Effects of sodom apple (*Calotropis procera)* leaf powder as additive in a total mixed ration on *in vitro* rumen fermentation characteristics and methane production

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Abstract

Ruminants contribute to the emission of anthropogenic methane which is one of the important greenhouse gases that cause global warming. Natural forms of additives are considered safe in reducing methane emission by ruminants. Using in vitro study, five levels (0, 5, 10, 15 and 20 mg/g) of Calotropis procera leaf (CLP) powder were added to a total mixed ration to form five different treatments. Each treatment was replicated ten times and were incubated with 30 mL of inoculum for 48 hours. Inclusion of Calotropis procera leaf powder in the diet reduced the net gas and methane production. Methane production was reduced by 29.67% and 28.89% with addition of 15 and 20 mg/g CLP in the diets respectively. Protozoal count reduced significantly ($p < 0.05$) with inclusion of 10 to 20 mg/g CLP. Diets containing 15 and 20 mg/g CLP recorded lower potential gas production (b) and rate of gas production (c) with higher lag time. In vitro organic matter digestibility, metabolizable energy and short chain fatty acids were unaffected with the inclusion of up to 15 mg/g CLP. Inclusion of 20 mg/g CLP in the diets gave the least dry matter digestibility. Higher partitioning factor was recorded in 10 mg/g CLP diet while microbial yield was higher in 0 mg/g and 10 mg/g CLP diet. Ammonia nitrogen concentration was significantly reduced ($P < 0.05$) in diets with 15 and 20 mg/g CLP. In conclusion, addition of 10 mg/g and 15 mg/g Calotropis procera leaf powder is encouraged in the diet of ruminants for enteric methane and ammonia nitrogen reduction with mild effect on nutrient digestibility. This study therefore was carried out to investigate the effects of sodom apple leaf powder as additive in a total mixed ration on in vitro rumen fermentation characteristics and methane production.

Keywords: Fermentation pattern, gas production, methane, additive, *Calotropis procera*, ruminants

Running title: Rumen fermentation and Sodom apple leaf

Effets de la poudre de feuilles de pomme de sodome (*Calotropis procera*) comme additif dans une ration totale mélangée sur les caractéristiques de fermentation du rumen in vitro et la production de méthane

Résumé

Les ruminants contribuent aux émissions de méthane d'origine anthropique, qui est l'un des gaz à effet de serre importants responsables du réchauffement climatique. Les formes naturelles d'additifs sont considérées comme sûres pour réduire les émissions de méthane chez les ruminants. À l'aide d'une étude in vitro, cinq niveaux (0, 5, 10, 15 et 20 mg/g) de poudre de feuilles de Calotropis procera (CLP) ont été ajoutés à une ration totale mélangée pour former cinq traitements différents. Chaque traitement a été répliqué dix fois et incubé avec 30 mL d'inoculum pendant 48 heures. L'inclusion de poudre de feuilles de Calotropis procera dans le régime a réduit la production nette de gaz et de méthane. La production de méthane a été réduite de 29,67 % et de 28,89 % avec l'ajout de 15 et 20 mg/g de CLP dans les régimes respectivement. Le comptage des protozoaires a diminué de manière

significative (p < 0,05) avec l'inclusion de 10 à 20 mg/g de CLP. Les régimes contenant 15 et 20 mg/g de CLP ont enregistré une production de gaz potentielle (b) et un taux de production de gaz (c) plus faibles avec un temps de latence plus élevé. La digestibilité in vitro de la matière organique, l'énergie métabolisable et les acides gras à chaîne courte n'ont pas été affectés par l'inclusion de CLP jusqu'à 15 mg/g. L'inclusion de 20 mg/g de CLP dans les régimes a donné la digestibilité de la matière sèche la plus faible. Un facteur de partitionnement plus élevé a été enregistré dans le régime avec 10 mg/g de CLP, tandis que le rendement microbien était plus élevé dans les régimes contenant 0 mg/g et 10 mg/g de CLP. La concentration en azote ammoniacal a été réduite de manière significative (p < 0,05) dans les régimes contenant 15 et 20 mg/g de CLP. En conclusion, l'ajout de 10 mg/g et 15 mg/g de poudre de feuilles de Calotropis procera est recommandé dans le régime des ruminants pour réduire le méthane entérique et l'azote ammoniacal avec un effet modéré sur la digestibilité des nutriments. Cette étude a donc été réalisée pour examiner les effets de la poudre de feuilles de pomme de sodome comme additif dans une ration totale mélangée sur les caractéristiques de fermentation du rumen in vitro et la production de méthane.

