



Moringa Leaves and Rumen Fermentation  
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## Effect of Different Levels of Moringa Leaves (*Moringa oleifera*) on Rumen Fermentation Pattern: An *In Vitro* Study

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### ABSTRACT

A study was conducted to determine the potential of different levels of *Moringa oleifera* leaves powder as an additive in total mixed ration (TMR) by assessing its effects on *in vitro* rumen fermentation characteristics. Concentrate mixture was replaced by different levels of Moringa leaves (ML) (0, 5, 10, 15, 25, 30, 40 and 50%) in a TMR (Roughage: Concentrate 60:40). They were incubated for 24 h at 39°C in 100 ml calibrated glass syringes containing buffered rumen fluid. For every treatment, rumen fermentation parameters and methanogenesis was studied. It was observed that ML could improve the degradability of dry matter and organic matter, gas production, short-chain fatty acids production and reduce methane production compared to TMR when fermented alone. The presence of ML in the total mixed ration exerted a positive associative effect on rumen fermentation parameters which resulted in an increase in net gas production, true degradability of dry matter, true degradability of organic matter and microbial biomass production with the simultaneous reduction in methanogenesis when compared TMR alone. The addition of higher levels of ML resulted in increased volatile fatty acid production and ammonia nitrogen. The study revealed that the incorporation of ML improved the fermentation pattern of TMR and reduced methane emission.

**KEYWORDS:** *In vitro* study, *Moringa oleifera* leaves, Methanogenesis, Net gas production, Total mixed ration

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### INTRODUCTION

Nutrient deficiency is a major constraint in animal production systems, especially in low output and low input systems. In low production systems, farmers depend on poor quality roughages available as the sole source of nutrition for animals. Improvement in the activity of rumen microbiota to degrade plant cell wall is highly desirable and usually leads to improved animal performance. For the rumen ecosystem, the priorities are fermentable nitrogen, together with trace quantities of microbial growth stimulants such as peptides/amino acids, minerals, vitamins and highly digestible fibre (Leng and

Preston, 1983; Krause et al., 2003). Rumen microbes predominantly ferment fibre and benefits animal with the fermentation end products such as volatile fatty acids (VFA) and microbial protein. Improving the capacity of fermentation of rumen microbes has been a core subject in ruminant nutrition for many decades. Supplementing such poor quality roughages with balanced concentrate mixture can improve the nutrition of animal's decreases activity of cellulolytic rumen micro-organisms and frequently depresses feed intake and fibre digestibility (Calsamiglia et al., 2008). However, high cost of concentrate mixture limits its use for small-holder farmers. *Moringa oleifera* (Moringa)

is a rapidly growing perennial tree belonging to Moringaceae family. Moringa leaves (ML) with various bioactive compounds act as remedy to fight malnutrition and also possesses many pharmacological properties such as anti-cancer, anti-diabetic, anti-inflammatory and antioxidant (Ma et al., 2020). ML and seeds are also used for wastewater treatment with positive improvement in environmental and human health (Boulaadjoul et al., 2018; Vunain et al., 2019; Ang and Mohammad, 2020). ML are rich source of proteins, minerals, vitamins and some growth promoting factors (Makkar and Becker, 1996). It is a tropical multipurpose tree with the potential to create favourable rumen environment by alleviating deficiencies impeding microbial fermentation. This study, therefore, aimed to examine the potential of ML addition on *in vitro* rumen fermentation characteristics and methanogenesis.

## MATERIALS AND METHODS

### Source of Moringa leaves

The ML were harvested from a plantation in farm section of National Dairy Research Institute, Karnal. Other lots of leaves were obtained from different locations.

