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Moringa oleifera L.: A Potential Plant for Greenhouse Gas Mitigation in Temperate Agriculture Systems

Adrian Mangar [1,2], Stefan Muetzel [3], Anurag Malik [4,5], Axay Bhuker [4], Virender Mor [4], Adrian Molenaar [3], Svetla Sofkova-Bobcheva [1], Sarah Pain [1] and Craig McGill [1,*]

[1] School of Agriculture and the Environment, Massey University, Palmerston North 4442, New Zealand; amangar@narei.gov.gy (A.M.); s.sofkova-bobcheva@massey.ac.nz (S.S.-B.); s.j.pain@massey.ac.nz (S.P.)
[2] National Agricultural Research and Extension Institute (NAREI), Georgetown, Guyana
[3] Resilient Agriculture, AgResearch Ltd., Palmerston North 4410, New Zealand; stefan.muetzel@agresearch.co.nz (S.M.); adrian.molenaar@agresearch.co.nz (A.M.)
[4] Department of Seed Science and Technology, CCS Haryana Agricultural University, Hisar 125004, Haryana, India; anuragmalikseed@hau.ac.in (A.M.); bhuker.axay@hau.ac.in (A.B.); virendermor@hau.ac.in (V.M.)
[5] Chandigarh School of Business, Department of Agriculture, Chandigarh Group of Colleges, Jhanjheri 140307, Mohali, India
* Correspondence: c.r.mcgill@massey.ac.nz; Tel.: +64-69517803

Abstract: The earth's climate is changing because of the increase in greenhouse gas (GHG) concentration, to which livestock is a major contributor. Methane produced from cattle can be reduced by using high quality forages. This study compared the GHG produced from *M. oleifera* in an artificial ruminant system with two high quality pasture species, ryegrass and white clover. Methane and total gas production were measured using an in vitro batch culture system. A preliminary screening using oven dried *M. oleifera* planted in field and greenhouse, and a main experiment using six provenances of *M. oleifera*, a composite sample and *M. oleifera* leaves from greenhouse was undertaken. Both experiments compared the *M. oleifera* from different sources with high quality ryegrass and white clover. Real time gas production was recorded for 48 h, total gas production, methane analysed at 12 and 24 h. Short chain fatty acids concentration were also determined at the end of the fermentation. Preliminary results showed that *M. oleifera* leaves grown in field and greenhouse have lower gas and methane production compared with ryegrass, but similar to white clover. The differences were driven by a high production of propionic and butyric acids. The six *M. oleifera* provenances also produced less methane than ryegrass but were similar to white clover at 12 and 24 h after the start of fermentation. *M. oleifera* fermented faster than ryegrass or white clover. Hydrogen production from fermentation of *M. oleifera* might not have been diverted to methane production but removed by other compounds. In vitro fermentation showed differences in methane production across provenances. This suggests that it may be possible to select for low methane genotypes.

Keywords: *Moringa oleifera*; methane; greenhouse; cattle; provenances

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1. Introduction

The climate has changed over time because of the changes in the concentration and proportions of gasses in the atmosphere. These changes are particularly due to the actions of humans [1]. Climate change is causing the earth's average temperature to increase, and this effect is termed the greenhouse effect. Any gas that absorbs infrared radiation emitted by the earth's surface and in turn emits radiation to the colder atmospheric temperatures is termed a greenhouse gas [2].

Globally, livestock emits 7.1 gigatonnes of carbon dioxide (CO_2) equivalent of greenhouse gas each year, with methane contributing about 3.1 gigatonnes of CO_2 equivalent. This amount will increase as the demand for meat grows because of a growing

global population [3]. As such, reducing methane emission from ruminant livestock is important [4]. A key strategy to help reduce methane emissions is to increase ruminant efficiency by providing better quality diets, because forage quality has been associated with methane emissions [5,6].

The methane emissions from forage diets are most times 6–9% of the gross energy that the animal consumes [7]. This has prompted investigations into methane emission in major forages, as reducing methane emission by ruminants would help lower greenhouse gas emissions and may also make more energy available to animals.

In New Zealand, the dairy industry is one of the largest contributors to agricultural greenhouse gas emissions. In 2020 New Zealand's gross greenhouse emissions were 78,778 kilotonnes carbon dioxide equivalent (kt CO_2-e) of which 44% was methane. Agriculture contributed 50% of gross greenhouse emissions. The major source of methane in the agriculture sector is enteric fermentation (28,831.5 kt CO_2-e) at 84.1% of New Zealand's gross methane emissions). Dairy cattle and non-dairy cattle (beef) contribute 48.7% and 20.7% per cent to enteric fermentation emissions respectively [8]. For this reason, the New Zealand dairy industry, which is based on pastoral grazing of pasture that consists mainly of ryegrass mixed with white clover, chicory, plantain and sometimes red clover pastures, have been under investigation for their methane emissions [9,10].