Mots-clés : Mode de fermentation, production de gaz, méthane, additif, *Calotropis procera*, ruminants

Introduction

Rumen fermentation is the main cause of emission of anthropogenic greenhouse gases in ruminants. It is an important process which helps in the conversion of ingested feed into energy, microbial biomass and ammonia that are utilized by the host animal. Carbondioxide (CO_2) and hydrogen (H_2) are major gases released during fermentation process. These gases must be removed from the animal system to facilitate continuous fermentation of feed to produce volatile fatty acids which supply energy to the animals. One of the most important means of removing H_2 from the rumen is through methanogenesis; which is the production of methane by methanogenic archaea (methanogens) via reduction of CO_2 to methane (CH_4) (Martin et al., 2010). Methane production results in about 2 -12% reduction in gross energy production due to depriving carbon resources which ought to have been used for energy production (Blaxland et al., 2021), this impairs the effective nutrient utilization. Apart from this, methane affects the environment negatively as it traps atmospheric heat 23 times higher than CO_2 making it more harmful than CO_2 for 20 years after it is released (UN, 2022) and it is therefore considered one of the most important greenhouse gases that contribute immensely to global warming which have negative impacts on humans and animal production. Methane emission worldwide by domestic animals through enteric fermentation has been reported to be 95 million tons with increasing emission rate of 0.90% (Patra, 2014). It has also been established that the rate of climate change is faster compared to the past 1000 years and there is possibility of increase in average global temperature by 1.8 to 4°C within the next 90 years (Yatoo et al., 2012).

Ammonia, which is also one of the products released in the rumen during fermentation process is a major source of nitrogen for microbial growth. Its production always exceeds the capacity utilized by rumen microorganisms (Flachowsky and Lebzien, 2006). Based on this, ruminants are inefficient nitrogen utilizer with about 25% compared

with other productive animals (Kohn et al., 2005; Huhtanen and Hristov, 2009). The excess ammonia production in the rumen serves as pollutants to the environment when excreted by the animals.

In Africa, small ruminant production plays a critical role in the socio-economic development of the nation. The industry supports about one billion small-holder farmers in low and middle income countries (Alders et al., 2021). It has been estimated that demand for both mutton and goat meat will increase by 216% while supply is estimated to increase by 159% in 2050 (FAO, 2018) which may be due to increase in population growth. However, small ruminant production systems have been investigated for their environmental and social impact (FAO, 2006). For instance, about 6.5% of the world emission is from sheep and goats, corresponding to 429 thousands Gg $CO_{2\text{-eq}}$ (FAO, 2016). Attempts have been made by ruminant nutritionists to reduce emissions by ruminants using chemicals and antibiotics to manipulate the rumen fermentation process (Broderick et al., 1991). However, increase in the cost of synthetic antibiotics and chemicals as well as potential hazards of residual effects on both the animals and humans limits their application. Therefore, there is increase interest in the use of natural plants which are rich source of bioactive compounds such as tannins, saponins and essential oils to manipulate rumen fermentation for efficient nutrient utilization and eco-friendly ruminant production. Most of these bioactive compounds have anti-microbial properties and have been confirmed to influence rumen fermentation patterns positively (Wang et al., 2019). Tannin containing plants have been demonstrated with the capacity to reduce methane emission (Hristov et al., 2013) and rumen ammonia production (Broderick et al., 2017). Inclusion of Acacia mearnsii extract up to 2.5% DM has been shown to reduce methane production by 33% in Zebu cattle on high quality diet. Similarly, quebracho tannins in high-roughage diet reduced ammonia nitrogen concentration and urinary nitrogen in cattle (Norris et al., 2020).

