



Assessing the combination efficiency of some unconventional feed resources with concentrates and *Chloris gayana* grass in mitigating ruminal methane production in vitro

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Abstract

In a preliminary in vitro study, leaves of *Acacia nilotica*, *Prosopis juliflora*, *Cajanus cajan*, *Leucaena leucocephala* and seed kernel of *Mangifera indica* were identified as potential candidates in mitigating ruminal methane (CH₄) production. The objective of the current study was to investigate the combination efficiency of these unconventional feeds with concentrate mix (CM) or *Chloris gayana* grass in CH₄ reduction. Two feed combinations in different proportions were incubated in vitro with buffered rumen fluid at Hohenheim Gas test. In combination 1, *C. gayana* and CM were included as basal substrates, while in combination 2, *A. nilotica*, *P. juliflora*, *C. cajan*, *L. leucocephala* or *M. indica* seed kernel were included as CH₄ reducing supplements at different proportions. The CH₄ reducing potentials of feed combinations were presented as the ratio of CH₄ to net gas production and expressed as percentage (pCH₄). The pCH₄ for CM and *C. gayana* was 16.7% and 16.9%, respectively, while it ranged from 3.18% in *A. nilotica* to 13.1% in *C. cajan*. The pCH₄ was reduced ($p < 0.05$) from 14.6% to 9.39% when *A. nilotica* was combined with CM. In combination of *L. leucocephala* or *C. cajan* with CM, the pCH₄ ($p < 0.05$) was reduced from 16.5% and 16.6% with the lowest proportion to 15.1% and 15.2% with the highest inclusion rate respectively. The combination of *C. gayana* with *L. leucocephala* or *C. cajan* reduced ($p < 0.05$) the pCH₄ from 16.3% and 16.4% to 15.1% and 14.9% respectively. The pCH₄ was reduced ($p < 0.05$) from 13.4% to 7.60% when *A. nilotica* was combined with *C. gayana*. Estimated digestible organic matter (dOM) and metabolizable energy (ME) increased ($p < 0.05$) with increasing proportions of *M. indica* seed kernel with CM or *C. gayana*. In conclusion, the combination of the basal substrates with unconventional supplements resulted in CH₄ reduction without affecting the dOM and ME at lower inclusion rates. Animal-based experiments await to validate in vitro findings.

KEYWORDS

Chloris gayana grass, combination, concentrate mix, in vitro, methane, unconventional feeds

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1 | INTRODUCTION

In tropical regions of the world, methane (CH₄) production from ruminants is mainly caused by poor feed resources that are characterized by high fibre content. In addition, CH₄ production considerably affects the efficiency of energy utilization of the feed by ruminants which is directly associated with its economic benefit. Over the last several years, various scholars have been interested in the use of tropical plants especially as rumen manipulating agents. Although results are not always conclusive, studies conducted so far have indicated partial defaunation and CH₄-suppressing effects of various multipurpose trees (MPTs) (Melesse, Steingass, Schollenberger, Holstein, & Rodehutschord, 2019; Soliva et al., 2008). Reducing ruminal CH₄ production might have the dual advantage of increasing production efficiency and lessening its atmospheric accumulation (Tadesse et al., 2022; Zeleke et al., 2006).

Several screening assays have been reported in which a large number of plant species have been examined in an in vitro batch culture of mixed ruminal microorganisms to examine their potential to reduce CH₄ production (Melesse et al., 2017, 2018, 2019; Melesse, Steingass, Schollenberger, Holstein, & Rodehutschord, 2019; Soliva et al., 2008). An in vitro gas production (GP) technique has been widely used over that of in vivo, allowing several diets and diet combinations to be evaluated simultaneously within a short period of time with limited expenditures. Among these, identifying available tropical feed resources that produce minimum CH₄ emissions when consumed by ruminants is the most cost-effective strategy. If the methanogenic potential and their enhancing properties have been identified, this knowledge can be applied to determine combinations of dietary supplements (e.g., when added to other diets) by targeting both goals, limiting ruminal methanogenesis while improving energy supply that will enhance animal productivity (Tadesse et al., 2022).

Supplementation of low-quality diets with protein and energy could be highly efficient in improving productivity of ruminants and thus reducing relative methane release (Tadesse et al., 2022). In tropical livestock husbandry practices, protein deficiency is by far the most important cause of low performance of ruminants reared in low-quality forages. Thus, ensuring adequate ammonia levels in the rumen for microbial growth should be the first priority in optimizing fermentative digestion of poor-quality forages. In this respect, unconventional feed resources such as plant parts of the MPTs and fruit byproducts could serve as a source of easily available supplements.

In a preliminary study, leaves of MPTs, forage grasses, fruit and agro-industrial by-products were screened in vitro for their properties to mitigate CH₄ production (Melesse et al., 2017, 2018, 2019; Melesse, Steingass, Schollenberger, Holstein, & Rodehutschord, 2019). Among the investigated feed materials, leaves of *Acacia nilotica*, *Prosopis juliflora*, *Cajanus cajan* and seed kernels of *Mangifera indica* were found to have the potential of reducing CH₄ methane production when tested in vitro. Nevertheless, the efficiency of

these unconventional feed resources in reducing CH₄ when combined with conventional feeds has not been determined.

The objective of this study was thus to investigate combination efficiency of the identified candidate unconventional feeds with concentrate mix (CM) or *Chloris gayana* grass in mitigating methane emission in vitro. It was hypothesized that the inhibitory effect of some unconventional feed additives on ruminal CH₄ production would persist and reduce microbial CH₄ production from a concentrate mixture and *C. gayana* grass when added in increasing proportions without affecting metabolizable energy (ME) and digestible organic matter (dOM).

2 | MATERIALS AND METHODS

2.1 | Feed sample collection and preparation

In this study, leaves or *M. indica* seed kernels of five unconventional feed resources that demonstrated reduced CH₄ production from Hohenheim Gas test screening studies were tested by combining them with either a CM or *C. gayana* (*G. gayana*) grass at different proportions. These were leaves of *A. nilotica*, *P. juliflora*, *Leucaena leucocephala* and *C. cajan* as well as seed kernels of *M. indica*. The CM used in this experiment comprised of 50% wheat bran, 35% maize grain, 14% Noug seed (*Guizotia abyssinica*) cake and 1% salt. Details on sample collection procedures of all these unconventional feed resources have been reported by Melesse et al. (2017, 2018, 2019) and Melesse, Steingass, Schollenberger, Holstein, and Rodehutschord (2019). All (except CM) samples were dried on plastic sheets kept under shade, ground to pass a 1 mm sieve and transported in air-tight plastic containers to the University of Hohenheim, Germany, for analyses.