Common methods to measure the methane and total gas produced from feeds involve the use of in vitro incubation systems. In in vitro incubation systems, feeds are mixed with rumen fluid and incubated to mimic the animal's digestive system. Early systems were designed to measure endpoint measurements of fermentation [11]. In the 1970s the Hohenheim gas test was developed. This system uses syringes made out of glass as incubation chambers, the feed and rumen fluid are then added and gas fermented is measured by the movement of the pistons inside the cylinders [12]. In the 1990s automated systems were developed to provide real time measurements of gas from fermentation [13]. Some of the first systems measured gas production for an entire 24 h releasing pressure build up using solenoid valve [14]. Most recent a fully automated system was designed and developed to measure methane, hydrogen and total gas production for screening feeds for their methane emission [15].

M. oleifera is already being used as feed for animals in some countries [16]. In Nicaragua it is used as a supplemental feed for cattle and research has proven it to be beneficial for both beef and dairy cattle when comprising of 50–60% of the total feed intake [17]. It is also used in Zimbabwe by subsistence farmers to feed cattle [18], and has been proposed as a source of protein for cows in tropical areas [19]. Various in vitro fermentation experiments have shown *M. oleifera* can lower methane production. *M. oleifera* was added either as an extract or dried forage; others used extracted root bark. For example, *M. oleifera* leaf extract was used in combination with soyabean hulls to feed Holstein steers. The *M. oleifera* leaf extract was made from leaves randomly collected from plants growing in Veracruz, Mexico. The leaves were crushed, and one gram mixed with eight millilitres of water. They were then left for seventy-two hours at 28 °C then for one hour at 39 °C. Three diets were used containing 0, 75 and 150 g/kg dry matter soya bean hull, and to each diet, *M. oleifera* was added at concentrations of 0.6, 1.2, 1.8 mL/g. Results showed that as the concentration of *M. oleifera* leaf extract increased in the diets, the asymptotic methane production decreased. This occurred in diets that contained 0 and 75 g/kg DM of soya bean hull, but was kept constant for the diet containing 150 g/kg DM. Nevertheless, in diets containing 150 g/kg DM of soya bean hull, the rate of methane production was reduced from 0.027 $(mL/g\ DM)^2$ to 0.012 $(mL/g\ DM)^2$ using 0 and 1.8 mL/g leaf extract respectively, demonstrating a reduction in the rate by more than half [20]. Similarly, research on *M. oleifera* was found to lower methane production in goats. The leaf extract was prepared by mixing ten grams of ground leaf with 90 mL of water and treatments included, 0, 0.6 and 1.8 mL/g DM. However, in this experiment live yeast cultures were also added at 0, 2 and 4 mg/g DM. The basal diet to which these treatments were added contained oat straw, corn, soya bean paste, urea, molasses, sunflower oil, and vitamins and

minerals. The results showed that *M. oleifera* leaf extract as a feed supplement in male goats (Nubia × Criollo) along with *Saccharomyces cerevisiae* (live yeast culture) at various doses, decreased methane production [21].

Moreover, Soltan, Morsy [22], contrary to the previous two experiments, used *M. oleifera* root bark extract. In this study, the root bark was collected from five to six months old *M. oleifera* plants grown in Alexandria, Egypt. The root bark extract was made by scraping the outer layer of the roots by hand, drying the material for 72 h at 40 °C and then making an ethanol extract using 100 g of dried material in 1000 mL of water and ethanol mix (700 mL/L). Results using data from the in vitro experiment showed that *M. oleifera* root bark extract added to the basal diet (clover hay, ground maize, soyabean meal, wheat bran and cotton seed meal) at 50 g/kg DM significantly reduced methane production at the end of 24 h when compared with using *M. oleifera* extract at 25 g/kg DM and monensin (an ionophore antibiotic that modifies rumen microbes) [22].

Another study showed that ethanol extract of *M. oleifera* leaf and stalk from mature two-year-old plants growing in Nicaragua lowers methane production. In this study the ethanol extraction was done by mixing 1 kg of fresh *M. oleifera* leaves with 3 litres of ethanol in a food blender. Afterwards, the mixture was filtered until the outflow was clear and the residue (ethanol extracted *M. oleifera*) was then extracted with acetone, dried and sieved. Four dietary treatments were used, soya bean meal, rapeseed meal, unextracted Moringa leaves and extracted Moringa leaves. These treatments were then added separately to diets consisting of hay, barley and straw meal. All the diets and their treatments were formulated to have the same amount of protein. The results showed diets containing *M. oleifera* had significantly lower methane production (mL per day) compared with diets of soyabean and rapeseed meal [23]. These studies provide evidence that *M. oleifera* can help mitigate methane emissions from cattle.

M. oleifera is widely cultivated [24]. If *M. oleifera* is to be used as a forage crop to feed cattle, the quantification of methane emission during ruminal fermentation can be part of an important strategy to help New Zealand and other countries lower their greenhouse gas emissions to combat climate change and meet reduction targets.

Therefore, the objective of this study was to compare total gas and methane production of *M. oleifera* across a range of *M. oleifera* provenances with that of ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) using an in vitro incubation system.

2. Materials and Methods

2.1. Location

The analyses were undertaken at the AgResearch Grasslands Research Centre, Tennent Drive, 11 Dairy Farm Road, Palmerston North, New Zealand, in May 2021 and in January 2022.

