In vitro Assessment of *Morinda lucida* Benth on Methanogenesis and Fermentation Parameters in West African Dwarf Goats

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Summary

Ruminants produce methane gas which is an energy loss to them, and also one of the greenhouse gases which cause global warming. This study assessed the effect of varying levels of *Morinda lucida* Benth leaf powder (MLLP) on methane production and rumen fermentation parameters using *in vitro* technique. Six concentrate diets were formulated to contain MLLP as an additive at levels 0, 2, 4, 6, 8 and 10mg g^{-1} DM. Fresh *Panicum maximum* Jacq. leaves were air dried to constant weight, milled and combined with each of the concentrate diet in the ratio 60 : 40 to serve as the substrate for the *in vitro* study. Incubation was carried out for 48 hours with 12 replicates per treatment in a completely randomized design. Data obtained were subjected to one-way analysis of variance and significant means were separated using the Duncan multiple range test. Results obtained showed that MLLP contained saponin (8.62%), alkaloids (3.48%), tannin (511.4 mg 100 g^{-1}), phenolic acids (328.15 mg 100 g^{-1}), oxalate (110.58 mg 100 g^{-1}), cyanogenic glycoside (4.34 mg 100 g^{-1}), flavonoids (595.63 mg 100 g^{-1}) and trypsin inhibitor (4.35 TIU g^{-1}). Inclusion of MLLP up to 10 mg g^{-1} in the diet did not ($P > 0.05$) affect metabolizable energy, short chain fatty acids, total volatile fatty acids production and pH. However, net gas production, methane production, *in vitro* organic matter digestibility, *in vitro* dry matter digestibility and ammonia nitrogen concentration decreased ($P < 0.05$) at 8 and 10 mg g^{-1} DM. The study therefore concluded that MLLP could be used to manipulate rumen fermentation at 2 mg g^{-1} DM to depress methanogenesis without negative effect on dry matter digestibility and ammonia nitrogen concentration.

Key words

methane, phytogenic additive, rumen fermentation, *in vitro*, ruminants

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Introduction

Digestion in ruminant is majorly a microbial process which leads to the production of compounds that animals can absorb and utilize as well as methane gas which is eructated into the air ((Bassett, 2013). Feed degradation in the rumen results in the production of volatile fatty acids, ammonia nitrogen, carbondioxide (CO_2) and methane (CH_4). Methane emission and ammonia release are seen as a disadvantage (Wallace, 2004) while volatile fatty acids (VFAs) production is an advantage of rumen fermentation of feed as VFAs are the major sources of energy for ruminants. Enteric methane production by ruminants contributes to the increasing problem of global warming due to accumulation of greenhouse gases (Tseu et al., 2021; Bamikole et al., 2019) and also reduces the efficiency of nutrient utilization by ruminants (Moss et al., 2000). Methane production contributes to a loss of about 2 - 15% of the gross energy of animal feed (Flachowsky et al. 2011) which otherwise could have been utilized by the ruminant. The livestock sector is responsible for 18% of global anthropogenic greenhouse gas (GHG) emissions (Steinfeld et al., 2006) while methane production by ruminants is about 15% of total atmospheric methane emissions (Takahashi et al., 2005). It is worth noting that in addition to concerns about GHG emissions, methane represents a significant loss of energy that could be potentially repartitioned towards tissues or the mammary gland (Bayat and Shingfield, 2012). The efficiency of energy and protein utilization in the rumen may be improved through the manipulation of rumen fermentation process. Several strategies have been used to manipulate rumen fermentation to improve nutrient utilization and mitigate methane production by ruminants. Use of medicinal plants as feed additives for livestock has received attention in recent years as alternative to antibiotics which have been banned in some countries due to safety concerns (Adebayo et al., 2021; Adebayo et al., 2022).