2.2 | Experimental protocols

Two components were weighed in different proportions together and incubated in vitro using a rotary incubator at the Department of Animal Nutrition, University of Hohenheim, Germany. In combination 1, *C. gayana* and CM were included as basal substrates. In Combination 2, either leaves of *A. nilotica*, *P. juliflora*, *C. cajan*, *L. leucocephala* or seed kernels of *M. indica* were included as CH₄ reducing supplements. The proportions of supplements were determined based on their in vitro CH₄ production potentials. Accordingly, the proportion of *M. indica*, *C. cajan* and *L. leucocephala* combination with either *C. gayana* or CM was the same being set at 15%, 30% and 60% (Table 1). However, the proportion of *A. nilotica* and *P. juliflora* combination with either *C. gayana* or CM was set differently due to their lower CH₄ production potential. Thus, the proportion for *A. nilotica* was set at 22%, 41% and 71% while that of *P. juliflora* was set at 32%, 53% and 80%.

In addition to the combinations, the individual feed samples were assessed for their CH₄ production potentials separately. Due to the

TABLE 1 Proportions of methane reducing feed materials (combination 2) in concentrate mix and *Chloris gayana* grass (Combination 1).

Combination 1 (basal substrates)	Combination 2 (methane reducing feed materials)				
	<i>M. indica</i>	<i>C. cajan</i>	<i>L. leucocephala</i>	<i>A. nilotica</i>	<i>P. juliflora</i>
Concentrate mix	15	15	15	22	32
	30	30	30	41	53
	60	60	60	71	80
<i>Chloris gayana</i> grass	15	15	15	22	32
	30	30	30	41	53
	60	60	60	71	80

capacity of the in vitro gas test incubator, two independent experiments were performed. In the first experiment, four runs were conducted in which *A. nilotica*, *P. juliflora*, *M. indica* seed kernels, CM and *C. gayana* and their combinations with either of the basal substrates (CM or *C. gayana*) were considered. In the second experiment, three runs were carried out by including *C. cajan*, *L. leucocephala*, CM and *C. gayana* and their combinations with either of the basal substrates. Each run contained two syringes per combination and individual feed.

2.3 | Chemical analyses

Chemical analyses of proximate nutrients and fibre fractions were performed as outlined by Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) (VDLUFA, 2007). The samples were analysed at the Department of Animal Nutrition (Institute of Animal Science, University of Hohenheim) for dry matter (DM, method 3.1), ash (method 8.1), crude protein (CP, method 4.1.1; $N \times 6.25$), petroleum ether extract (EE, method 5.1.1) and crude fibre (CF, method 6.1.1). Neutral detergent fibre (aNDFom) was assayed on an organic matter basis after pretreatment with heat-stable amylase (method 6.5.1) and acid detergent fibre (ADFom) on an organic matter basis (method 6.5.2). Acid detergent lignin was analysed according to method 6.5.3.

The tested feed samples were further analysed for concentrations of total phenols and nontannin phenols using the Folin-Ciocalteu method (Jayanegara et al., 2011) with modifications as described by Wischer et al. (2013). Soluble condensed tannins were analysed according to Jayanegara et al. (2011). Concentrations of tannin phenols were then calculated as differences between total phenol and nontannin phenol concentrations. The absorbance of total phenols and nontannin phenols was recorded at 725 nm using an ultraviolet-visible spectrophotometer (UV-VIS) (Perkin Elmer Instruments). Condensed tannins were analysed by the butanol-HCl-iron method. The absorbance was read at 550 nm using the same UV-VIS spectrophotometer as for total phenols and nontannin phenols and was expressed as leucocyanidin equivalents. All analyses were performed in duplicate and were averaged. Readers are referred to Melesse, Steingass, Schollenberger, Holstein, and Rodehutsord (2019) for detailed description of the protocols.

Melesse et al. (2018, 2019) and Melesse, Steingass, Schollenberger, Holstein, and Rodehutsord (2019) provided detailed information on the contents of crude nutrients and antinutritional factors of feed materials used in this experiment. In brief, as shown in Table 2, *M. indica* seed kernels had the lowest CP content followed by *C. gayana*. Among MPTs, *A. nilotica* leaf had the lowest CP while that of *P. juliflora* the highest. *C. gayana* grass and *C. cajan* leaf had the highest aNDFom and ADFom contents. The content of crude fat in all feed materials was generally low. The highest concentrations of total phenols and tannin phenols were observed in *A. nilotica* leaf. The concentration of soluble condensed tannin was highest in the leaf of *C. cajan* while that of *P. juliflora* had the lowest.

2.4 | In vitro GP procedures

The in vitro gas and methane production measurements were carried out at the Department of Animal Nutrition, University of Hohenheim, according to the procedure of VDLUFA official method (VDLUFA, 2007) and Menke and Steingass (1988) as described by Melesse, Steingass, Schollenberger, Holstein, and Rodehutsord (2019). The individual experimental feed sample was weighed and transferred into 100 mL calibrated glass syringes, fitted with white Vaseline lubricated glass-made plungers. A mixture of rumen fluid was used which was collected from two rumen fistulated, lactating Jersey cows fed a total mixed ration consisting of 20% maize silage, 20% grass silage, 20% hay and 40% dairy concentrate mixture on DM basis. The filtered rumen fluid was added to the buffer solution (1:2 v/v) under constant stirring. In total, 30 mL of buffered rumen fluid was transferred into each syringe containing weighted feed samples, which was then immediately placed into a rotating incubator maintained at constant temperature of 39°C. Three syringes with only buffered rumen fluid (blanks), three syringes with hay standard and another three with concentrate standard with established GP references were also included with each run. The GP of samples, blanks and standards was recorded at 0, 6 and 24 h of incubation. Finally, the total GP of the feed samples was corrected by the results of blanks (syringes containing only the rumen fluid), the sample weight and its DM concentration, and the corrected total GP is referred to here as net gas production (NGP). For the estimation of ME and dOM, the NGP of the feed samples was further corrected by the standards of hay and

TABLE 2 Chemical compositions and antinutritional factors (g/kg dry matter) of feed components (Source: Melesse et al., 2017, 2018, 2019; Melesse, Steingass, Schollenberger, Holstein, & Rodehutschord, 2019).