Calotropis procera (giant milk weed) is an evergreen perennial shrub that is widely distributed and can strive in poor or arid soil without fertilizer. It belongs to Asclepiadaceae family which have wide range of therapeutic activities. The different parts of the plant such as the leaves, stem, root, bark and fruits possess various phytochemicals for different pharmacological activities which include anti-microbial, anthelmintic, insecticidal, anti-inflammatory, anti-oxidant and antidiarrheal with other beneficial properties (Ahmed et al., 2005). Calotropis procera leaf has been reported to contain Cardenolide, terpenes, flavonoids, tannins, alkaloids, saponin, glycosides and phenols (Murti et al., 2010). However, there is paucity of information on the use of Calotropis procera leaf to modify rumen fermentation and its effects on methanogenesis. This study therefore, investigated the influence of Calotropis procera leaf powder on rumen fermentation and methane production in vitro.

Materials and methods

Experimental location and preparation of experimental diets

The experiment was carried out at the Laboratory of Animal Nutrition Department, Federal University of Agriculture, Abeokuta, Nigeria, at 7°13’57°N and 3°26’12°E (Google Earth, 2024). Calotropis procera leaves were harvested at the premises of Federal University of Agriculture, Abeokuta and were properly cleaned to remove sand and dust;

sun-dried for 7 days and ground to pass through 1mm mesh. The ground leaves were added to a total mixed ration (consisting of 16% crude protein) at different levels of 0, 5, 10, 15 and 20 mg/g resulting in five experimental diets as shown in Table 1.

Table 1: Gross composition of total mixed ration

	Levels of *Calotropis procera* leaf powder (mg/g)				
Ingredients	0	5	10	15	20
Maize stover	17.00	17.00	17.00	17.00	17.00
Palm kernel cake	14.00	14.00	14.00	14.00	14.00
Soyabean meal	6.00	6.00	6.00	6.00	6.00
Wheat offal	30.00	30.00	30.00	30.00	30.00
Rice bran	30.00	30.00	30.00	30.00	30.00
Bone meal	1.50	1.50	1.50	1.50	1.50
Premix	0.50	0.50	0.50	0.50	0.50
Common salt	1.0	1.0	1.0	1.0	1.0
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Calotropis procera leaf powder	-	+	++	+++	++++

0 mg/g (-), 5 mg/g (+), 10 mg/g (++), 15 mg/g (+++), 20 mg/g (++++)

***In vitro* gas production procedure**

The *in vitro* gas production procedure was carried out as described by Menke and Steingass (1988). Rumen fluid was collected from six mature West African dwarf goats fed a typical total mixed ration (TMR) through the use of suction tube as described by Babayemi and Bamikole (2006). The fluid was collected before morning feeding into a pre-warmed insulated flask at 39˚C and was immediately taken to the laboratory. Collected fluid was strained through four layers of muslin cloth and the required amount was used to prepare the inoculum together with the prepared artificial saliva in the ratio 2:1. Two hundred milligrams (200 mg) of each experimental diet (treatment) was added into 100 mL plastic syringes. Inoculum (30 mL) was dispensed anaerobically (continuous flushing with CO_2) into each syringe. Air in the syringes was eliminated by tapping and pushing the syringes upward by a piston. Three blank syringes (control) which contained 30 mL inoculum only were included for correction of gas produced from rumen fluid alone. Each treatment was replicated ten times in a completely randomized design. Incubation was done for 48 hours and volume of gas produced was measured at 3 hours interval. The total gas volume was corrected for blanks across the treatments. Cumulative gas production data were fitted to the non-linear regression model of France *et al.* (2002) as follows:

$A = b(1 - e^{-c(t-L)})$ France *et al.* (2002)

Where A = gas produced at time "t", b = potential gas production from the fermentable fraction (ml), c = fractional rate of gas production (ml/hr), t = incubation time (h), L = Lag time (hr)

Methane production measurement

Methane gas in the total gas produced was determined at the end of incubation period (48 h) by introducing 4 mL of 10 M NaOH into

the incubated syringes (5 per treatment). A pop sound was given up immediately the NaOH was introduced which is an indication of CO_2 absorption and the remaining gas was measured and recorded as methane gas (Fievez *et al*., 2005).