Morinda lucida Benth, a medium-sized tropical rainforest tree also called Brimstone tree is widely distributed in Africa. It is a versatile plant used in traditional medicine of many countries for the treatment of a variety of ailments and the claims of efficacy are particularly remarkable in the treatment of infections and immuno-inflammatory disorder (Nworu et al., 2012). The leaves are used as an ingredient of "fever teas," usually taken for the traditional treatment of malaria (Nworu et al., 2012). Different parts of the plant are attributed with diverse therapeutic benefits. In most parts of West Africa, it is used among traditional healers to treat fever, dysentery, abdominal colic and intestinal worm infestation, diabetes, hypertension, and gonorrhoea. Decoctions and infusions of root, bark and leaves are recognized remedies against different types of fever, including yellow fever, malaria, trypanosomiasis and feverish condition during childbirth (Adejo et al., 2015). Phytochemicals associated with *Morinda lucida* include anthraquinones, alkaloids, tannins, flavonoids, saponins, glucosides, and triterpenoids (Adeyemi et al., 2014; Adeleye et al., 2018a). Plants rich in phytochemicals otherwise known as secondary metabolites have been reported (Yusuf et al., 2018; Bamikole et al., 2019; Adebayo et al., 2021; Oni et al., 2021) to be effective in rumen manipulation and invariable depression of methanogenesis.

Studies have been carried out on chemical composition (Adesogan, 1973; Koumaglo et al., 2007; Adeyemi et al., 2014; Osuntokun et al., 2016) and anthelmintic property (Adewunmi and Adesogan, 1984) of *Morinda lucida* as well as its effects on health status of Wister rats/mice (Makinde and Obih, 1985; Adeneye and Agbaje, 2008; Anofi and Olugbenga, 2011; Nworu et al., 2012; Adeleye et al., 2018b), broiler chickens (Ola-Fadunsin and Ademola, 2014; Olayemi et al., 2016; Lala et al., 2018), sheep (Osakwe and Drochner, 2006) and humans (Appiah-Opong et al., 2016). There is a dearth of information on the use of *Morinda lucida* in mitigating methane production and as feed resource for ruminants. Hence, this study investigated the effect of diets containing varying levels of *Morinda lucida* leaf powder on rumen fermentation parameters and methane production using *in vitro* technique.

Materials and Methods

Experimental Site

The experiment was carried out at the Department of Animal Nutrition laboratory of the Federal University of Agriculture, Abeokuta (FUNAAB) Ogun State, Nigeria. Ogun State is in the rainforest zone of South West Nigeria. The area has an annual mean temperature of 34.7 °C, a relative humidity of 82% and an annual mean rainfall of 1,037 mm. It is about 70 m above sea level and lies at latitude 7°5'-7°8'N and longitude 3°11.2'E (Google Earth, 2020).

Collection of Samples and Formulation of Diets

Morinda lucida plant was harvested in and around the premises of the University and was identified at the Department of Pure and Applied Botany, FUNAAB. The leaves were detached from the stem and air-dried for 7 days to constant weight. The dried leaves were later milled to pass through 2 mm sieve, bulked and stored for later use. The leaf powder was used to formulate six (6) concentrate diets (Table 1) at levels 0, 2, 4, 6, 8 and 10 mg g^{-1} DM. *Panicum maximum* was harvested fresh from an established pasture after 7 weeks of re-growth following a cut-back, then air dried to constant weight and milled to pass through 1 mm sieve. The concentrate diets were combined with *Panicum maximum* in ratio 40:60 (200 mg of substrate was incubated in each syringe out of which 40% was made up of concentrate diet and the remaining 60% was *P. maximum*) and served as the substrates for this *in vitro* study.

In vitro Gas Production Study

The *in vitro* gas production procedure was conducted as reported by Menke and Steingass (1988). Rumen fluid needed for the experiment was collected from four (4) West African dwarf bucks under the same feeding regime using suction tube as described by Babayemi and Bamikole, (2006), prior to morning feeding. The fluid was collected in a pre-warmed thermos flask and taken to the laboratory immediately. Coarse materials were removed from the rumen fluid by filtering through four-layered cheese cloth. Handling of the rumen fluid was done under a continuous flow of CO_2. Two hundred milligrams (200 mg) of the substrate (consisting of 60% milled *Panicun maximum* Jscq.+ 40% concentrate diet containing 0, 2, 4, 6, 8 and 10 mg g^{-1} *M. lucida*) were weighed into 100 mL calibrated transparent glass

syringes fitted with silicon tube to make six treatments which were replicated twelve times each. Thereafter, 30 mL of medium consisting 10 mL of rumen fluid and 20 ml of buffer solution were added to each syringe. The syringes were tapped and pushed upward by piston to eliminate air in the inocula. The silicone tubes in the syringes were tightened by a metal clip so as to prevent escape of gas. Incubation was carried out at 39 °C and the volume of gas produced was measured at three-hour interval from 0-48 hours. Three blanks containing 30 ml of medium only were included in the run to correct for gas production not arising from degradation of substrate. The gas volume produced by the blanks was subtracted from the gas volume produced by samples to give the net gas production.