Feed components	Nutrient compositions							Antinutritional factors			
	Ash	Crude protein	Crude fat	aNDFom	ADFom	ADL	Starch	Total phenols	Nontannin phenols	Tannin phenols	Soluble condensed tannin
Concentrate mix	98.3	232	64.8	250	127	47.0	324	7.0	4.0	3.0	0
<i>Chloris gayana</i> grass	131	135	13.3	651	370	41.1	Na	7.0	6.0	1.0	Nd
<i>Acacia nilotica</i> leaf	47.9	168	18.4	140	90.4	42.6	Na	503	31.0	472	14.7
<i>Prosopis juliflora</i> leaf	102	261	33.3	331	245	119	28.8	18.9	14.6	4.30	0.10
<i>Mangifera indica</i> seed kernel	26.5	63.2	83.3	118	60.2	17.7	473	158	5.30	153	7.80
<i>Cajanus cajan</i> leaf	86.0	200	43.9	413	276	125	1.0	90.1	30.5	59.6	97.7
<i>Leucaena leucocephala</i> leaf	122	245	54.2	233	128	54.6	Na	65.4	19.1	46.3	23.1

Abbreviations: ADFom, acid detergent fibre on organic matter basis; ADL, acid detergent lignin; aNDFom, neutral detergent fibre on organic matter basis after amylase treatment; Na, not analysed; Nd, not detected; NFE, nitrogen free extract.

concentrate. The values of dOM and ME were estimated by applying the following equations (Menke & Steingass, 1988):

$$\begin{aligned} \text{ME (MJ/kg DM)} &= 1.68 + 0.1418 \times \text{GP} + 0.0073 \times \text{CP} \\ &\quad + 0.0217 \times \text{XL} - 0.0028 \times \text{XA} \\ \text{dOM (\%)} &= 14.88 + 0.889 \times \text{GP} + 0.0448 \times \text{CP} \\ &\quad + 0.0651 \times \text{XA} \end{aligned}$$

where GP (mL/200 mg DM), CP (g/kg DM), XL (g/kg DM) and XA (g/kg DM) are gas production, crude protein, crude fat and crude ash respectively.

2.5 | Determination of the methane production

To determine the CH₄ production, 100–400 mg sample material was weighted and transferred into calibrated glass syringes and mixed with 30 mL of buffered rumen fluid as described in the above section. The initial sample weight was adjusted depending on the proportion of inclusion and GP potentials of individual unconventional feed resources as reported earlier (Melesse et al., 2017, 2018, 2019; Melesse, Steingass, Schollenberger, Holstein, & Rodehutschord, 2019). Accordingly, for samples that showed a high GP potential, the initial sample weight was reduced. This adjustment was essential to avoid resetting syringes during the incubation process to control the gas escaping from such activities. For samples with low GP, the initial weight of the test sample was increased so that enough gas would be available for the later measurement of CH₄ production.

After an incubation period of 24 h, the individual glass syringe was taken out of the rotor incubator, the volume was recorded and the rumen fluid medium mixture was slowly drained by releasing the

clip of the elastic tube that is attached to syringe. This process was performed with utmost care to avoid escaping of gas from the syringes. To measure the methane content of the gas fraction, the infrared analyzer (Pronova Analysentechnik GmbH & KG) was calibrated with the help of a reference gas (13.0% CH₄, Munster Westfalen AG). Subsequently, each syringe was directly connected to the measuring instrument via the elastic tube. By gently pushing the plunger, the gas was transferred into the measuring instrument, whereupon the methane content of the gas mixture was read on a digital display. The CH₄ produced by each sample was then corrected by the amount of CH₄ produced by the blank syringes, the sample weight and its DM concentration, as well as by the factors of reference hay and concentrate feed which were included in each run as stated before.

In addition to the concentrations (mL/g DM), the ratio of the CH₄ to NGP was presented as a percentage of the net gas produced. This is of greater importance with regard to the methane-reducing potential of a feed sample as suggested by Soliva et al. (2008). Low percentages, therefore, indicate a low CH₄ production potential of the digestible part of the feed. The percentage of methane (pCH₄) in NGP was thus determined according to Melesse et al. (2018) using the following formula:

$$p\text{CH}_4 = \frac{\text{CH}_4 \text{ sample}}{24 \text{ hr net gas production}} * 100$$

2.6 | Statistical analysis

The analysis of time series measurements was carried out using Microsoft Excel 2013. Data on NGP and methane production as well as dOM and ME were subjected to two-way ANOVA by fitting feed combination and proportions as independent variables and analysed using the Statistical Analysis System (SAS 2012, Ver. 9.4, Institute).



Analysis of variance was conducted according to the following statistical model: $y_{ijk} = \mu + A_i + B_j + A_i * B_j + e_{ijk}$, where y_{ijk} is the response of the observed k th variables; μ is the overall mean of observed k th variables; A_i is the effect due to i th feed combinations; B_j is the effect due to j th proportions of feed combinations; $A_i * B_j$ is the effect due interaction of i th combinations with j th proportions of combinations and e_{ijk} is the residual random error. Means were compared using Duncan's multiple range test and differences were considered significant at $p < 0.05$ level unless indicated otherwise.

3 | RESULTS

3.1 | Estimated parameters and GP

As shown in Table 3, the CM had higher ($p < 0.05$) absolute CH₄, ME and dOM than the other feed materials studied. The NGP and pCH₄

values of CM were comparable with that of *M. indica* and *C. gayana* grass respectively. The lowest values for NGP, absolute CH₄, pCH₄, ME and dOM were observed in *P. juliflora* leaves and differed significantly from the other feed materials. The dOM was comparable among *C. Gayana*, *P. juliflora* and *L. leucocephala* and did not differ with each other statistically.