2.2. Source of Dried M. oleifera Leaves

Seeds of *M. oleifera* were sourced from King Seeds (Katikati, New Zealand), Guyana and India. There were seven provenances used in this experiment (King Seeds considered a provenance). Moringa seed from King Seeds (a local New Zealand seed company) is of unknown origin but was sourced from the United States (King Seeds, pers. Comm.), four provenances were sourced from Guyana and two from India. The four provenances sourced from Guyana were Benab, Bush Lot, Mon Repos and Queenstown, and the two from India were PKM-1 (g) from Gujrat and PKM1 (t) from Tamil Nādu.

2.3. Preliminary Analysis

Dried leaves of *M. oleifera* were obtained from plants of King Seeds. Two hundred seeds from King Seeds were sown in potting bags filled with Dalton's potting mix on the 29 September 2020, at the Plant Growth Unit, Massey University, Batchelar Road, Palmerston North. The seeds were germinated under glass house conditions. The temperature range inside the glasshouse was 25 to 27 degrees Celsius; humidity was not measured. The

plants were left to grow for five months. No fertilizer was added to the potting mixture after the plants were germinated. Afterwards one hundred plants were transplanted in an open field located on Poultry Farm Road, Palmerston North. The plants were transplanted into the field using a spacing of 1 m by 1 m. The remaining plants were left in the greenhouse.

2.4. Main Analysis

The dry leaf material to carry out the main analysis was obtained from plants used in a previous salinity trial [25], along with dried leaves of plants grown from seeds sourced from King Seeds New Zealand. Seeds originated from Guyana and India were sown in the first week of August 2021 as part of a salinity screening trial. These seeds were sown directly in 9-litre plant pots with a 24 cm diameter. The potting mixture used in all the pots was Daltons Base mix + Osmocote Pro from New Zealand.

2.5. Age of Plants and Dried Plant Material

Dried *M. oleifera* plant material used in this experiment was harvested from plants of different ages. At the time of harvesting fresh plant material, plants of King seeds were seven months old (from the time of sowing the seeds). The fresh material obtained from the other provenances was taken from three-month-old plants. However, all leaf material was from the first fully expanded mature leaf at the apex of the plant, irrespective of plant age.

The fresh leaves of plants grown from seed from King Seeds were harvested from plants that were in the field and in the greenhouse in April 2021 so that enough leaf material was obtained. The leaf material was dried separately. For the main analysis (provenances that originated from Guyana and India) leaf material from the untreated controls (i.e., no salinity treatment applied) were harvested from the 0 mM NaCl (nil NaCl) treatments at the end of the salinity trial in October 2021.

All fresh plant material for both analyses were oven-dried for 72 h in a convection oven set to 60 °C. Dried plant material was then ground finely using a coffee and spice grinder, labelled and stored in airtight plastic containers.

2.6. Substrate Collection

For the preliminary analyses eight substrates were used in addition to the control. *M. oleifera* transplanted in the field were planted in ten rows, each consisting of ten plants. Young fully expanded mature leaves were collected from the first three rows of plants, then another four rows and the last three rows and pooled separately forming three composite samples. This procedure was also used for the plants in the greenhouse since the plants were in pots and consisted of ten rows. Dried grounded ryegrass and white clover were supplied by AgResearch Grasslands. These were harvested in spring 2010 from Aorangi Experimental Station, 315 Lockwood Road, Palmerston North and stored at −20 °C for long-term use.

For the main analyses, there were 34 different sources of *M. oleifera* used; *M. oleifera* leaves from the preliminary experiment were re-tested with the exception that leaves originating from the field and greenhouse were pooled into one composite sample and mixed thoroughly by placing into a square container and rotating the container twelve times on its edges. Young leaves were collected from all the plants in the greenhouse (plants grown from seeds sourced from King Seeds that remained in the greenhouse) and pooled into one composite sample. The salinity trial consisted of 18 treatments and 5 blocks, set up in a randomised complete block design. Within each block there were 6 treatments of 0 mM NaCl that corresponded to the six provenances. From each 0 mM NaCl treatment, dried leaves from all the plants were combined, forming six samples within each block that represents the six provenances and thus making 30 samples in total (6 from each block × 5 blocks). Ryegrass and white clover were the same as for the preliminary analysis.

2.7. Rumen Fluid

For this study, rumen fluid was collected from three fistulated seven-year-old dry Holstein Friesian cows. In all the experiments rumen fluid was collected in the morning before the animals were allowed to graze on pasture (around 8 am) on the day of the incubation. Rumen fluid was collected from two fistulated cows grazed on ryegrass-based pasture (Grasslands Animal Ethics Committee (approval AE15154)). The rumen contents were squeezed into a pre-warmed Thermos flask and transported to the laboratory within 15 min where they were filtered through one layer of cheesecloth. Equal amounts from each cow were mixed with the pre-warmed (39 °C) chemically reduced carbonate-based buffer [26] equilibrated with CO_2.

2.8. In Vitro Fermentation

The in vitro fermentation was done using a fully automated batch culture system according to the procedures outlined in Muetzel, Hunt [15]. For each experiment, each substrate (dried leaf material to be used in the in vitro incubation) was duplicated and then replicated three biological times, with each biological replicate a mixture of rumen fluid from two different donor animals.