Determination of rumen fermentation parameters and protozoa population

The pH of the incubation fluid was immediately determined with the use of a pH meter (PH-200). Total volatile fatty acids (mM) concentration of the same fluid was estimated according to Barnett and Reid (1956) using Markham apparatus. According to the method, 2 mL of incubated fluid together with 1 mL of 10% Potassium oxalate and 1 mL oxalic acid were injected into Markham apparatus. Distillate collected from each sample was titrated against 0.01 N NaOH using 2 drops of phenolphthalein as indicator. The volume of NaOH was used in calculating the concentration of the total volatile acids produced. Ammonia nitrogen concentration was determined by adding 10 mL concentrated NaOH to a 10 mL incubation fluid under a Kjeldahl distillation system. The ammonia distillate collected in boric acid solution was then titrated against a standard 0.1 N HCl (AOAC, 2000). Ammonia nitrogen concentration in the fluid was then calculated.

$$\text{TVFA (mM)} = \frac{\text{Volume of NaOH x Normality of NaOH}}{\text{Inoculum volume}} \times 100$$

$$\text{Ammonia-N (mg/dL)} = \frac{\text{Volume of HCl x Normality of HCl}}{\text{Inoculum volume}} \times 100$$

Protozoa population in the incubation fluid was determined by diluting 1 mL of the fluid with 1mL of 18.5% formaldehyde with addition of 3-4 drops of brilliant green. This was incubated for 24 hours at room temperature. The stained protozoa were counted by haemocytometer as described by Baker and Breech (1986).

Estimation of post incubation parameters of substrate

Organic matter digestibility (OMD), metabolizable energy (ME) and short chain fatty acid (SCFA) of the substrate were estimated using volume of gas produced at 24 hours as an index and proximate composition of the substrate as follow:

OMD% = 14.88 + 0.889GV + 0.45CP + 0.651A (Menke and Steingass, 1988)

ME (MJ/Kg) = 2.20 + 0.136GV + 0.057CP + 0.0029 $(CF)^2$ (Menke and Steingass, 1988)

SCFA (mmol/g DM) = 0.0239GV - 0.0601 (Getachew *et al*., 2002)

where GV = volume of gas produced at 24 h of incubation period; CP = crude protein content of the substrate (g/kg DM), CF = crude fibre content (g/kg DM) and A = Ash content of the substrate (g/kg DM)

Partitioning factor and microbial biomass yield of substrate incubated

Partitioning factor (PF) was calculated as the ratio of substrate (mg) organic matter digestibility (OMD) to volume of gas produced *in vitro* while microbial biomass yield (MBY) of the substrate was estimated according to the method of Blummel *et al*. (1997) as follows:

$$\text{PF} = \frac{\text{IVOMD}}{\text{Gas volume (24hr)}}$$

$$\text{MBY (mg)} = \text{OMD} + \text{Gas volume (24hr)} \times 2.25$$

Chemical analysis

The proximate composition of *Calotropis procera* leaf and total mixed ration was carried out according to AOAC (2000). Fibre fractions (NDF, ADF and ADL) was also determined (Van Soest *et al*., 1991). Total

tannin content was determined according to Folin-Ciocalteu method of Makkar (2003). Determination of total phenol was done according to Do et al. (2014) while flavonoids was analysed using a modified Aluminium chloride by Nasseri *et al*. (2019). Saponin and alkaloids content were determined according to the methods reported by Mir *et al*. (2016) and Adeniyi *et al*. (2009) respectively.

Statistical analysis

All data collected in this study were subjected to one way Analysis of Variance and significant differences among means were separated using Duncan's Multiple Range Test (SPSS, 2023)

Results

The chemical composition of experimental diet and *Calotropis procera* leaf used for the study is presented in Table 2. The diet contained 88.57% dry matter, 16% crude protein, 14% ash, 77.23% NDF, 41.23% ADF and 47.59% ADL while the *Calotropis procera* leaf contained 80.10, 14.53, 15.50, 68.03, 33.10 and 36.63% for the same parameters, respectively. In addition, the result also revealed that *Calotropis procera* leaf contained bioactive compounds such as tannin, total phenolic compounds, flavonoids, oxalate and cyanogenic glycosides.