Table 4 shows the CH₄ and GP values and estimates of ME and dOM for the combination of CM or *C. gayana* with *A. nilotica*. The effects of combination and proportion of feed supplements were highly significant ($p < 0.001$) for all parameters, although not the interactions. Accordingly, the combination of *A. nilotica* leaf with CM and *C. gayana* resulted in considerable differences between the proportions for all parameters. No effect was observed for NGP, dOM, ME and CH₄ production when the proportions of *A. nilotica* mixture with *C. gayana* increased from 22% to 41%. However, there was a reduction ($p < 0.05$) in these parameters as the proportion of *A. nilotica* to *C. gayana* increased from 41% to 71% except for dOM. In the combination with

TABLE 3 In vitro gas and methane production and estimates of metabolizable energy (ME) and digestible organic matter (dOM) of individual feed ingredients.

Feed components	NGP (mL/g DM)	CH ₄ (mL/g DM)	CH ₄ (% to NGP)	ME (MJ/kg DM)	dOM (%)
Concentrate mix	248 ^a	41.4 ^a	16.7 ^a	12.7 ^a	80.8 ^a
<i>Chloris gayana</i>	194 ^b	32.8 ^b	16.9 ^a	7.94 ^d	62.9 ^b
<i>Mangifera indica</i>	230 ^a	22.3 ^c	9.69 ^b	10.7 ^b	62.5 ^b
<i>Leucaena leucocephala</i>	151 ^{b,c}	17.9 ^c	11.9 ^b	9.12 ^c	64.0 ^b
<i>Cajanus cajan</i>	88.5 ^d	11.7 ^{d,e}	13.1 ^b	6.67 ^f	47.2 ^d
<i>Acacia nilotica</i>	140 ^c	4.70 ^e	3.82 ^c	7.35 ^e	51.7 ^c
<i>Prosopis juliflora</i>	46.7 ^d	0.01 [*]	0.003 [*]	5.42 ^g	42.0 ^e

Abbreviations: CH₄, methane; DM, dry matter; NGP, net gas production at 24 h feed sample incubation.

*These values were omitted from the statistical analysis due to extreme deviations from the rest of the data.

^{a-g}Row means with different superscript letters are significant at $p < 0.05$.

TABLE 4 Changes in CH₄ and gas production and estimates of metabolizable energy (ME) and digestible organic matter (dOM) for combinations of *A. nilotica* leaves with concentrate mix or *Chloris gayana* at various additive proportions.

Proportions C1/C2	NGP (mL/g DM)		CH ₄ (mL/g DM)		CH ₄ (% to NGP)		ME (MJ/kg DM)		dOM (%)	
	CM	CG	CM	CG	CM	CG	CM	CG	CM	CG
78/22	238 ^a	189 ^a	34.7 ^a	25.2 ^a	14.6 ^a	13.4 ^a	11.3 ^a	8.35 ^a	74.7 ^a	63.9 ^a
59/41	226 ^a	182 ^a	29.3 ^b	22.4 ^a	13.0 ^b	11.6 ^a	10.7 ^a	8.56 ^a	71.6 ^a	62.8 ^a
29/71	207 ^b	169 ^b	19.0 ^c	12.9 ^b	9.39 ^c	7.60 ^b	9.61 ^b	8.06 ^b	65.2 ^b	58.4 ^a
SEM	9.64	5.86	4.61	3.72	1.54	1.71	0.32	0.21	2.00	1.38
Sources of variation										
Proportions (P)	0.003	0.009	<0.001	0.002	<0.001	0.002	0.002	0.003	0.003	0.002
Combinations (C)	<0.001		<0.001		<0.001		<0.001		<0.001	
C × P	0.322		0.423		0.777		0.168		0.236	

Abbreviations: C1, Combinations 1: Concentrate mix or *Chloris gayana*; C2, Combinations 2: *Acacia nilotica*; CG, *Chloris gayana*; CH₄, methane; CM, concentrate mix; DM; dry matter; NGP, net gas production; SEM, standard error of the mean.

^{a-c}Column means between proportions within each combination with different superscript letters differ significantly at $p < 0.05$.



the CM, increasing the proportions of *A. nilotica* reduced the CH₄ production and the pCH₄ values ($p < 0.05$) at each proportion (Table 3). In general, the CH₄ production and other parameters were reduced ($p < 0.05$) when *A. nilotica* leaf was combined with either of the two basal substrates. A significant difference was also observed in NGP, ME, dOM and CH₄ production values between combinations of *A. nilotica* with either CM or *C. gayana*. The combination of *A. nilotica* with CM showed higher values of these parameters than its combination with *C. gayana*. Conversely, the pCH₄ did not vary between CM and *C. gayana* when combined with *A. nilotica* at 22% and 41% proportions, while it was higher ($p < 0.05$) for CM at 71% as compared to *C. gayana*. The interaction effect between combination and proportion remained insignificant.

In the combination of *P. juliflora* leaf with either of the CM or *C. gayana*, the effect of combination and proportion as well as their interactions was highly significant ($p < 0.001$) for all parameters

except that of pCH₄ (Table 5). Consequently, all studied parameters reduced ($p < 0.001$) as the proportion of *P. juliflora* to CM or *C. gayana* increased from 32% to 53%. Moreover, the combination of *P. juliflora* with CM at a ratio of 80% resulted in significantly lower values for all parameters than those observed at 32% and 53%. However, no further significant difference was observed as the ratio of *P. juliflora* to *C. gayana* increased from 53% to 80%. There has been a difference ($p < 0.05$) observed between combinations of *P. juliflora* with CM for NGP, absolute CH₄, ME and dOM at all proportions. Except for the pCH₄, the interaction effect of combination and proportion was significant ($p < 0.01$) for all parameters.

The effect of proportions and combinations of *M. indica* seed kernel with either of CM or *C. gayana* was significant for most parameters except for the absolute CH₄ production (Table 6). No interaction effect of combination and proportion was observed for all parameters. Higher ($p < 0.05$) NGP, dOM, ME and lower pCH₄ values

TABLE 5 In vitro gas and methane production and estimates of metabolizable energy (ME) and digestible organic matter (dOM) for combinations of *Prosopis juliflora* leaves with concentrate mix or *Chloris gayana* at different additive proportions.