Preliminary Experiment: Comparing *M. oleifera* leaves from plants grown from King Seed's seed in the field and greenhouse with leaves from ryegrass and white clover.

The first experiment consisted of nine substrates; six substrates of *M. oleifera* (three from the field and three from greenhouse), white clover, rye grass and the internal control ryegrass sample that had been used repeatedly in incubations for over eight years. The preliminary experiment began on 17 May 2021.

Main Experiment—Comparing different provenances of *M. oleifera* used in the salinity trial (from Guyana and India), a composite sample of King Seeds from the field and greenhouse, leaves of King Seeds from the greenhouse and comparing them with ryegrass and white clover. Three separate incubations were undertaken, each time using a mixture of different donor animals to yield the three biological replicates. The first incubation was undertaken on 8 December 2021, the second on 15 December 2021 and the third on 17 January 2022, and making 5 litres of batch culture (BC) buffer for each incubation.

2.9. Incubation

Before the incubation, 0.5 g of substrate was placed in 100-mL serum bottles. The bottles were randomized, then placed on a reciprocal shaker in a fan-driven incubator and pre warmed to 39 °C. The incubation medium was prepared as shown in Table 1. The composition of the buffer and macromineral solutions (listed in Table 2 [26]) was used. Each incubation medium contained a mixture of two different rumen fluids from different donor animals. The buffers were gassed with CO_2 for at least 30 min before the reducing agent (NaOH 2.5 mM and cysteine-HCl 2.5 mM) was added just prior to collection of rumen fluid. Before rumen fluid collection, calibration gases were injected to the gas chromatograph.

Table 1. The quantity of each compound used in one litre of medium.

BC Buffer	Volume
10× Buffer 1	80 mL
10× Buffer 2	80 mL
dH_2O	640 mL
Cystein HCL	0.316 g
10 N NaOH	0.2 mL
Rumen Fluid	200 mL

Table 2. The quantity of each component used in one litre (a) buffer 1 and (b) buffer 2.

(a)			
10× Macrominerals			Volume (1 L)
Buffer component	MW	Conc. (mM)	amount (g)
Na_2HPO_4	142.0	60	8.51
KH_2PO_4	136.1	96	13.02
$MgCl_2$ 6×H_2O	203.3	5	1.05
(b)			
10× Buffer			Volume (1 L)
Buffer component	MW	Conc. (mM)	amount (g)
$NaHCO_3$	84.0	645	54.18
NH_4CHO_3	79.0	178	14.07

The rumen fluid was collected in pre-warmed thermos flasks. The flasks were filled completely with rumen fluid, leaving no head space to reduce oxygen contamination. The rumen fluid was then filtered through one layer of cheese cloth and added immediately to the buffer. CO_2 was then bubbled through the medium for 15 more minutes to equilibrate the medium. A sample of the medium without any substrate was collected for the analysis of short chain fatty acids (0 h sample).

The serum bottles were flushed with CO_2 and 50 mL of medium was then added to each prewarmed bottle and sealed with a butyl rubber stopper. The bottles were filled one at a time and returned to the incubator where each bottle was connected to the automated gas measurement and release system by inserting a 23-gauge needle through the rubber stopper. When all the bottles were filled, and needles inserted, the oscillating shaker was set to 120 rpm and turned on.

Gas accumulation was measured every minute by the increase in pressure in the bottles. When a threshold pressure representing a gas volume to flush the system was reached, the corresponding solenoid valve was opened, and the gases were injected in a gas chromatograph via a sampling loop attached to a six-port valve. If more than 1 bottle had reached the threshold the bottle with the highest pressure was prioritised. Because of the sequential nature of the GC analysis, data for methane and total gas production have to be estimated for any given timepoint analysis. Analysis was carried out for the cumulative gas and methane production at 12 and 24 h of incubation.

2.10. Short-Chain Fatty Acids

After incubation, a sample for short-chain fatty acids analysis was taken from each bottle. Each serum bottle was shaken by hand before pipetting 1.8 mL of sample from each into 2 mL Eppendorf tubes. The samples were centrifuged (21,000× *g* for 10 min at 4 °C) and 900 µL of the supernatant was transferred into new 1.5 µL tubes and 100 µL of internal standard solution (19 mM ethylbutyrate in 20% (*v*/*v*) phosphoric acid) added.

The samples were kept in a −20 °C freezer until the following day, thawed and centrifuged as above. An aliquot of 750 µL of the supernatant was transferred into a 2 mL crimp cap gas chromatography vial and crimped immediately. Short-chain fatty acids were analysed by gas chromatography as described by Attwood, Klieve [27].

2.11. Experimental Design

In both experiments, each substrate was incubated in duplicate bottles which served as technical replicates. For statistical evaluations, each incubation was repeated three times. The substrates were completely randomised in the incubator. For the preliminary experiment, three *M. oleifera* samples from a field trial and three from the greenhouse were compared to high quality perennial ryegrass and white clover.