Table 2: Chemical composition of total mixed ration and *Calotropis procera* leaf

Parameters	Total mixed ration	*Calotropis procera* leaf
Proximate composition (%)		
Dry matter	88.57	80.10
Crude protein	16.00	14.53
Ash	14.00	15.50
Fibre analysis (%)		
Neutral detergent fibre	77.23	68.03
Acid detergent fibre	41.23	33.10
Acid detergent lignin	47.59	36.63
Phytochemical composition (mg/100g)		
Tannin	ND	438.137
Total phenolic	ND	223.256
Flavonoids	ND	803.342
Oxalate	ND	75.820
Cyanogenic glycosides	ND	1.089
Alkaloids (%)	ND	8.11
Saponin (%)	ND	6.65

In vitro gas production parameters and protozoa population of diets as influenced by *Calotropis procera* leaf powder is presented in Table 3. The total gas and methane volume produced at the end of incubation period reduced significantly ($P < 0.05$) with addition of *Calotropis procera* leaf powder in the diets. Percentage methane reduction was higher in diets containing up to 15 mg/g CLP. The protozoal count was also reduced across the treatments.

Table 3: *In vitro* gas production and protozoa population of diets containing *Calotropis procera* leaf powder

	Levels of *Calotropis procera* leaf powder (mg/g)						
Parameters	CLP 0	CLP 5	CLP 10	CLP 15	CLP 20	SEM	P-value
Total gas volume (mL/200 mgDM)	43.67^{a}	40.67^{b}	34.67^{c}	32.33^{cd}	30.00^{d}	1.41	<0.001
Methane volume (mL)	15.33^{a}	12.00^{b}	9.67^{c}	8.00^{cd}	7.67^{d}	0.79	<0.001
%methane production	35.15^{a}	29.56^{b}	27.85^{b}	24.81^{b}	25.56^{b}	1.15	0.006
% methane reduction	0.00^{d}	15.90^{c}	20.86^{b}	29.67^{a}	28.89^{a}	4.99	<0.001
Protozoa (x10^{5}cfu/ml)	1.65^{a}	1.46^{ab}	1.12^{b}	0.75^{b}	0.77^{b}	0.14	0.002

^{abc}*Means on the same row with different superscripts are statistically different (P < 0.05), SEM- Standard error of mean,*

Fermentation kinetics, organic matter digestibility, metabolizable energy and short chain fatty acids of the diets were affected (P< 0.05) by the inclusion of Calotropis leaf powder in the diet (Table 4). The potential gas production (b) and the rate of gas production was unaffected (P > 0.05) with addition of 5 and 10 mg/g CLP in the diet but reduced drastically in diets containing 15 and 20 mg/g CLM. Higher lag time was observed in diets containing 15 and 20 mg/g CLP (3.36 and 3.64 hr respectively).

In vitro dry matter digestibilities of diets containing 5 and 10 mg/g CLP were not different (P > 0.05) from that of the control diet. Organic matter digestibility, metabolizable energy and short chain fatty acid values were similar across the treatments except in diet with 20mg/g CLP which recorded the least value. Higher partitioning factor was recorded in diet with 10 mg/g CLP. Microbial yield of the diets was significantly (p<0.05) higher in control and CLP10 diets while diet containing CLP 5 had the least value.