### Experimental design and substrate preparation

The leaves of moringa were dried at field shed on a plastic sheet. Green maize and wheat straw were dried in hot air oven at 60°C for 48 h till a constant weight was attained. The dried samples were ground through 1mm sieve using electrically operated laboratory Willey mill. The nutritional worth of various TMRs formulated by mixing of green maize, wheat straw and various concentrate mixtures in 45:15:40 ratios, respectively was determined by using *in vitro* gas production technique (Menke and Steingass, 1988). ML was incorporated by replacing concentrate mixture at the incremental levels of 5, 10, 15, 20, 25, 30, 40 and 50% of dry matter. Ingredient composition of concentrate mixture was maize grain 28%, bajra grain 5%, ground nut cake 10%, soyabean meal 15%, mustard oil cake 13%, wheat bran 15%, rice polish 11% mineral mixture

and vitamins premix 2% and salt 1%. The control TMRs was devoid of Moringa leaves otherwise with similar forage to concentrate ratio (60:40). Total mixed rations with similar incremental levels were balanced for CP.

### Donor animals and rumen liquor

Two rumen cannulated adult Murrah buffalo steers, fitted with permanent rumen cannula, served as donor of rumen inoculum for *in vitro* studies. Animals were fed on maintenance ration consisting of wheat straw, green maize and concentrate mixture. Clean drinking water was provided free choice to the animals housed in well-ventilated shed with provision of individual feeding. Rumen liquor collected before the morning feeding was pooled and brought to the laboratory in a pre-warmed thermo flask (preheated at 39°C) for further use. All laboratory handling of strained rumen liquor was done under continuous flushing with CO<sub>2</sub> to maintain anaerobic conditions.

### *In vitro* incubation of substrate

Each TMR (200 mg±5mg) was incubated in 100 ml calibrated glass syringes filled with 30 ml of buffered rumen inoculum and placed in the water bath at 39±0.5°C for 24 h (Menke et al., 1979; Menke and Steingass, 1988). The syringes were shaken after every 30 minutes for initial 2 h from the start of the incubation and thereafter at every 2 h interval up to 24 h of incubation to prevent the plunger from picking up substrate and proper mixing of feeds with rumen inoculum. The substrate was incubated for 24h and at the end of incubation, the level of piston was again recorded (final reading). Incubations were also carried out along with respective blanks and standard in triplicate.

### Estimation of total gas and methane production

Gas produced (mL/200 mg substrate) by fermentation during 24 h was measured from movement of piston upside by visual assessment of the calibrated scale on the syringe and net gas produced by the substrate was calculated from corresponding blank values. After 24 h incubation, suitable (200µl) aliquot of gas was withdrawn from

the tip of the incubation syringe using air tight Hamilton syringe and analyzed for its methane with the help of Gas chromatograph (Nucon 5700, India) fitted with stainless steel column packed with Porapak-N and Flame Ionization Detector (FID). The temperature of injector, column and detector was 40°, 50° and 100°C, respectively. The flow rate of carrier gas (nitrogen), air and fuel gas (IOLAR grade hydrogen) through the column was 30, 300 and 30 mL/min. Gas sample (2mL) from the syringe (5mL) was injected into the GC through injection port. The standard gas used for methane estimation (Spantech House, Surrey, England) composed of 50% methane and 50% CO<sub>2</sub>. The peak of methane gas was identified on the basis of retention time of standard methane gas and the response factor obtained was used to calculate methane percentage in the gas sample.

#### Individual volatile fatty acid estimation (IVFA)

Individual VFAs were estimated using gas chromatography (Nucon 5700, Nucon Engineers, New Delhi) equipped with flame ionization detector and stainless steel column packed with chromosorb-101. After 24 hours of incubation, 5 mL of supernatant was treated with 1 mL of 25% metaphosphoric acid and kept overnight at 4°C (Erwin et al., 1961). The mixture was then centrifuged at 3000 rpm for 10 min and used for IVFA estimation. Temperature of injection port, column and detector was set at 210, 180 and 230°C, respectively. The flow rate of the carrier gas (nitrogen) was 40 mL/min. 2 µL of sample was injected through the injection port using Hamilton syringe. Different VFAs were identified on the basis of their retention time as well as the peak area of standards after deducting the corresponding blank values.