Proportions C1/C2	NGP (mL/g DM)		CH ₄ (mL/g DM)		CH ₄ (% to NGP)		ME (MJ/kg DM)		dOM (%)	
	CM	CG	CM	CG	CM	CG	CM	CG	CM	CG
68/32	186 ^a	95.5 ^a	26.9 ^a	12.8 ^a	14.4 ^a	13.3 ^a	9.91 ^a	5.89 ^a	67.0 ^a	48.5 ^a
47/53	124 ^b	57.5 ^b	7.80 ^b	1.40 ^b	6.00 ^b	2.31 ^b	7.95 ^b	5.08 ^b	55.8 ^b	42.3 ^b
20/80	75.5 ^c	46.6 ^b	0.05 ^c	0.10 ^b	0.02 ^c	0.01 ^b	6.38 ^c	5.13 ^b	47.1 ^c	41.2 ^b
SEM	32.0	14.8	7.98	4.03	4.17	4.10	0.96	0.42	5.96	2.71
Sources of variation										
Proportions (P)	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Combinations (C)	<0.001		<0.001		0.113		<0.001		<0.001	
C × P	<0.001		0.004		0.135		<0.001		<0.001	

Abbreviations: C1, Combinations 1: Concentrate mix or *Chloris gayana*; C2, Combinations 2: *Prosopis juliflora*; CG, *Chloris gayana*; CH₄, methane; CM, concentrate mix; DM, dry matter; NGP, net gas production; SEM, standard error of the mean.

^{a-c}Column means between proportions within each combination with different superscript letters differ significantly at $p < 0.05$.

TABLE 6 In vitro gas and methane production and estimates of metabolizable energy (ME) and digestible organic matter (dOM) for combinations of *Mangifera indica* seed kernel with concentrate mix or *Chloris gayana* at different additive proportions.

Proportions C1/C2	NGP (mL/g DM)		CH ₄ (mL/g DM)		CH ₄ (% to NGP)		ME (MJ/kg DM)		dOM (%)	
	CM	CG	CM	CG	CM	CG	CM	CG	CM	CG
85/15	236 ^b	210 ^b	35.2	34.4	14.9 ^a	16.4 ^a	11.0 ^b	9.06 ^b	74.0 ^b	67.3 ^b
70/30	254 ^a	228 ^a	36.2	35.9	14.3 ^a	15.8 ^a	11.9 ^a	9.77 ^a	75.4 ^a	69.1 ^a
40/60	254 ^a	232 ^a	33.3	31.7	13.1 ^b	13.7 ^b	11.7 ^a	10.3 ^a	71.9 ^a	68.8 ^a
SEM	6.01	6.77	0.85	1.23	0.53	0.82	0.17	0.21	1.04	1.11
Sources of variation										
Proportion (P)	0.011	0.005	0.126	0.154	0.007	0.009	0.004	0.004	0.006	0.002
Combination (C)	<0.001		0.070		0.005		<0.001		<0.001	
C × P	0.912		0.898		0.428		0.7095		0.8578	

Abbreviations: C1, Combinations 1: Concentrate mix or *Chloris gayana*; C2, Combinations 2: *Mangifera indica*; CG, *Chloris gayana*; CH₄, methane; CM, concentrate mix; DM, dry matter; NGP, net gas production; SEM, standard error of the mean.

^{a,b}Column means between proportions within each combination with different superscript letters differ significantly at $p < 0.05$.



were observed for the combination of *M. indica* with CM than that of *C. gayana*. A higher ($p < 0.05$) pCH_4 was also noted when *M. indica* was combined with *C. gayana* at 30% than that of CM. The pCH_4 production decreased ($p < 0.05$) on the combination of *M. indica* seed kernel with the proportion of 60% to CM and *C. gayana*. The NGP, dOM and ME values increased ($p < 0.05$) with increasing proportions of *M. indica* seed kernel from 15% to 30% to both CM and *C. gayana*.

As shown in Table 7, the effect of combination of *C. cajan* with either CM or *C. gayana* was significant ($p < 0.001$) for all parameters except pCH_4 . In the combination of *C. cajan* leaf with CM, the effect of proportion was significant for all parameters. Similarly, although not for pCH_4 , the proportion exerted a significant effect on all parameters when the *C. cajan* leaf was combined with *C. gayana* grass. The interaction of both factors remained insignificant. Apart from the pCH_4 , the combination of *C. cajan* leaf with CM produced higher ($p < 0.05$) values for all parameters than those of *C. gayana*. Although it

was significant only at 60%, all parameters consistently reduced with increasing proportion of *C. cajan* to CM and *C. gayana*.

As indicated in Table 8, the effect of combination of *L. leucocephala* leaf with CM or *C. gayana* was significant for all parameters except for pCH_4 . Similarly, except for pCH_4 , the effect of proportion was significant for all parameters when *L. leucocephala* leaf was combined with CM. Proportion had significant effect for the combination of *L. leucocephala* with *C. gayana* on all parameters except for absolute CH_4 and pCH_4 production. An interaction effect ($p < 0.05$) was also observed for NGP, ME and dOM. Except for pCH_4 , all other parameters were higher ($p < 0.05$) when *L. leucocephala* leaf was combined with CM at 15% and 30% proportions as compared to *C. gayana*. The NGP, dOM and ME values were reduced ($p < 0.05$) with increasing proportion of *L. leucocephala* with CM. However, these parameters did not vary ($p > 0.05$) between proportions when *L. leucocephala* was combined with *C. gayana*. In the combination of

TABLE 7 Effect of combinations and additive proportions of *Cajanus cajan* leaves with concentrate mix or *Chloris gayana* on in vitro gas and methane production and estimates of metabolizable energy (ME) and digestible organic matter (dOM).

Proportions C1/C2	NGP (mL/g/DM)		CH ₄ (mL/g DM)		CH ₄ (% to NGP)		ME (MJ/kg DM)		dOM (%)	
	CM	CG	CM	CG	CM	CG	CM	CG	CM	CG
85/15	208 ^a	167 ^a	34.5 ^a	27.3 ^a	16.6 ^a	16.4	10.2 ^a	7.77 ^a	73.1 ^a	62.9 ^a
70/30	199 ^a	153 ^a	32.9 ^a	25.0 ^a	16.5 ^a	16.3	9.90 ^a	7.48 ^a	71.0 ^a	60.2 ^a
40/60	156 ^b	124 ^b	23.7 ^b	18.5 ^b	15.2 ^b	14.9	8.38 ^b	6.80 ^b	61.7 ^b	54.3 ^b
SEM	16.1	12.7	3.37	2.64	0.45	0.48	0.52	0.38	3.22	2.51
Sources of variation										
Proportions (P)	0.002	0.001	0.002	0.003	0.019	0.126	0.011	0.002	0.011	0.001
Combinations (C)	<0.001		<0.001		0.457		<0.001		<0.001	
C × P	0.278		0.3836		0.998		0.5084		0.5818	

Abbreviations: C1, Combinations 1: Concentrate mix or *Chloris gayana*; C2, Combinations 2: *Cajanus cajan*; CG, *Chloris gayana*; CH₄, methane; CM, concentrate mix; DM, dry matter; NGP, net gas production; SEM, standard error of the mean.