In the main experiment, each provenance from Guyana and India sourced from a salinity experiment (0 mM NaCl) from within each block was considered as a separate substrate, hence there were 6 substrates from each block (6 × 5 blocks). Those along with young *M. oleifera* leaves from plants in the greenhouse, a composite *M. oleifera* sample from the first experiment, were compared again with perennial ryegrass and white clover.

2.12. Statistical Analysis

Total gas and methane production in millilitre per gram (mL/g) of substrate at 12 and 24 h were calculated. Total gas and methane production at 12 and 24 h were calculated and extracted from the real-time data obtained from the fermentation. These time periods were selected to reduce the variability of time. Real-time gas production started as soon as the rumen fluid was added to the substrate, and it varied for all the substrates because rumen fluid was not added to all the samples at once but rather one at a time. The biological replicates (rumen fluid from different cows) were used as blocks. For each experiment there were three blocks, each block was a mixture of rumen fluid from two cows. Because gas production started at a different time for the substrates, unbalanced data were produced from both incubations. The data were analysed using analysis of variance for unbalanced designs using GenStat regression in GenStat 21st edition [28]. Total gas, methane, proportion of methane, average standard error of the differences of means and *p*-value were also calculated.

3. Results

3.1. Initial Comparison of M. oleifera with Perennial Ryegrass and White Clover

Real-time gas production showed a lower gas production by *M. oleifera* and white clover than in ryegrass (Figures 1 and 2). These differences, however, became obvious only after 12 h of incubation. Raw data for methane production showed ryegrass with a higher production throughout the entire incubation period followed by white clover and *M. oleifera*.

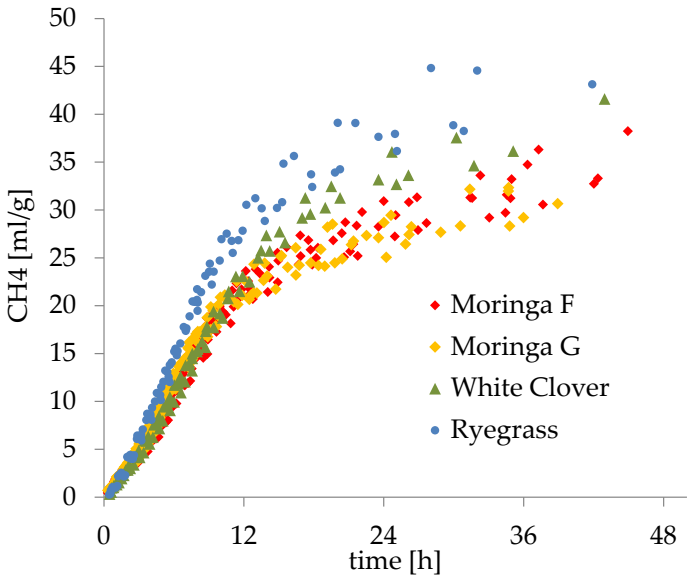

Figure 1. Real-time production of methane (mL/g) for *M. oleifera* in field (Moringa F) and in greenhouse (Moringa G), ryegrass and white clover.

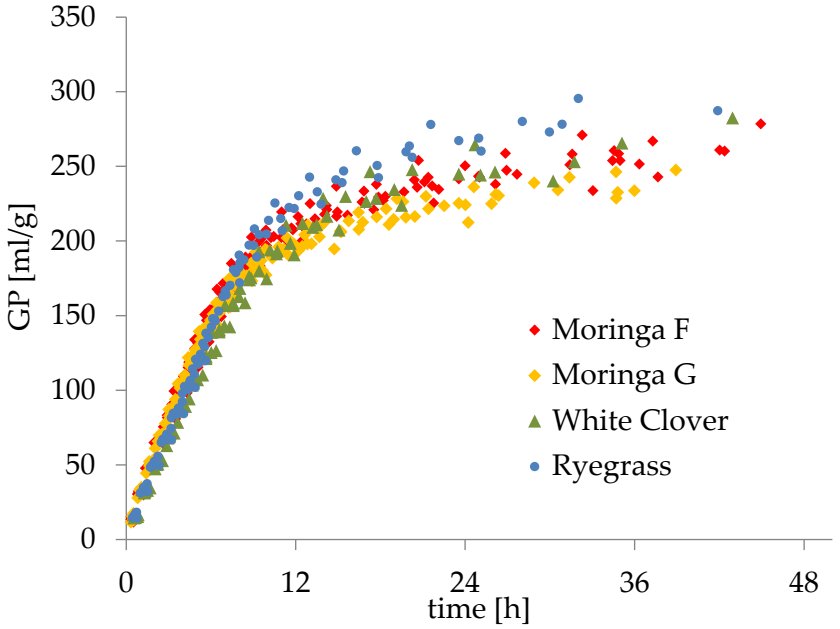

Figure 2. Real-time production of gas production (mL/g) for *M. oleifera* in field (Moringa F) and in greenhouse (Moringa G), ryegrass and white clover.