#### *In vitro* DM and OM digestibility

After incubation of 24 h, the syringe contents were transferred to centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and the pellets were used for estimation of *in vitro* digestibility of DM and OM. The pellets were then transferred to

500 mL spoutless beakers by dissolving with 100 mL of neutral detergent solution (NDS). Beakers were kept on heater and refluxing was done at 100°C for 1 h from start of boiling. The contents in the beakers were filtered under vacuum through pre-weighed (G1) crucibles and washed with hot water. Crucibles containing residue was oven dried till constant weight (65°C, 48 h), then weighed and the dry residues was ashed at 550°C. *In vitro* DM digestibility (IVDMD) and true OM digestibility (TOMD) were calculated from the disappearances of DM and OM.

#### Partitioning factor and microbial biomass production

Partitioning factor (PF) and microbial biomass production (MBP) were calculated based on truly degraded organic matter (TDOM) as described by Blummel et al. (1997) and Blummel et al. (2005), respectively. For Ammonia nitrogen (NH<sub>3</sub>-N), 5 mL of acidified supernatant was mixed with 10 mL of NaOH (1N) and immediately steam distilled using KEL PLUS® - N analyzer (Pelican, India). The NH<sub>3</sub> evolved was collected in boric acid solution (20% w/v) having mixed indicator and titrated against N/100 H<sub>2</sub>SO<sub>4</sub>.

#### Chemical and statistical analysis

Samples of ML, different TMRs and fermentation residue were analyzed for DM, organic matter, crude protein, ether extract and total ash as per AOAC (2005) and neutral detergent fibre (NDF) and acid detergent fibre (ADF) were also determined as per Van Soest et al. (1991). Statistical analysis was carried out by One-way analysis of variance (ANOVA) using SAS (2012) software, version 9.3. Post-hoc comparison of means was carried out using Tukey's Studentised Range Test. Data are presented as mean ± SE. The significance level of all the parameters were <0.001 and hence, it is not mentioned in the table.

## RESULTS AND DISCUSSION

The ML had 20.2% crude protein and 10.6% TA (Table 1). The results are in agreement with Makkar and Becker (1996), Kakengi et al. (2005) and

Olugbemi et al. (2010) who reported that CP of ML varies from 15 to 30%. Whereas Mutayoba et al. (2011) reported higher crude protein in *Moringa oleifera* leaves (30.6%) and the composition varies depending on the maturity and respective proportions of leaves to stems plant variety and climatic condition. In present study crude protein content of ML (20.2 %) was similar with the

concentrate mixture (20.01%) and these findings were comparable with reported by various researchers (Moyo et al., 2011; Njidda et al., 2010; Kasiga and Lochmann, 2014) who reported that CP of ML in range of 19-33%. Gas production reflects fermentation of all nutrients, soluble as well as insoluble fractions and carbohydrates to acetate, propionate and butyrate (Table 1).

Table 1. Chemical composition of feedstuffs (% DM basis)

Attributes	<i>Moringa oleifera</i> leaves	Maize fodder	Wheat straw	Concentrate mixture
OM	89.3	89.7	87.8	89.9
CP	20.2	10.1	3.20	20.0
EE	4.08	1.50	0.99	4.64
TA	10.6	10.3	12.2	10.1
TCHO	62.8	78.0	83.6	65.2
NFC	39.4	15.0	7.20	40.5
NDF	23.4	63.0	77.4	24.7
ADF	16.1	38.0	53.9	11.9
NDICP	1.45	5.84	0.95	1.95
ADICP	1.05	1.42	0.73	0.72
Hemicellulose	12.2	24.9	23.0	12.7
Cellulose	19.3	27.3	45.1	6.43
ADL	6.28	4.94	7.05	2.33
TDN	67.8	54.6	43.8	74.5
DE (MJ/kg)	12.5	10.0	8.10	13.7
ME (MJ/kg)	10.7	8.31	6.29	12.0