^{a,b}Column means between proportions within each combination with different superscript letters differ significantly at $p < 0.05$.

TABLE 8 In vitro gas and methane production and estimates of metabolizable energy (ME) and digestible organic matter (dOM) for combinations of *L. leucocephala* with concentrate or *Chloris gayana* at various additive proportions.

Proportions C1/C2	NGP (mL/g DM)		CH ₄ (mL/g DM)		CH ₄ (% to NGP)		ME (MJ/kg DM)		dOM (%)	
	CM	CG	CM	CG	CM	CG	CM	CG	CM	CG
85/15	230 ^a	176	38.1 ^a	27.9 ^a	16.5	16.3 ^a	11.0 ^a	7.97	78.2 ^a	64.5
70/30	210 ^{ab}	172	34.2 ^a	27.7 ^a	16.2	15.8 ^a	10.3 ^{ab}	8.30	74.5 ^{ab}	66.1
40/60	186 ^b	165	28.2 ^b	23.3 ^b	15.1	14.1 ^b	9.92 ^b	8.31	72.3 ^b	65.1
SEM	12.7	3.21	2.88	1.50	0.43	0.67	0.42	0.09	2.57	0.64
Sources of variation										
Proportions (P)	0.022	0.205	0.013	0.022	0.107	0.022	0.018	0.443	0.020	0.372
Combinations (C)	<0.001		<0.001		0.102		<0.001		<0.001	
C × P	0.0342		0.1557		0.6312		0.0249		0.0349	

Abbreviations: C1, Combinations 1: Concentrate mix or *Chloris gayana*; C2, Combinations 2: *Leucaena leucocephala*; CG, *Chloris gayana*; CH₄, methane; CM, concentrate mix; DM, dry matter; NGP, net gas production; SEM, standard error of the mean.

^{a,b}Column means between proportions within each combination with different superscript letters differ significantly at $p < 0.05$.



L. leucocephala with CM, the pCH₄ did not differ among proportions, although it consistently reduced with increasing proportions of *L. leucocephala* from 15% to 60%.

4 | DISCUSSION

4.1 | *P. juliflora* leaf

The extremely low GP and the resulting low values of ME and dOM in *P. juliflora* leaves appeared to be surprising because the crude nutrient contents were in the range of the other MPT leaves (Melesse, Steingass, Schollenberger, Holstein, & Rodehutschord, 2019). As a result, the *P. juliflora* leaf might be considered as less favourable supplements to ruminants. However, these drawbacks were considerably minimized when *P. juliflora* leaf was combined with CM and *C. gayana* grass. This combination approach has further contributed to the reduction of CH₄.

The higher the proportion of the CH₄ reducing supplements the better the reduction of methane production observed. Similar works have been reported by Castro-Montoya et al. (2012) in which different methane-reducing additives in different concentrations had reduced the CH₄ production. The results of the combination of CM or *C. gayana* with *P. juliflora* leaf showed a strong interaction for all parameters except for pCH₄ indicating its nonadditive effect. At higher proportion of *P. juliflora* leaf (80%), the CH₄ production considerably decreased in which it was no longer detectable. Although *P. juliflora* leaf contained negligible amounts of tannins, they still produced the lowest CH₄. It is thus suggested that the leaves may contain secondary metabolites other than tannins which might have suppressed the activity of methanogenesis resulting in a low CH₄ production. Nakano (2004) and Wamburu et al. (2013) reported the presence of alkaloids in *P. juliflora* plants which might be considered as plausible explanation for such a dramatic reduction in CH₄ production. However, it still remains unclear whether these all stated antinutritional factors alone or also other substances that are not investigated here are responsible for the complete inhibition of methanogenesis.

4.2 | *A. nilotica* leaf

The antinutritional factors present in MPT leaves, particularly plants of the genus *Acacia* that often have high levels of tannins might have contributed to the low CH₄ production (Aganga & Tshwenyane, 2003; Dube et al., 2001). Some in vitro studies have also reported that tannins in acacia, chestnut or valonea have reduced the CH₄ production compared with the control (Hassanat & Benchaar, 2013; Wischer et al., 2013). The effects of combination and proportions were highly significant for *A. nilotica* leaf when combined with either CM or *C. gayana*, which suggests that the level of tannin content could have had an impact on possible interactions between the combinations and the proportions as described hereabove. However, the interaction was not significant for all parameters, and thus, the mechanisms underlying these effects are not fully understood at this stage and could be a

future research topic. The CH₄ production significantly reduced as the level of *A. nilotica* increased with either of CM or *C. gayana* which suggests the potential of the plant material to reduce the activity of the methanogens (Hassanat & Benchaar, 2013; Wischer et al., 2013). Studies conducted by Pal et al. (2015) revealed that the *A. nilotica* leaves not only produced low methane but also had generally greater organic matter degradability and favoured production of microbial biomass compared with other tropical tree leaves. In the current study, the *A. nilotica* leaf had better ME and dOM as compared to the leaves of *P. juliflora* and *C. cajan* which is in line with the reports of Pal et al. (2015). These observations suggest that the *A. nilotica* leaf could be used as potential supplement to poor quality forages along with its additional benefit in reducing methane production.

4.3 | *L. leucocephala* leaf

As a fodder, leucaena is highly palatable and rich in many micro- and macro-nutrients, including iron, fibre and protein (Melesse, Steingass, Schollenberger, Holstein, & Rodehutschord, 2019). While the interaction values of combination of *L. leucocephala* with CM or *C. gayana* were not significant for CH₄ production, the presumption arises that those nonadditive effects between the two combinations significantly influenced the NGP, dOM and ME productions. The NGP, dOM and ME values were reduced with increasing proportion of *L. leucocephala* leaf combination with CM. On the other hand, these parameters (NGP, dOM and ME) did not vary ($p > 0.05$) between proportions when *L. leucocephala* leaf was combined with *C. gayana*. This observation suggests that supplementation of *C. gayana* grass with *L. leucocephala* leaves might be an alternative strategy to enhance the protein supply to ruminants.