Estimation of Methane Gas Production

The volume of methane gas from each sample was determined by dispensing 4 ml of sodium hydroxide (NaOH, 10M) into the sample at the end of 48 hours of incubation. The tip of a 5ml syringe used for collection of 2 ml NaOH was used to introduce it into the incubated syringe. The clip was then unscrewed and the reagent (NaOH) introduced was added to absorb CO_2 produced during fermentation and the remaining gas was recorded as methane according to (Fievez et al., 2005). The syringe was turned upside down for the reading of the methane level. The average of the volume of methane produced from the blanks was deducted from the volume of methane produced per samples to give the net methane production from substrates.

Determination of *In vitro* Dry Matter Digestibility

After 48 hours of incubation, samples were centrifuged at a speed of 2500 rpm for 10 mins. The residue was washed with warm water until the water was clear. Each sample was dried at 65 °C and the weight of the oven dried residue was recorded. The percentage of *in vitro* dry matter digestibility (IVDMD) was calculated as:

IVDMD% = ((DM of samples incubated – DM of residues) × 100) / DM of samples incubated

Estimation of Post Incubation Parameters

Post incubation parameters such as metabolizable energy, organic matter digestibility and short chain fatty acids were estimated at the end of the incubation process as follows:

- Metabolizable Energy (ME) (MJ kg^{-1} DM) = 2.20 + 0.136GP + 0.0574CP + 0.0029 CF (Menke and Steingass, 1988)
- Organic Matter Digestibility (OMD) (%) = 14.88 +0.889GP + 0.45CP + 0.651Ash (Menke and Steingass, 1988)
- Short Chain Fatty Acids (SCFA) (µmol g^{-1} DM) = 0.0239GP – 0.0601 (Getachew et al., 2002),

where GP is the net gas production at 24hours, CP is the crude protein content of substrate and CF is crude fibre content of substrate.

Determination of Total Volatile Fatty Acids, Ammonia Concentration and pH of Substrates

At post incubation period 20 ml of incubation fluid from three syringes per treatment was transferred into plastic bottles and pH was measured immediately using portable pH meter (HANNA Instruments HI 98153). Thereafter, 1 mL of metaphosphoric acid was added to stop microbial fermentation and centrifuged at 2500 rpm for 10 min. The supernatant was decanted to determine total volatile fatty acids (TVFAs) using Markham apparatus (Barnett and Reid 1956). This was carried out by adding 2 mL of rumen fluid together with 1 mL 10 % Potassium oxalate buffer and 1 mL oxalic acid injected into the Markham apparatus, where a distillate of 100 ml was collected. This was then titrated against a standard 0.01N NaOH with 2 drops of phenolphthalein as indicator. Concentration of TVFA was then calculated using the following equation:

TVFA (Mm) = (NaoH volume × NaOH normality × 1000) / rumen inoculum volume

Ammonia-N concentration was determined using steam distillation procedures (Ogubai and Sereke, 1997).

Table 1. Ingredients composition (%) of concentrate diets

Ingredients	Levels of inclusion of *Morinda lucida* leaf powder (mg g^{-1})					
	0	2	4	6	8	10
Wheat offal	30.00	30.00	30.00	30.00	30.00	30.00
Maize bran	30.00	30.00	30.00	30.00	30.00	30.00
Palm kernel cake	20.00	20.00	20.00	20.00	20.00	20.00
Rice bran	17.00	17.00	17.00	17.00	17.00	17.00
Bone meal	2.00	2.00	2.00	2.00	2.00	2.00
Salt	1.00	1.00	1.00	1.00	1.00	1.00
Morinda lucida leaf powder	-	+	++	+++	++++	+++++
Total	100	100	100	100	100	100

Chemical Analyses

Oven-dried samples of the substrates were analyzed for their proximate composition according to AOAC (2000). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to Van Soest et al. (1991). Tannin was determined following the procedure of Marinova et al. (2005), Alkaloid (Harborne, 1973), Phenolic acid (Singh et al., 2012). Flavonoids were analyzed according to Mahajan and Badujar (2008), cyanogenic glycosides according to method published by Onwuka (2005), oxalate according to the method published by Munro (2000), saponin was analyzed according to the method published by Obadoni and Ochuko (2001), phytic acid was analyzed according to the method published by Oboh et al. (2002) and trypsin inhibitor according to the method published by Kakade et al. (1974) as modified by Hammerstrand et al. (1981). Metabolizable energy was calculated using Pauzenga (1985) equation.