Table 4: Fermentation kinetics and post incubation parameters of diets containing *Calotropis procera* leaf powder

	Levels of *Calotropis procera* leaf powder (mg/g)						
Parameters	CLP0	CLP5	CLP10	CLP15	CLP20	SEM	P-value
Fermentation kinetics							
b (mL)	97.32^{a}	78.19^{a}	46.43^{a}	46.42^{b}	44.29^{b}	6.41	0.002
c (mL/hr)	0.06^{a}	0.05^{a}	0.05^{a}	0.02^{b}	0.01^{c}	0.01	<0.001
Lag (hr)	1.08^{b}	1.10^{b}	1.96^{b}	3.36^{a}	3.64^{a}	0.33	0.004
Post-incubation parameters							
IVDMD (%)	73.00^{a}	68.00^{ab}	65.00^{ab}	60.00^{b}	46.00^{c}	0.75	0.006
OMD (%)	58.69^{a}	55.13^{ab}	55.25^{ab}	53.95^{ab}	50.10^{b}	0.86	0.008
ME (MJ/kg DM)	8.08^{a}	7.54^{ab}	7.40^{ab}	7.35^{ab}	6.77^{b}	0.13	0.008
SCFA (µmol/200mg DM)	0.61^{a}	0.51^{ab}	0.49^{ab}	0.48^{ab}	0.38^{b}	0.02	0.008

Partitioning factor	2.11[b]	2.38[b]	2.76[a]	2.38[b]	2.30[b]	0.07	0.008
Microbial yield (mg)	195.05[a]	153.97[c]	178.05[ab]	173.80[b]	172.38[b]	4.13	0.008

[ab]Means on the same row with different superscripts are statistically different (P < 0.05), SEM- Standard error of mean, IVDMD - in vitro dry matter digestibility, OMD - Organic matter digestibility, ME- Metabolizable energy, SCFA – short chain fatty acids

The pH and total volatile fatty acids concentration were not affected (P > 0.05) by the inclusion of CLP in the diets. However, there was significant difference (P < 0.05) in the ammonia nitrogen concentration across the treatments with diets containing 10, 15 and 20 mg/g CLP recording the lower values.

Table 5: Fermentation parameters of diets containing *Calotropis procera* leaf powder

	Levels of *Calotropis procera* leaf powder (mg/g)						
Parameters	CLP0	CLP5	CLP10	CLP15	CLP20	SEM	P-value
pH	6.43	6.44	6.55	6.50	6.52	0.04	0.869
Total volatile fatty acids (mM)	57.30	61.00	53.40	56.90	55.00	1.42	0.578
Ammonia N conc. (mg/dL)	31.47[a]	28.94[ab]	25.92[b]	24.67[b]	23.81[b]	1.08	0.045

[ab]Means on the same row with different superscripts are statistically different (P < 0.05), SEM- Standard error of mean

Discussion

Leaves of *Calotropis procera* have been characterized by high nutritional and medicinal values (Mazen *et al*., 2020). This study has clearly shown that leaves of *Calotropis procera* are good source of nutrients considering its proximate composition with crude protein content of 14.53%. The result of phytochemical composition has revealed that the leaf contained phenols, tannins, flavonoids, oxalate, glycosides, and saponins. This is consistent with the findings in the previous studies (Murti *et al*., 2010**;** Shrivastava *et al*., 2013). These phytochemicals are important rumen modifiers that improved rumen fermentation and promote animal health and performance (Li *et al*., 2022). A study by Moustafa *et al*. (2010) indicated that flavonoids and polyphenols are the main constituents of *C. procera*. Phenolic compounds are important for their antioxidant properties. Flavonoids are known for their anti-microbial and anti-oxidative properties and could affect volatile fatty acids production along with reduction of rumen methane concentration (Kalantar, 2018).

In vitro total gas production of a diet can be used to detect the extent of organic matter digestibility (Marlida *et al*., 2023) which reflect the value of the feed. The reduction in total gas and methane production with inclusion of Calotropis leaf powder in the diets was in agreement with the findings of Akinbode *et al*. (2023) which showed that dietary substrates containing bioactive compounds have the potential to inhibit the growth or activity of some rumen microbes responsible for degradability of substrate and hence total gas production. Some studies have reported the inhibitory effects of bioactive compounds such as phenols, tannins and

polyphenols on methanogenesis accompanied with improved fermentation efficiency (Bodas *et al*., 2012). This is evident in this study as inclusion of 10 mg/g *Calotropis procera* leaf powder in the diets had the highest partitioning factor (PF) which means that a higher proportion of digested organic matter was used for synthesis of microbial biomass. The implication of this in ruminant feeding is that, diets with greater PF would be utilized more efficiently for production of animal products along with positive impact on the environment. The partitioning factor value obtained in diet with 10 mg/g Calotropis leaf powder was within the theoretical range of PF for most feeds according to Blummel *et al*. (1997).