Further analysis of data revealed that *M. oleifera* had a lower ($p < 0.001$) methane and total gas production in milliliters of gas per gram of substrate (mL/g) (Table 3) at 12 and 24 h. Methane production (mL/g) at 12 and 24 h was significantly lower ($p < 0.001$) in all *M. oleifera* substrates than ryegrass (Table 3), but similar to white clover. In addition, analysis of short-chain fatty acids at the end of incubation showed *M. oleifera* had a higher percentage butyrate and propionate but lower acetate than ryegrass and white clover (Table 4). Importantly, *M. oleifera* took a shorter time to reach half the maximum gas production (GP t 1/2 max) (Table 3).

Table 3. Total gas production, methane and proportion of methane at 12 and 24 h for perennial ryegrass (*L. perenne*), white clover (*T. repens*) and *M. oleifera.* The average standard error of the differences of means (SED) and *p*-value are presented. GP t 1/2 max(h) and CH_4 t 1/2 max(h) (half for *M. oleifera* in open field and greenhouse are the average for F1, F2, F3 and G1, G2, G3 respectively. t 1/2 max signifies half the maximum time.

	Time	**12 h**			**24 h**				
	Substrate	**GP mL/g**	**CH_4 mL/g**	**CH_4% GP**	**GP mL/g**	**CH_4 mL/g**	**CH_4% GP**	**GP t1/2 max(h)**	**CH_4 t1/2 max(h)**
	Ryegrass	223.7	28.17	12.58	267.13	37.60	14.05	5.81	8.11
	White clover	202.3	22.78	11.25	244.83	33.13	13.54	5.91	9.56
M. oleifera Open field	F1	205.6	21.88	10.63	237.11	28.66	12.08		
	F2	210.1	21.41	10.16	243.70	28.46	11.66	4.97	8.51
	F3	210.6	20.89	9.90	243.82	27.57	11.28		
M. oleifera Greenhouse	G1	196.2	21.53	11.03	224.62	27.79	12.35		
	G2	198.6	21.42	10.76	227.52	27.52	11.90	4.48	7.13
	G3	195.3	21.30	10.88	223.59	26.82	11.99		
	SED	3.903	0.323	0.138	4.947	0.506	0.181	0.084	0.296
	p-value	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***

*** Significant $p < 0.001$.

Table 4. Short chain fatty acids at the end of fermentation. Moringa F and G are the mean for *M. oleifera* samples, F1, F2, F3 and G1, G2 and G3 respectively. Acetate, propionate and butyrate are presented along with total short chain fatty acids. The mean concentration in millimoles (mM), average standard error of the differences (SED) and *p*-value are presented.

	Acetate	Propionate	Butyrate	Total SCFA			
Substrate	**[mM]**	**[mM]**	**[mM]**	**[mM]**	**Acetate [%]**	**Propionate [%]**	**Butyrate [%]**
Ryegrass	53.42	15.05	8.19	81.26	65.75	18.52	10.07
White Clover	49.44	12.89	7.16	72.98	67.75	17.66	9.81
Moringa F	49.03	15.56	8.48	77.48	63.27	20.08	10.94
Moringa G	49.21	14.55	8.14	76.19	64.59	19.10	10.69
SED	0.881	0.273	0.150	1.365			
p-value	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***			

*** Significant $p < 0.001$.

The results also showed that *M. oleifera* leaves from the greenhouse had overall lower gas production at 12 and 24 h (Table 3). This was significantly different from ryegrass but not when compared with *M. oleifera* grown in the field.

3.2. Comparison of M. oleifera Provenances with Perennial Ryegrass and White Clover

3.2.1. Gas Production

Data analysis for methane and gas production at 12 and 24 h showed significant ($p < 0.001$, Table 5) differences. Methane production of the composite sample (King Seeds) of *M. oleifera* at 12 h differed significantly from white clover, and both were significantly lower than ryegrass and all other *M. oleifera* provenances. However, at 24 h the methane production was similar for Bush Lot, Mon Repos, Queenstown, and the composite sample. However, the composite sample was lower ($P < 0.001$) than white clover and both were significantly lower than ryegrass (Table 5). The mean methane production at 24 h was 30.5 mL/g; all samples tested were below this mean, except for white clover, PKM-1 (t), ryegrass, benab, PKM-1 (g) and young leaves (King Seeds) from the greenhouse. Bush Lot had the lowest gas production at 24 h which was not significantly different from Mon Repos, Benab and Queenstown, but significantly lower than ryegrass which showed a higher ($p < 0.001$) gas production than all other substrates.

Table 5. Total gas production, methane and proportion of methane at 12 and 24 h for *M. oleifera* composite sample, young leaves of *M. oleifera*, Queenstown, Benab, PKM-1 (t), PKM-1 (g), Mon Repos, Bush lot, Ryegrass (*L. perenne*) and white clover (*T. repens*). Gas and methane production rate is shown (GP t 1/2 max and CH_4 t 1/2 max). The average standard error of the differences of means (SED) and *p*-value are presented.