OM: organic matter; CP: crude protein; EE: ether extract; TA: total ash; TCHO: total carbohydrates; NFC: non fibrous carbohydrates; NDF: neutral detergent fibre; ADF: acid detergent fibre; NDICP: neutral detergent insoluble crude protein; ADICP: acid detergent insoluble crude protein; ADL: acid detergent lignin; TDN: total digestible nutrients; DE: digestible energy; ME: metabolizable energy

As roughage content of diet increases simultaneously  $CH_4$  production increases (Ebeid et al., 2020). Gas production reflects nutritional values that are closely related to chemical composition (Cerrillo and Juaraz, 2004). The net gas production increased ( $P < 0.05$ ) exponentially with incorporation of graded levels of ML. Significant effect was found

at 10, 20, 30, 40 and 50% ( $P < 0.05$ ) level of incorporation. Similar results were also observed by Meale et al. (2012) where, ML could increase cumulative gas production due to higher digestibility among the different grasses, leguminous and non-leguminous shrubs, respectively indicating that these species are fermentative in nature. Likewise, Dey et

al. (2014) also reported positive associative effect of ML with wheat straw on rumen fermentation. Chaudhary et al. (2006) reported that higher total gas production with wheat straw and concentrate mixture as compared to ML alone. In present study it was observed that by increasing ML in TMRs, methane production was reduced. Reduction in methane emission is always beneficial in the aspect of sound environment and the energy is diverted towards improvement of dietary nutrients utilization efficiency. Results of CH<sub>4</sub> (ml) showed difference at 15, 20, 25 and 50% (P<0.05) replacement levels. Soliva et al. (2005) also observed decrease in *in-vitro* methane gas, when ML was incubated with meadow hay diet. Dey et al. (2014) also found a declining trend in the methane replaced by wheat straw. The positive associative effect of ML concurrently reduced methane up to 18.11. This could be due to ML being rich in linolenic acid (Machmuller et al., 1988; Machmuller et al., 2000) or presence of saponins and tannins (Hess et al., 2003). The TDMD significantly increased with the graded level of ML (Table 2). However highest

TDMD (%) was found at 50% incorporation level (67.21). Nouala et al. (2006) reported a significant improvement in the digestibility of nutrient by supplementation of ML with ground nut hay. The observed values for TDDM and TDOM were greater than those from substrates fermented alone. Dey et al. (2014) reported increasing trend in dry matter and organic matter digestibility by supplementation of Moringa. Increase in digestibility of ML supplemented material could be explained by high degradable capacity high energy concentration of ML (Aregheore, 2002; Soliva et al., 2005). PF within the range at different level of replacement, it is the ratio of *in-vitro* substrate truly digested to gas volume (Blummel et al., 1997) and it varies from 2.75 to 4.41 reflecting YATP from 10-40. Hence, in the current study PF values indicate proper partitioning of nutrients to meet microbial protein synthesis. ML resulted in higher substrate degradation and microbial protein synthesis thereby, improved partitioning of nutrients for mixed diets. Microbial biomass production (MBP) significantly (P<0.05) increased with increasing level of ML (Table 2).

Table 2. *In vitro* net gas, methane production, digestibility, PF and MBP of total mixed rations containing graded levels (%) of *Moringa oleifera* leaves