The reduction in the volume of methane with inclusion of Calotropis leaf powder in diets as observed in this study makes this plant a promising specie that can be used as rumen modifier for effective nutrient utilization with lower impact on the environment. Percentage methane production determined in this study showed the proportion of methane produced per unit of organic matter degradation which was lower in all diets containing CLP. Percentage methane reduction of 29.67% obtained in diet with 15 mg/g CLP could be a direct effect of bioactive compounds on the methanogens and protozoans as organic matter digestibility, metabolizable energy and short chain fatty acids contents at this level of inclusion were not affected. Malik *et al*. (2017) reported 15-17% methane reduction by incorporating Tamarind seed husk at up to 5% of diet and associated the effect to direct inhibition of rumen methanogens, defanuation and modified fermentation pattern. It has also been established that medicinal plants could reduce enteric methane by up to 8 to 50% by regulating the rumen fermentation pathway (Lambo *et al*., 2024) The significant reduction of ME and SCFA in diet with 20 mg/g CLP could be attributed to higher concentration of tannic acids as reported by Hristov *et al*. (2003). Several factors could affect the production and composition of SCFA in a diet; for instance, inhibition of fibre degradation may lead to production of more propionate than acetate and hence lesser hydrogen production and methane formation. Phytogenic feed additive is desirable in ruminal fermentation if the SCFA of the feed increased or remains unaffected and methane production decreased (Bhatta *et al*., 2014). Similar observation was established in this study as SCFA at 5 - 15 mg/g CLP was not different from that of control but caused a significant reduction in methane production. The findings of this study were in agreement with the report of Chalchissa *et al*. (2023) who recorded over 20% methane reduction without a significant effect on SCFA when evaluating the methane reduction potential of some medicinal plants.

Calotropis leaf powder caused a significant reduction in protozoal count. This could be as a result of inhibitory effects of bioactive compounds such as saponin and tannin as defanuation agents that can supress protozoa activity by forming bonds with sterols found in protozoan cell wall and hence influencing the surface tension of protozoan cell membranes (Ayemele *et al*., 2020). This enhances bacteria growth and development for effective breakdown of feed materials. Saponin from the leaves of herbal plants have been reported to increase the efficiency of the fermentation process by reducing the protozoan predatory properties against bacteria due to their reduced population (Fagundes *et al*., 2020; Roca-Fernández *et al*. 2020). This is in agreement with the study of

Akinbode *et al*. (2023) and Antonius *et al*. (2023) when diets were supplemented with *Cassia fistula* leaves and herbal plants respectively. When the population of protozoa is reduced in the rumen, there will be disruption in the rumen environment and substrates (hydrogen and carbon dioxide) availability for methanogens since protozoa help to supply methanogens with hydrogen for the production of methane. Furthermore, production of methane during the anaerobic fermentation process of ration in the rumen is reflecting a loss of energy from the feed. A higher production of methane gas implies less energy use efficiency (McDonald *et al*., 2020). It has also been scientifically established that removal of protozoan from the rumen results to 9-37% reduction in methane emission (Hook *et al*., 2010) and Wenner *et al*. (2020) submitted that defaunation reduces the number of methane producing microorganisms (methanogens).

