation of nitrate in dairy cows, did not correspond to any significant increase in milk production. These findings indicate that cows were unable to utilize energy that became available through reduction of methane (Zijderveld *et al*. 2011). No previous studies regarding dietary energy balance with reduction of methane through MSC substitution is available to date, therefore it is unknown whether dietary energy saved through methane reduction could be utilized for production practically as theoretically suggested. However, *in vivo* studies with appropriate levels of MSC in ruminants can provide insights on its prospects to reduce methane emission while improving energetic efficiency.

Inadequate NFC contents in a ration usually reduce energy for rumen microbes thereby subsequently decreasing fiber digestion, ultimately leading to reduced volatile fatty acids and microbial protein synthesis (NRC 2001). The recommended levels of NFC range from 30 to 40% of the DM in a ration designed for high producing lactating cows (Nocek, 1997). This suggests that the relationship between NFC and fiber fractions of a ration is critical for optimum rumen function. Interestingly, only the 10% MSC diet contained less than 30% NFC, but that did not show any adverse effects on microbial activity (as evidenced from gas production) as compared to control (0% MSC). Non-fiber carbohydrate (NFC) is calculated using the total NDF content, including hemi, which may not be singularly degraded. The linear relationship of DM degradation as a function of MSC level (fiber) observed in our data, show that the level of MSC in the ration (on DM basis) as well as the ADF contents (Fig. 1(A&B) and 2) can be used to predict dDM. However, *in vivo* trials are required to refine these relationships and confirm potential activity of MSC to reduce methane emission from ruminants and its scope as feed ingredient to replace costly dietary protein sources.

Conclusion

Our study showed that inclusion of 12.5% MSC in a ration with 1:1 roughage to concentrate ratio reduces dietary value of the ration. The actual and predicted reduction in either degraded DM or NDF could be a useful tool in ration planning based on MSC cost, availability and production needs. Moringa whole seed cake significantly reduced methane production above inclusion level of 7.5% in the diet which shows its potential to improve dietary energetic efficiency. However, hemicelluose and/or their interactions with lignin in MSC may inhibit fiber degradation which needs further studies with different dietary fiber sources. Our findings suggest a need to investigate potential effects of MSC, and/or its major phytochemical contents, on rumen microbial populations, specifically methanogens, to understand the mechanisms of methane reduction and fiber degradation. Moreover, our findings show that 5% MSC could be recommended to replace soybean meal without negative effects on fiber degradation, rumen fermentation or methane production.

Acknowledgements

Thanks are given to Lotte Ørbæk, Majbritt Toldbod Nielsen and Konstantinos Manolopoulos for their help in the laboratory. Dr. Hossam Ebeid gratefully acknowledges the scholarship from the Danish Agency for Higher Education for his stay in Denmark.

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