Treatments	Control	5	10	15	20	25	30	40	50
Net Gas (ml/200mg)	26.3 <sup>a</sup> ±0.85	27.6 <sup>a</sup> ±0.85	28.5 <sup>b</sup> ±0.41	30.8 <sup>b</sup> ±0.48	33.1 <sup>d</sup> ±0.48	34.0 <sup>c</sup> ±0.41	35.0 <sup>bc</sup> ±0.41	36.22 <sup>c</sup> ±0.61	38.37 <sup>d</sup> ±0.85
CH <sub>4</sub> (%)	38.8 <sup>a</sup> ±0.69	37.6 <sup>a</sup> ±0.71	35.5 <sup>ab</sup> ±0.68	34.6 <sup>b</sup> ±0.67	33.1 <sup>c</sup> ±0.66	31.3 <sup>d</sup> ±0.87	30.1 <sup>d</sup> ±0.52	29.56 <sup>d</sup> ±0.49	27.02 <sup>e</sup> ±0.14
CH <sub>4</sub> (mmol/L)	10.8 <sup>a</sup> ±0.09	10.1 <sup>a</sup> ±0.08	9.89 <sup>ab</sup> ±0.08	9.02 <sup>b</sup> ±0.08	8.51 <sup>c</sup> ±0.07	8.29 <sup>d</sup> ±0.18	8.21 <sup>d</sup> ±0.22	8.19 <sup>e</sup> ±0.34	7.05 <sup>f</sup> ±0.39
CH <sub>4</sub> (mmol/100mgDDM)	9.50 <sup>a</sup> ±0.09	9.09 <sup>a</sup> ±0.13	8.80 <sup>ab</sup> ±0.15	8.10 <sup>b</sup> ±0.44	7.95 <sup>b</sup> ±0.03	7.65 <sup>c</sup> ±0.05	7.49 <sup>d</sup> ±0.47	7.30 <sup>d</sup> ±0.19	6.30 <sup>e</sup> ±0.23
TDMD (%)	50.2 <sup>a</sup> ±1.47	51.5 <sup>b</sup> ±0.93	53.7 <sup>c</sup> ±0.66	56.2 <sup>d</sup> ±0.38	56.7 <sup>d</sup> ±0.41	57.8 <sup>d</sup> ±0.30	62.0 <sup>e</sup> ±1.33	64.49 <sup>f</sup> ±0.49	67.21 <sup>g</sup> ±0.66
TOMD (%)	52.0 <sup>a</sup> ±0.41	53.9 <sup>b</sup> ±0.39	55.9 <sup>c</sup> ±0.81	57.5 <sup>d</sup> ±0.73	57.5 <sup>d</sup> ±0.51	58.3 <sup>d</sup> ±0.47	63.2 <sup>e</sup> ±0.88	66.43 <sup>f</sup> ±0.67	68.86 <sup>g</sup> ±0.25
PF	3.86 <sup>f</sup> ±0.11	3.68 <sup>de</sup> ±0.07	3.71 <sup>e</sup> ±0.05	3.63 <sup>cd</sup> ±0.02	3.44 <sup>b</sup> ±0.03	3.30 <sup>a</sup> ±0.02	3.55 <sup>bc</sup> ±0.08	3.58 <sup>cd</sup> ±0.03	3.55 <sup>bc</sup> ±0.03
MBP (mg)	57.1 <sup>a</sup> ±0.06	61.6 <sup>b</sup> ±0.06	63.7 <sup>c</sup> ±0.05	68.2 <sup>d</sup> ±0.36	72.4 <sup>e</sup> ±0.30	77.4 <sup>f</sup> ±0.41	77.3 <sup>f</sup> ±0.42	78.84 <sup>g</sup> ±0.50	85.19 <sup>h</sup> ±0.72

Means in a row with different superscripts differ significantly (P<0.05)

The readily available carbohydrate proportion of Moringa could improve rumen micro biome to enhance the fermentation of substrate present in rumen so MBP value significantly increased by incorporating graded level of ML. Similar trend was observed by Dey et al. (2014). The high CP content, in conjunction with greater ruminal degradability of ML protein (Makkar and Becker, 1997) and high proportion of non-ammonia N (Soliva et al., 2005) suggested to increase the microbial protein synthesis by ML. The fermentable N and readily available carbohydrates supplied by ML (Melesse, 2012) could have improved the rumen microbiome to stimulate the fermentation of wheat straw for higher MBP from composite diet. With NH<sub>3</sub>-N values, significant (P<0.05) difference was found up to 25% level replacement whereas at 30, 40, 50% level similar result was observed. The acetate and propionate concentration was significantly (P<0.05) increased with increasing graded levels of ML (Table 3).