In vitro fermentation kinetics describes the degradation characteristics of feedstuffs in ruminants. The higher potential gas production (b) recorded in the control (CLP0), CLP5 and CLP10 treatments indicated higher degradability of the diets with more gas production. This indicated that the concentration of bioactive compounds in these treatments was not high enough to affect the activities of microorganisms involved in degradation. This was also reflected in the volume of total gas produced at the end of incubation period. The significant reduction in the values of b at 15 and 20 mg/g levels of inclusion could be as a result of higher concentration of bioactive compounds in these treatments which can influence the fermentation process. Tannins and Saponins can bind to proteins and carbohydrates, reducing their availability for microbial fermentation. It could also be that Calotropis leaf powder has modified the fermentation process by changing the patterns toward more efficient pathways such as promoting propionate production over acetate. The rate of gas production (c) which describes how fast the substrate is fermented by microbes over time was unaffected up to 10 mg/g inclusion level but significantly reduced by the inclusion of 15 and 20 mg/g Calotropis leaf powder in the diet. A low rate of gas production in ruminants may sometimes indicate a more gradual release of nutrients and it can also suggest challenges in nutrient utilization if microbial activity is hindered. However, the values obtained for c in this study was within the range reported by Maduro-Dias *et al*. (2023) when several non-conventional plants were tested for ruminant feeding. Rate of gas production may depend on several factors such as seasonal variation and plant composition. A longer lag time observed in diet with 15 and 20 mg/g Calotropis leaf powder suggests that it takes more time for microbes to access and begin breaking down of the feed which may be due to binding of bioactive compounds to protein and carbohydrates. This may delay the onset of fermentation leading to lowering the initial digestibility but potentially better protein utilization later on. In addition, the kinetics of ruminal degradation sometimes depends on the types of carbohydrate, protein profile and ether extract content of the feed (Lee *et al*., 2016).

In vitro dry matter digestibility is an important criterion for assessing the value of feeds used in livestock production as it reflects the amount of plant material that can be digested by the ruminants (Leng, 1997). Inclusion of 15 and 20 mg/g Calotropis leaf powder reduced the dry matter digestibility significantly. This

may be attributed to the concentration of the phytochemicals in the feed at these levels of inclusion (Abd'quadri-Abojukoro, 2022). Suppression of nutrient degradability has been reported in feed samples fermented with plant extract containing higher concentration of condensed tannins (Naumann *et al*., 2017; Abd'quadri-Abojukoro and Nsahlai, 2023). Meanwhile, the range of dry matter digestibility recorded in this study was above the recommended minimum DMD value required for the maintenance needs of animals (Arzani *et al*., 2006). Organic matter digestibility of substrates was unaffected up to 15 mg/g CLP compared with the control. This shows that CLP at this level do not interfere with rumen microorganisms involved in organic matter digestion. However, a previous study reported increased dry matter and organic matter digestibility of substrate *in vitro* with herbal plant supplementation (Muchlas *et al.,* 2014)

The pH of the rumen determines the optimal functioning of the rumen microbes. The pH can affect the population of rumen microbes involved in fermentation process. A pH range of 6 -7 is required to maintain a normal rumen metabolism for optimal microbial activity while pH value above 7 or below 5.5 is detrimental to the rumen health and microbes. Meanwhile, the pH range observed in this study falls within the normal range of 6-7 which means that inclusion of *Calotropis procera* leaf powder in the diet has no effect on the bacteria growth and cellulolysis.

Ammonia is one of the important products of fermentation and measuring its concentration in the rumen can be used to determine the efficiency of nitrogen utilization by rumen microbes because the microbes use ammonia for their protein synthesis. Decreased ammonia nitrogen concentration observed in diets with Calotropis leaf powder may be an indication of reduced proteolysis which may be attributed to direct inhibition of high ammonia-producing bacteria (HAB) which are known to produce ammonia at a much faster rate than other microbes. The inhibition could also be due primarily to the bioactive composition of Calotropis with anti-microbial properties that can specifically target and modulate rumen microbial populations including HAB. Excess ammonia production in the rumen can be lost through urea excretion, which is energetically costly for the animal (Shen *et al*., 2023) and hence ineffective nitrogen utilization.

Conclusion

In conclusion, the study revealed that *Calotropis procera* leaf contained tannin, phenols, flavonoids, alkaloids and saponin which inhibit production of methane. Inclusion of 15 mg/g CLP reduced methane, protozoa count and ammonia nitrogen concentration without affecting the organic matter digestibility, energy value of the feed and pH of the incubation fluid. Based on this results, *Calotropis procera* leaf powder (10 - 15 mg/g) is a potential natural feed additive for ruminants with a view of reducing the production of environmentally threatening greenhouse gases without destructing the digestibility process.

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