Time		**12 h**			**24 h**			
Substrate	**GP mL/g**	**CH_4 mL/g**	**CH_4%**	**GP mL/g**	**CH_4 mL/g**	**CH_4 %**	**GP t1/2 max(h)**	**CH_4 t1/2 max(h)**
Ryegrass	215.5	25.69	11.91	260	32.84	13.13	6.30	8.30
White clover	199.2	21.63	10.79	241.1	31.60	13.08	5.90	9.82
Composite F + G	206.31	20.19	9.74	241.3	28.27	11.11	5.07	8.18
leaves from greenhouse	200.5	22.74	10.98	241.7	31.17	12.78	4.84	8.47
Queenstown	197.1	22.79	11.52	229.3	29.50	12.81	4.63	7.11
Mon Repos	195.7	22.10	11.23	229.9	29.99	12.74	4.75	7.73
Bush Lot	194.6	22.93	11.73	225.5	29.82	13.20	4.63	7.66
Benab	202.3	24.06	11.86	235	30.79	13.22	4.64	7.60
PKM-1 (t)	205.6	23.71	11.50	240.5	30.87	12.64	4.67	7.46
PKM-1 (g)	208.6	24.76	11.58	243	31.76	12.82	4.72	7.44
SED	5.094	0.770	0.3293	5.675	1.082	0.3961	0.121	0.556
p-value	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***

*** Significant $p < 0.001$.

3.2.2. Short-Chain Fatty Acids

PKM-1 (g) and leaves from plants from seeds sourced from King Seeds had the highest concentration of short-chain fatty acids which was significantly higher than white clover but not for ryegrass (Table 6). PKM-1 (g) and ryegrass had similar acetate, propionate and butyrate concentration which were not significantly different.

Table 6. Short-chain fatty acids at the end of fermentation for different *M. oleifera* sources. Acetate, propionate and butyrate are presented along with total short-chain fatty acids. The mean concentration in mM, average standard error of the differences (SED) and *p*-value are presented.

	Acetate	Propionate	Butyrate				
Substrate	**[mM]**	**[mM]**	**[mM]**	**SCFA [mM]**	**Acetate [%]**	**Butyrate [%]**	**Propionate [%]**
Ryegrass	56.40	15.02	8.02	83.39	67.64	9.62	18.01
White clover	52.95	12.98	7.35	75.09	70.52	9.79	17.29
leaves from greenhouse	55.27	14.98	9.31	85.45	64.68	10.90	17.53
F + G Composite	49.55	14.47	8.66	76.27	64.97	11.35	18.98
Queenstown	55.10	13.56	8.46	82.49	66.79	10.25	16.43
Benab	54.49	14.09	8.62	83.18	65.51	10.36	16.94
PKM-1 (t)	52.75	13.27	8.49	77.87	67.74	10.90	17.04
PKM-1 (g)	54.89	14.34	9.21	84.48	64.98	10.91	16.97
Mon Repos	52.60	13.63	8.22	78.30	67.17	10.49	17.41
Bush Lot	53.55	13.64	8.14	80.51	66.52	10.11	16.94
SED	1.814	0.483	0.332	2.635			
p-value	0.015 *	<0.001 ***	<0.001 ***	0.005 *			

*** Significant $p < 0.001$, * Significant $p < 0.05$.

4. Discussion

In vitro fermentation results showed variations in gas production for provenances of *M. oleifera* (Table 5). Variations in gas production during fermentation can be caused by the nutritional composition of the plant. Plant nutrition is affected by genetics [29] and soil fertility. The different provenances may have genetic variations, and these may cause variations in the plant nutritional content. Because fermentation is a function of the cellulose content [30], a change in cellulose content can affect methane production. In fact, a study done by Lima, Abdalla [31] reported that high cellulose content observed in *Megathyrsus* species led to an increase in methane production and species with low cellulose content showed lower methane production.

In this study the cellulose content of the leaves of the different provenances of *M. oleifera* was not measured but Dhakad, Ikram [32] had reported the fibre content differences in plants from different geographic locations. Fibre content ranges as low as 7 g per 100 g of leaf material in Nigeria to as high as 37 g per 100 g in Haiti. This implies that the geographical locations of the *M. oleifera* used in this study may have influenced the gas production and may explain the variation among provenances from Guyana and India.

In this study, ryegrass had a significantly higher gas production ($p < 0.001$) when compared with *M. oleifera* and white clover. This is most likely explained by its high digestibility (70–85%) [33].

Ulyatt, Lassey [34] had reported grass nutritional content changes with climate, which affects the amount of methane produced during fermentation. This may be the reason for different methane emission from plants in field compared with plants in greenhouses, i.e., different contrasting environmental conditions. Plants in open fields are exposed to the weather and plants in greenhouses are in a relatively controlled environment. Conversely, raw data and analysis after 12 and 24 hours' fermentation from this study showed *M. oleifera* from the field and the greenhouse performed similarly for methane and total gas production (Table 3 and Figures 1 and 2). In the preliminary analyses, the composition of the plants was not measured because of the lack of sufficient plant material, but previous literature

has shown nutritional composition of *M. oleifera* to vary [35]. The results obtained may suggest that methane emission from ruminants fed *M. oleifera* grown in greenhouses may differ from ruminants fed *M. oleifera* grown in open fields.