Table 3. *In vitro* NH<sub>3</sub>-N and VFA of total mixed rations (TMR<sub>s</sub>) containing graded levels (%) of *Moringa oleifera* leaves

Treatments	Control	5	10	15	20	25	30	40	50
NH <sub>3</sub> -N (mg/dl)	13.6 <sup>a</sup> ±0.42	14.7 <sup>ab</sup> ±0.32	14.6 <sup>ab</sup> ±0.78	15.2 <sup>ab</sup> ±0.37	16.4 <sup>b</sup> ±1.04	17.0 <sup>b</sup> ±2.58	20.0 <sup>c</sup> ±0.12	21.41 <sup>c</sup> ±1.88	21.49 <sup>c</sup> ±2.89
Acetate (mmol/L)	35.9 <sup>a</sup> ±0.81	38.5 <sup>b</sup> ±1.16	42.1 <sup>c</sup> ±0.70	43.8 <sup>b</sup> ±0.47	47.5 <sup>c</sup> ±0.64	48.1 <sup>ef</sup> ±0.87	48.5 <sup>ef</sup> ±0.49	49.96 <sup>fg</sup> ±0.78	50.54 <sup>g</sup> ±1.59
Propionate (mmol/L)	14.3 <sup>a</sup> ±0.35	15.7 <sup>b</sup> ±0.45	16.1 <sup>b</sup> ±0.59	16.4 <sup>b</sup> ±0.90	19.3 <sup>c</sup> ±0.59	19.1 <sup>c</sup> ±0.40	21.3 <sup>d</sup> ±0.84	22.13 <sup>d</sup> ±0.52	24.95 <sup>e</sup> ±1.05
Butyrate (mmol/L)	8.81 <sup>b</sup> ±0.28	8.89 <sup>b</sup> ±0.76	8.92 <sup>b</sup> ±0.59	8.94 <sup>b</sup> ±0.33	8.40 <sup>b</sup> ±1.00	8.75 <sup>b</sup> ±0.95	6.74 <sup>a</sup> ±0.42	6.68 <sup>a</sup> ±0.24	6.86 <sup>a</sup> ±0.71
A:P ratio	2.52 <sup>cd</sup> ±0.10	2.45 <sup>c</sup> ±0.04	2.60 <sup>e</sup> ±0.12	2.67 <sup>e</sup> ±0.15	2.46 <sup>cd</sup> ±0.04	2.51 <sup>ch</sup> ±0.04	2.28 <sup>b</sup> ±0.09	2.26 <sup>b</sup> ±0.08	2.03 <sup>a</sup> ±0.10

Whereas, significant decreasing trend was observed in butyrate concentration and a similar result was reported by Dey et al. (2014) and Soliva et al. (2005). Results were also observed by Ebeid et al. (2020) with the addition of *M. oleifera* seed oil at 3 and 4% in the ration. Microbial protein and propionate concentration were found increased but acetate concentration was decreased. Higher propionate and lesser acetate production revealed that less hydrogen (H<sub>2</sub>) produced by the degradation of structural carbohydrates in rumen in turn prevented methanogenic archaea to produce more CH<sub>4</sub> (Haque, 2018). Increase in IVFA concentration was observed due to high digestibility of ML (Dey et al., 2014). Nouala et al. (2006) reported a significant improvement in digestibility of groundnut hay upon supplementation of ML. Higher short chain fatty acids production associated with the sample of ML alone could be attributed to the presence of readily available carbohydrate and higher degradability of leaf fibre (Soliva et al., 2005). The acceleration in VFA production of wheat straw due to the presence of ML could be due to stimulation of fibrolytic activity in rumen fluid by providing favourable environment through supply of N and growth promoting factors (Makkar and Becker, 1997).

## CONCLUSION

Results of chemical composition, *in vitro* gas production parameters, digestibility and individual volatile fatty acids indicated that the *Moringa* leaves can be considered as a feed supplement to ruminants. Mitigation in methane emission advocated the potentiality of ML as a supplement to diets based on crop residues/poor roughages. Further researches envisaged with *in vivo* studies to examine the nutritive value of ML to improve ruminant production and reduce environmental pollution.

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