In animal-based experiments, Montoya-Flores et al. (2020) reported that the inclusion of dried *L. leucocephala* leaf up to 12% (on DM basis) enhanced digestible CP and reduced daily production of enteric CH₄ without affecting the DM intake, rumen microbial population and fermentation parameters in crossbred heifers. Supplementation with *L. leucacephala* leaf during the dry season has shown a positive effect on the growth rate and reproductive performance of goats (Mataveia et al., 2019). In a recent study conducted by Tadesse et al. (2022), the replacement of CM with dried *L. leucocephala* leaf improved the growth performance of yearling rams and substantially reduced the CH₄ production by 68% in vitro. It should be, however, noted that leucaena foliage contains high levels of mimosine, a toxic nonprotein amino acid that can cause alopecia, goitre and other thyroid problems, infertility and foetal death in farm animals when fed at high levels (Honda & Borthakur, 2019). Hence, care must be taken while supplementing leucaena leaves to farm animals.

4.4 | *C. cajan* leaf

The *C. cajan* leaf is rich in protein and well-suited for feeding small ruminants and was also reported as potential candidates in mitigating



methane production (Melesse, Steingass, Schollenberger, Holstein, & Rodehutscord, 2019). The partial replacement of concentrate mixture with increasing level of *C. cajan* leaf resulted in increased total protein intake in yearling rams (Tadesse et al., unpublished data).

Among the residues of the leguminous feed crops studied, Soliva et al. (2008) reported that *C. cajan* appeared to be the most promising feed due to its low CH₄ production potential at concurrently high ruminal ammonia release. The observed reduction in CH₄ production in *C. cajan* leaf could be associated with the highest content of condensed tannins (98 g/kg DM) as discussed earlier for *P. juliflora* (Patra & Saxena, 2010). Moreover, the low CH₄ in *C. cajan* leaf might be due to high concentration of phenolic compounds (as shown in Table 1) and their antimicrobial properties as suggested by Kong et al. (2009).

4.5 | *M. indica* seed kernel

The *M. indica* seed kernel contained in the seed is a good source of carbohydrates (58%–80%) (Melesse et al., 2018; Mwaurah et al., 2020). According to these authors, the protein of the *M. indica* seed kernel has also a relatively higher essential amino acid profile, and its oil is a good source of stearic and linoleic acids compared with the other unconventional feed materials used in this experiment. Animal-based experiments conducted by Omer et al. (2019) revealed that replacement up to 50% of yellow corn by *M. indica* seed kernel was an alternative source of energy in sheep without any notable effect on digestion and fermentation.

In the current study, high tannin phenol contents were detected in *M. indica* seeds kernel (153 g/kg DM), which could account for the observed low pCH₄ production. The measured methane production showed only slight deviations from the expected values when *M. indica* seed kernel was combined with CM. These observations suggest that the supplementation of *C. gayana* with *M. indica* seed kernel might be more beneficial in formulating rations than combining it with CM.

Since these unconventional feed resources grow naturally under tropical climatic conditions, they are usually available in bulk and do not directly compete with human food production. Thus, using the leaves of the studied unconventional feed resources as a supplement to either of the conventional feeds (CM and *C. gayana* grass) may have similar effect on the CH₄ mitigation strategy in the livestock production system mainly in the tropical regions. Nevertheless, it should be noted that their palatability might be reduced with increased rate of supplementation due to the presence of antinutritional substances observed in some of them.

5 | CONCLUSIONS

Based on chemical composition, leaves and *M. indica* seed kernels of unconventional feed resources were found to have a better potential as supplements to poor quality forages. On the other hand, higher content of antinutritional factors such as high phenolics in *A. nilotica*

leaf and fibre bound condensed tannins in *C. cajan* leaf may limit their potential as supplement. Moreover, although *P. juliflora* leaf contained high CP, its low NGP, poor ME and dOM contents may make it less favourable supplements to ruminants. However, these drawbacks have been considerably improved when *A. nilotica* and *P. juliflora* leaves were combined with CM or *C. gayana* grass at various proportions. The reduction of the pCH₄ was not affected when leaves of *C. cajan* and *L. leucocephala* were combined with *C. gayana* grass and CM, respectively, at increasing proportions. However, the combination of *A. nilotica* and *P. juliflora* leaves with either *C. gayana* grass or CM with increased proportions has significantly reduced the CH₄ production. Nonetheless, it would be worthwhile to note that increasing the supplementation level of the unconventional feeds may affect their palatability with possible determinantal effects on ME and dOM values specifically with high rates of inclusion. The authors recommend animal-based experiments for leaves of *C. cajan*, *A. nilotica* and *P. juliflora* to validate the in vitro findings.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data collected during this study will become available to interested researchers upon request. Please kindly contact the corresponding author.

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REFERENCES

- Aganga, A. A., & Tshwenyane, S. O. (2003). Feeding values and antinutritive factors of forage tree legumes. *Pakistan Journal of Nutrition*, 2(3), 170–177.
- Castro-Montoya, J., De Campeneere, S., Van Ranst, G., & Fievez, V. (2012). Interactions between methane mitigation additives and basal substrates on in vitro methane and VFA production. *Animal Feed Science and Technology*, 176(1–4), 47–60. <https://doi.org/10.1016/j.anifeeds.2012.07.007>
- Dube, J. S., Reed, J. D., & Ndlovu, L. R. (2001). Proanthocyanidins and other phenolics in Acacia leaves of Southern Africa. *Animal Feed Science and Technology*, 91(1–2), 59–67. [https://doi.org/10.1016/S0377-8401\(01\)00229-2](https://doi.org/10.1016/S0377-8401(01)00229-2)