Statistical Analysis

Data obtained were subjected to one-way analysis of variance in a completely randomized design using version 9.1 of SAS software (SAS, 2003) with the following model: $Y_{ij} = \mu + T_i + e_{ij}$, where Y_{ij} = observed variation, μ = population mean, T_i = effect of varying levels of leaf powder and e_{ij} = error term. Means were separated using the Duncan procedure of the same software.

Results

Chemical Composition of Experimental Diets (Substrate)

The chemical composition (on dry matter basis) of the concentrate diet and *Panicum maximum* are presented in Table 2. The dry matter (93.77%) and crude protein (14.18%) content of the concentrate diet were higher than those of *P. maximum* which were 44.51% and 8.05% respectively. Crude fibre (20.00%), neutral detergent fibre (64.33%), acid detergent fibre (29.00%) and acid detergent lignin (27.33%) values of *P. maximum* were higher than 12.67%, 59.00%, 21.00% and 19.33% obtained for the concentrate diet. Metabolizable energy value obtained for *P. maximum* was 11.91 MJ kg^{-1} while that of the concentrate diet was12.24 MJ kg^{-1}.

Table 2. Chemical composition (% DM) of experimental diets

Parameters	Concentrate	*Panicum maximum*
Dry matter	93.77	44.51
Crude Protein	14.18	8.05
Crude fibre	12.67	20.00
Ether extract	4.50	3.00
Ash	9.83	9.25
Nitrogen free extract	58.82	59.7
Neutral Detergent fibre	59.00	64.33
Acid Detergent Fibre	21.00	29.00
Acid Detergent Lignin	19.33	27.33
Metabolizable Energy (MJ kg^{-1})	12.24	11.91

Phytochemical Composition of *Morinda lucida* Leaf Powder

The phytochemical constituents of *M. lucida* leaf powder (MLLP) is presented in Table 3. Tannin, phenolic acid, oxalate, glycoside, flavonoid, trypsin inhibitor, saponin and alkaloid are present in MLLP. Saponin content was 8.62%, alkaloid 3.48%, flavonoids 595.63 mg 100 g^{-1}, tannin 511.41 mg 100 g^{-1}, phenolic acid 328.15 mg 100 g^{-1}, trypsin inhibitor 229.01 mg 100 g^{-1}, oxalate 110.58 mg 100 g^{-1} and glycoside 4.34 mg 100 g^{-1}.

Table 3. Phytochemical composition of *Morinda lucida* leaf powder

Parameter	Content
Tannin (mg 100 g^{-1} DM)	511.41
Phenolic acid (mg 100 g^{-1} DM)	328.15
Oxalate (mg 100 g^{-1} DM)	110.58
Cyanogenic glycoside (mg 100 g^{-1} DM)	4.34
Flavonoids (mg 100 g^{-1} DM)	595.63
Trypsin inhibitor (TUI mg^{-1} DM)	4.35
Saponin (%)	8.62
Alkaloids (%)	3.48

In vitro Gas Production and Post Incubation Parameters of Substrates Containing Varying Levels of *Morinda lucida* Leaf Powder

Metabolizable energy (ranging between 10.75-11.49 MJ kg^{-1}) and short chain fatty acids (ranging between 1.29-1.42 µmol) were not significantly ($P > 0.05$) influenced by the inclusion of *M. lucida* powder (MLLP) in the substrates (Table 4). Net gas production, methane production, organic matter digestibility and dry matter digestibility decreased ($P < 0.05$) as the level of MLLP increased in the substrate. Net gas production was the highest at 0, 2, 4, and 6 mg g^{-1} MLLP inclusion levels with the values ranging from 55.67-59.00 mL 200 mg^{-1} DM and the lowest at 8 and 10 mg g^{-1} MLLP inclusion levels with the values of 54.33 and 50.67 mL 200 mg^{-1} DM, respectively. Methane production was the highest (26.00 mL 200 mg^{-1} DM) at the control and the lowest (16.33 and 13.67 mL 200 mg^{-1} DM) at 8 and 10 mg g^{-1} MLLP inclusion levels. Methane percentage (expressed as a percentage of net gas production) was also the lowest ($P < 0.05$) at 8 and 10 mg g^{-1} inclusion levels of MLLP. Higher values (82.13, 80.94, 80.35, 79.16 and 77.98%) were obtained for *in vitro* organic matter digestibility (IVOMD) at 0, 2 ,4, 6, and 8 mg g^{-1} MLLP inclusion levels respectively, while lower (71.64%) value ($P < 0.05$) was obtained at 10 mg g^{-1} inclusion level of MLLP. *In vitro* dry matter digestibility (IVDMD) was the highest (63.33%) at the control and the lowest (36.67%) at 10 mg g^{-1} inclusion level of MLLP.