In this study, *M. oleifera* samples from the field and greenhouse had a pooled average methane and total gas production of 27.73 mL/g and 233.39 mL/g (millilitres of methane per gram of substrate) after 24 h, respectively. Work done by Meale, Chaves [36] reported total gas production of 187 mL/g DM (dry matter) incubated and a methane production of 6.4 mg/g digested DM after 24 h. The fresh material used was 60 days' old regrowth from mature trees grown in Ghana. However, no information was provided on whether the regrowth included leaves alone or leaves and stem. The dry material was obtained by oven-drying for 72 h at 55 °C. However, the technique used by [36] was not automated and gas production was not measured in real time, which may explain the extremely low methane production reported. This indicates that different techniques used to measure gas production during ruminal fermentation can cause variations in gas production. Hall and Mertens [37] showed that different in vitro fermentation methods affected results of gas production and fibre digestibility estimates. However, in this study, all *M. oleifera* dried leaves were measured using the same in vitro batch culture system by Muetzel, Hunt [15] and therefore the technique used here does not provide an explanation for variations in gas production among provenances.

All gas production results in this study are based on the fermentation of dried *M. oleifera* leaves only and exclude effects that may be caused by other feed additives. Other studies measured methane production from *M. oleifera* by using its leaf extract and incorporating it in diets. Adding *M. oleifera* leaf extract at a concentration of 1.8 mL/DM and 0.6 mL/g DM to balanced diets containing 75% forage and 25% concentrate resulted in a total gas production of 108.2 mL/0.5 g dry matter and 85 mL/0.5 g dry matter at 24 h, respectively. But this was not significantly different to providing feed with no additives, according to Pedraza-Hernández, Elghandour [21]. This suggests that the effect of lower gas and methane production from *M. oleifera* leaves is caused by the leaves and the variation is a result of their composition.

M. oleifera fermented faster than both ryegrass and white clover (Tables 3 and 5). Preliminary fermentation revealed that *M. oleifera* from the field and greenhouse had a higher percentage propionate and butyrate in relation to total short-chain fatty acids when compared with ryegrass and white clover, but lower acetate (Table 3). Short-chain fatty acids or volatile fatty acids are the product of anaerobic microbial fermentation of complex carbohydrates. The major short-chain fatty acids in abundance are acetate, propionate and butyrate. These are absorbed as nutrients which constitute up to 80% of the ruminant maintenance energy requirement [38]. This implies that they are important measures of the quality of feed for ruminants and should be included in in vitro fermentation studies. More propionate at the end of fermentation for *M. oleifera* could mean that the hydrogen produced is not diverted to methane production, but used for the formation of propionate [39]. This could also imply there are compounds in *M. oleifera* that drive alternative pathways to remove hydrogen and thereby produce less methane. *M. oleifera* has been known to contain condensed tannins [40,41] a compound in white clover, reported to reduce ammonia and methane in vitro [42].

In the main experiment some *M. oleifera* provenances also had lower acetate concentration after fermentation than high quality ryegrass and white clover but the values were not significant. Similarly, a lower acetate content was found when comparing *M. oleifera* with other pasture species such as *Andropodon gayanus*, *Brachiaria ruziziensis*, *Pennisetum purpureum*, *Cajanus cajan*, *Cratylia argentea*, *Gliricidia sepium*, *Leucaena leucocephala* and *Stylosanthes guianensis*, *Annona senegalensis*, *Securinega virosa* and *Vitellaria paradoxa* [36]. Ryegrass belongs to the *Poaceae* plant family [43] along with *Andropodon gayanus*, *Brachiaria ruziziensis* and *Pennisetum purpureum*, and are all called grasses. White clover, however, belongs to the *Fabaceae* plant family [44,45] together with *Stylosanthes guianensis* and *Cratylia argentea*. This relationship may be the reason why ryegrass and white clover had a higher

similar acetate concentration as the species reported. Similar results were found by Soliva, Kreuzer [23], and some authors did not quantify short-chain fatty acid content during their in vitro study [21].

A high acetate production may be caused by the high cellulose content. *M. oleifera*, ryegrass and white clover contain cellulose, and the high gas production obtained from the fermentation of ryegrass implies that it is more digestible than *M. oleifera* and white clover, and correlates with the acetate concentration obtained after fermentation. However, it has been reported that, plant-based diets are primarily constituted of cellulose and a satisfactory amount of sugars. As a result ruminants on plant based diets will have a microbial population that will consist mostly of cellulolytic and saccharolytic bacteria, causing more cellulose digestion and sugar formation that will result in a higher acetate production [46]. This implies that, generally, fermentation of plants high in digestible cellulose will yield a high acetate content.

5. Conclusions

This study has shown that dried *M. oleifera* leaves fermented faster than high quality ryegrass and produce less methane and total gas in vitro. The fermentation of *M. oleifera* was similar to that of white clover but there were also some variations in gas production among the different provenances evaluated, suggesting that there is potential to select for low methane genotypes. *M. oleifera* leaves also produced a higher concentration of short-chain fatty acids than ryegrass or white clover at the end of fermentation but this was not significant. In addition, growing location (greenhouse or field) did significantly affect methane production among *M. oleifera* plants. Further research is required to examine the performance and fermentation parameters of Moringa in comparison to other high-quality forages of similar nutritive value.

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