- Hassanat, F., & Benchaar, C. (2013). Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production in vitro. *Journal of the Science of Food and Agriculture*, 93(2), 332–339. <https://doi.org/10.1002/jsfa.5763>
- Honda, M. D. H., & Borthakur, D. (2019). Mimosine concentration in *Leucaena leucocephala* under various environmental conditions. *Tropical Grasslands-Forrajés Tropicales*, 7(2), 164–172. [https://doi.org/10.17138/TGFT\(7\)164-172](https://doi.org/10.17138/TGFT(7)164-172)
- Jayanegara, A., Wina, E., Soliva, C. R., Marquardt, S., Kreuzer, M., & Leiber, F. (2011). Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. *Animal Feed Science and Technology*, 163(2–4), 231–243. <https://doi.org/10.1016/j.anifeeds.2010.11.009>
- Kong, Y., Fu, Y. J., Zu, Y. G., Liu, W., Wang, W., Hua, X., & Yang, M. (2009). Ethanol modified supercritical fluid extraction and antioxidant activity of cajaninstilbene acid and pinostrobin from pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves. *Food Chemistry*, 117, 152–159. <https://doi.org/10.1016/j.foodchem.2009.03.091>
- Mataveia, G. A., Garrine, C. M. L. P., Pondja, A., Hassen, A., & Visser, C. (2019). Impact of supplementation of *Moringa oleifera* and *Leucaena leucocephala* tree fodder on the production performance of Indigenous goats in Mozambique. *Black Sea Journal of Agriculture*, 2(2), 93–102.
- Melesse, A., Steingass, H., Schollenberger, M., Holstein, J., & Rodehutschord, M. (2019). Nutrient compositions and in vitro methane production profiles of leaves and whole pods of twelve tropical multipurpose tree species cultivated in Ethiopia. *Agroforestry Systems*, 93(1), 135–147. <https://doi.org/10.1007/s10457-017-0110-9>
- Melesse, A., Steingass, H., Schollenberger, M., & Rodehutschord, M. (2017). Screening of common tropical grass and legume forages in Ethiopia for their nutrient composition and methane production profile in vitro. *Tropical Grasslands-Forrajés Tropicales*, 5(3), 163–175. [https://doi.org/10.17138/TGFT\(5\)163-175](https://doi.org/10.17138/TGFT(5)163-175)
- Melesse, A., Steingass, H., Schollenberger, M., & Rodehutschord, M. (2018). Component composition, in vitro gas and methane production profiles of fruit byproducts and leaves of root crops. *The Journal of Agricultural Science*, 156, 949–958. <https://doi.org/10.1017/S0021859618000928>
- Melesse, A., Steingass, H., Schollenberger, M., & Rodehutschord, M. (2019). Chemical, mineral, and amino acid compositions and methane mitigation potentials of agro-industrial by-products and crop residues. *Journal of Applied Sciences*, 19(8), 763–770. <https://doi.org/10.3923/jas.2019>
- Menke, K. H., & Steingass, H. (1988). Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Animal Research Development*, 28, 7–55.
- Montoya-Flores, M. D., Molina-Botero, I. C., Arango, J., Romano-Muñoz, J. L., Solorio-Sánchez, F. J., Aguilar-Pérez, C. F., & Ku-Vera, J. C. (2020). Effect of dried leaves of *Leucaena leucocephala* on rumen fermentation, rumen microbial population, and enteric methane production in crossbred heifers. *Animals*, 10(2), Article 300. <https://doi.org/10.3390/ani10020300>
- Mwaurah, P. W., Kumar, S., Kumar, N., Panghal, A., Attkan, A. K., Singh, V. K., & Garg, M. K. (2020). Physicochemical characteristics, bioactive compounds and industrial applications of mango kernel and its products: A review. *Comprehensive Reviews in Food Science and Food Safety*, 19(5), 2421–2446. <https://doi.org/10.1111/1541-4337.12598>
- Nakano, H. (2004). Growth inhibitory alkaloids from mesquite (*Prosopis juliflora* (Sw.) DC.) leaves. *Phytochemistry*, 65(5), 587–591. <https://doi.org/10.1016/j.phytochem.2004.01.006>
- Omer, H. A. A., Tawila, M. A., Gad, S. M., & Abdel-Magid, S. S. (2019). Mango (*Mangifera indica*) seed kernels as untraditional source of energy in Rahmani sheep rations. *Bulletin of the National Research Centre*, 43(1), Article 176. <https://doi.org/10.1186/s42269-019-0241-4>
- Pal, K., Patra, A. K., Sahoo, A., & Kumawat, P. K. (2015). Evaluation of several tropical tree leaves for methane production potential, degradability and rumen fermentation in vitro. *Livestock Science*, 180, 98–105. <https://doi.org/10.1016/j.livsci.2015.07.011>
- Patra, A. K., & Saxena, J. (2010). A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry*, 71, 1198–1222. <https://doi.org/10.1016/j.phytochem.2010.05.010>
- Soliva, C. R., Zeleke, A. B., Clément, C., Hess, H. D., Fievez, V., & Kreuzer, M. (2008). In vitro screening of various tropical foliages, seeds, fruits and medicinal plants for low methane and high ammonia generating potentials in the rumen. *Animal Feed Science and Technology*, 147(1–3), 53–71. <https://doi.org/10.1016/j.anifeeds.2007.09.009>
- Tadesse, A., Melesse, A., & Rodehutschord, M. (2022). Partial substitution of concentrate mix with dried *Leucaena leucocephala* leaf reduced in vitro methane production in rams without affecting the nutrient intake and performance traits. *Tropical and Subtropical Agroecosystems*, 25(2), 1–12.
- Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA). (2007). *Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA Methodenbuch)*, Bd. III: Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag.
- Wamburu, R. W., Kareru, P. G., Mbaria, J. M., Njonge, F. K., Nyaga, G., & Rechab, S. O. (2013). Acute and sub-acute toxicological evaluation of ethanolic leaves extract of *Prosopis juliflora* (Fabaceae). *Journal of Natural Sciences Research*, 3(1), 8–15.
- Wischer, G., Boguhn, J., Steingaß, H., Schollenberger, M., & Rodehutschord, M. (2013). Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis. *Animal*, 7, 1796–1805. <https://doi.org/10.1017/S1751731113001481>
- Zeleke, A. B., Clément, C., Hess, H. D., Kreuzer, M., & Soliva, C. R. (2006). Effect of foliage from multi-purpose trees and a leguminous crop residue on in vitro methanogenesis and ruminal N use. *International Congress Series*, 1293, 168–171. <https://doi.org/10.1016/j.ics.2006.01.017>

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