Table 4. *In vitro* gas production and post incubation parameters of substrates containing varying levels of *Morinda lucida* leaf powder

Parameters	Levels of inclusion of *Morinda lucida* leaf powder (mg g^{-1})							
	0	2	4	6	8	10	SEM	P
NGP	59.00[a]	57.63[a]	56.00[a]	55.67[a]	54.33[ab]	50.67[b]	1.56	0.04
CH_4	26.00[a]	23.33[ab]	20.00[ab]	20.00[ab]	16.33[b]	13.67[b]	1.67	0.04
CH_4 %	44.03[a]	40.45[a]	35.09[a]	35.92[a]	30.06[b]	25.47[b]	2.19	0.02
ME	11.49	11.30	11.21	11.03	10.85	10.75	0.22	0.95
IVOMD	82.13[a]	80.94[a]	80.35[a]	79.16[a]	77.98[a]	71.64[b]	1.58	0.04
SCFA	1.42	1.39	1.37	1.34	1.31	1.29	0.04	0.95
IVDMD	63.33[a]	56.67[ab]	50.00[ab]	50.00[ab]	46.67[b]	36.67[c]	3.75	0.03

Note: Means on the same row having different superscripts are significantly different ($P < 0.05$); NGP - net gas production (mL 200 mg^{-1} DM); CH_4 - methane ((mL 200 mg^{-1} DM); ME - metabolizable energy (MJ kg^{-1}); IVOMD - *in vitro* organic matter digestibility (%); SCFA - short chain fatty acid (µmol); IVDMD - *in vitro* dry matter digestibility (%); ME, IVOMD and SCFA were estimated using equations by Menke and Steingass (1988); SEM -standard error of mean; *P* – *P* - value

Table 5. Total volatile fatty acids, pH and ammonia-N concentrations of incubation fluid

Parameters	Levels of inclusion of *Morinda lucida* leaf powder (mg g^{-1})							
	0	2	4	6	8	10	SEM	P
pH	6.25	6.14	6.01	6.43	6.35	7.04	0.31	0.42
TVFA (Mm)	37.00	35.10	37.27	38.20	39.13	37.87	0.09	0.50
NH_3-N (mg dL^{-1})	51.88[a]	53.58[a]	46.78[a]	48.48[a]	44.27[b]	39.97[b]	0.49	0.04

Note: Means on the same row having different superscripts are significantly different ($P < 0.05$); SEM – standard error of mean; *P* – *P* - value

Total Volatile Fatty Acids, pH and Ammonia-N Concentrations of Incubation Fluid of Substrate Containing Varying Levels of *Morinda lucida* Leaf Powder

Inclusion of MLLP in the substrate did not significantly ($P > 0.05$) influence the pH (ranging between 6.01 - 7.04) and total volatile fatty acids (ranging between 35.10 and 39.13 mM) concentration (Table 5). However, ammonia nitrogen concentration reduced ($P < 0.05$) at 8 and 10 mg g^{-1} inclusion levels MLLP. Higher values ($P < 0.05$); 51.88. 53.88, 46.78 and 58.48 mg dL^{-1} were recorded at 0, 2, 4, and 6 mg g^{-1} inclusion levels while lower values of 44.27 and 39.97 mg dL^{-1} were obtained at 8 and 10 mg g^{-1} inclusion levels respectively.

Discussion

The dry matter obtained for *Panicum maximum* Jacq. in this study was higher than 25.6%, 30.70% and 35.46% reported by Oyaniran et al. (2018), Yusuf et al. (2013) and Eyoh et al. (2019) respectively. It was, however, lower than 65.50% and 74.4% obtained by Yusuf et al. (2016) and Adebayo et al. (2022) respectively for wilted *P. maximum*. The differences observed in the dry matter may be attributed to processing (whether fresh or wilted), stage of growth or season of harvest of the plant. Dry matter tends to increase with the age of the plant. According to Irwin et al. (2014) flowering and matured plants tend to have low moisture and high fibre compared to emerging plants and dry matter is higher during the rainy season than in the dry season. The dry matter of the concentrate supplement in this study was higher than 83.50% obtained by Yusuf et al. (2016). This suggests that the ingredients used were all properly dried. The crude protein value obtained for *P. maximum* was similar to the report of Aderinboye et al. (2011) and Eyoh et al. (2019) who reported 8.12% and 8.89% CP, respectively. The crude protein content of *P. maximum* and concentrate supplement was above 7% required for maintenance in ruminants (Pugh, 2020) but in line with the recommendation of NRC (1981) for growth and production. The crude fibre value of 20.00% obtained in this study was lower than 23.62% and 28.95% reported by Obua et al. (2012) and Eyoh et al. (2019) respectively. Ash represents the inorganic or mineral content of a feed. The ash and ether extract values recorded for *P. maximum* were comparable to 10.00% and 2.94% reported by Yusuf et al. (2013) and Eyoh et al. (2019) for ash and ether extract respectively. Fibre is essential for proper rumen function. The neutral detergent fibre value obtained was similar to 64.36% recorded by Aderinboye et al. (2011) but the acid detergent fibre value was lower than 43.26% and 36.00% reported by the Aderinboye et al. (2011) and Oyaniran et al. (2018) respectively. The disparity in the values obtained by the authors may be linked to different seasons and age of harvest.

The phytochemical analysis of *M. lucida* in this study revealed the presence of saponins, alkaloids, flavonoids, tannin, phenolic acid, trypsin inhibitor, oxalate, phytate and cyanogenic glucosides. This is similar to the report of Ogundare and Onifade (2009), Ugbeni and Osubor (2017) and Igwilo et al. (2018) that reported the presence of saponin, tannin, alkaloids, phytate, oxalate, and cyanogenic glycosides in *M. lucida* leaves. Phytochemicals associated with *M. lucida* include 10 anthraquinones, alkaloids, tannins, flavonoids, saponins, glucosides, and triterpenoids (Adeleye et al., 2018a). Adewumi and Adesogan (1984) also reported that tannins, flavonoids and saponins had been detected and isolated in *M. lucida*.

The lowest net gas production observed at 10 mg g^{-1} inclusion levels of MLLP can be attributed to the activity of secondary metabolites in MLLP which was able to manifest at higher inclusion level. This suggests that the metabolites were able to interfere with the activities of microorganisms involved in feed degradation. Yusuf et al. (2013) opined that anti nutritional factors inactivated some microorganisms in the rumen thereby inhibiting fermentation activities and hence decrease in gas production. Tannins and saponin have been reported to reduce feed degradation and invariably gas production (Min et al., 2003; Hess et al., 2003; Guo et al., 2008; Silivong, 2012). According to Adebayo et al. (2021) reduction in gas production is an indication of reduced digestibility of feed. The higher the digestibility of feed, the higher the gas production and vice versa. Thus the reduced gas production obtained at 10 mg g^{-1} MLLP is an indication of reduced digestibility of the substrate. This result supports the findings of some authors (Bamikole et al., 2019; Aderinboye et al., 2020; Oni et al., 2021; Adebayo et al., 2021) who also observed reduced gas production with the use of phytogenics/nutraceuticals. Methane production was the lowest at 8 and 10 mg g^{-1} inclusion levels of MLLP. This suggests that the phytochemicals in MLLP were able to suppress methanogenesis at these levels. Saponin and tannins reduced methanogens and protozoa population in the rumen (Kamra, 2008; Cieslak et al., 2009).

Protozoa produce large amount of hydrogen which is utilized by methanogens for methane production, hence a reduction in protozoa number will invariably depress methanogenesis. Tropical plants containing saponin and tannins have been reported to decrease methanogenesis (Patra et al., 2006; Bodas et al., 2009; Goel and Makkar, 2012; Tseu et al., 2021). Flavonoids (Kim et al., 2015) and Phenols (Asiegbu, 1995) have also been implicated in methane inhibition. A positive relationship seems to exist between total gas production and methane gas production. The higher the gas production, the higher the methane gas produced. Aderao et al. (2018) and Tseu et al. (2021) attributed methane reduction potentials of plant secondary metabolites to inhibition of fibre degradation. However, Babayemi et al. (2005) reported that the amount of methane produce did not relate to the extent of digestibility. Reduction in *in vitro* organic and dry matter digestibility at 10mg g^{-1} inclusion level of MLLP could also be attributed to the secondary metabolites in MLLP which hindered microbial degradation of feed. According to Pineiro-Vazquez et al. (2015) and Tseu et al. (2020) tannins decrease fibre digestibility by forming complexes with lignocellulose and inhibit microbial digestion. Min et al. (2002) reported that tannin decreased dry matter digestibility. According to Kongmun et al. (2010) *in vitro* dry matter digestibility is an indicator of microbial population and activity during substrate fermentation. The decreased *in vitro* organic and dry matter digestibility observed at 10 mg g^{-1} inclusion level of MLLP could be responsible for the reduced methane gas production recorded at this level. Some authors (Cobbellis et al., 2016; Joch et al., 2019; Aderinboye et al., 2020; Adebayo et al., 2021) have also reported reduction in methane gas production with associated decrease in feed digestibility. Goel and Makkar (2012) observe that substantial reduction in methane gas production seems unachievable without reduction in feed digestibility. However, Kawed et al. (2016) and Oni et al. (2021) recorded an increase in *in vitro* dry matter digestibility with *Chromolaena odorata* (L.) R.M.King & H.Rob. leaf meal. Kholif et al. (2017) reported that feeding lemon grass or rosemary treatment increased organic matter and fibre digestion in an *in vitro* study. Jimenez-Peralta et al. (2011) and Oni et al. (2021) also observed an increase in *in vitro* organic matter digestibility with Leucaena lucocephala and *C. odorata* leaf meals respectively. The differences in the results obtained could be attributed to different test ingredients (with varying compositions of phytochemicals) and levels of inclusions used by the authors.

Ammonia-nitrogen is an essential source of nitrogen for microbial protein synthesis (Wanapat, 2000). Excess of it is absorbed by the ruminal wall into the portal circulation where it is converted by the liver into urea and excreted in the urine. Ammonia nitrogen production in the rumen generally exceeds the capacity of use by microbes leading to accumulation and subsequent absorption and conversion to urea by the liver (Rodrigues, 2016). Inclusion of MLLP at 10 mg g^{-1} DM reduced ammonia nitrogen concentration. This is indicative of reduced protein degradability as a result of the phytochemicals in MLLP. This could also be the explanation for decreased dry matter digestibility observed. Concentration of ammonia nitrogen in the rumen fluid is the net result of ammonia nitrogen production from feed, protein digestion and absorption rate through the rumen wall and utilization by microorganisms (Okunade et al., 2020). Plants containing tannin and saponin have been reported to reduce ammonia production in the rumen (Patra and Saxena, 2010; Bodas et al., 2012). According to McSweeny et al. (2001) and Gurbuz et al. (2006), the effects of tannin are associated with their ability to combine with dietary proteins, cell wall polymers such as cellulose, hemicellulose and pectin as well as minerals thus either retarding or preventing their microbial digestion. Tannins are polyphenolic compounds which bind to proteins and can be used as chemical additives for protecting and decreasing ruminal fermentation of proteins in ruminant feeds (Makkar, 2003). Nigrant et al. (2017) also report that tannins have the ability to bind to protein rendering them inaccessible to rumen degradation and making them available post-ruminally. Lu and Jorgensen (1987) showed that saponins, isolated by ethanol extraction, hydrolyzed partially, and administered to sheep intra-ruminally, reduced microbial fermentation and total protozoa count was also significantly reduced.

Conclusion

The result of this study shows that addition of *Morinda lucida* leaf powder up to 10 mg g^{-1} DM had no effect on metabolizable energy, short chain fatty acids, pH and total volatile fatty acids production. It, however, reduced net gas production, *in vitro* dry matter digestibility, organic matter digestibility, methane gas production and ammonia nitrogen.

M. lucida leaf powder can be used at 2 mg g^{-1} DM for rumen manipulation to reduce methane gas production without detrimental effect on dry matter digestibility and ammonia nitrogen production. Inhibition of methanogenesis is advantageous in reducing global warming and improvement of productivity in ruminant animals.

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