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Screening of tree leaves for bioactive components and their impact on in vitro fermentability and methane production from total mixed ration

Pompi Deuri · N. Sood · M. Wadhwa · M. P. S. Bakshi · A. Z. M. Salem

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Abstract This study was taken up to assess the effect of supplementing tree leaves [*Eucalyptus globules* (Safeda), *Populus tremula* (Poplar), *Ficus bengalensis* (Banyan), *Saraca asoca* (Ashoka), *Acacia nilotica* (Kikar), *Phoenix dactylifera* (Khajoor), *Aegle marmelos* (Bael), *Murraya koenigii* (Curry), *Cassia fistula* (Amaltas), *Bauhinia variegata* (Kachnar), *Mangifera indica* (Mango) and *Psidium guajava* (Guava)] at 1–3% on fermentability and in vitro methane production from total mixed ration (TMR). The globulins content was highest in *Aegle*, while albumin was highest in *Psidium* leaves. Prolamin was highest in *Ficus* (17.3%), while glutelin was the highest in *Phoenix* (6.50%). *Ficus* (15.2%) and *Psidium* (10.7%) leaves contained highest level of condensed tannins. *Eucalyptus* and *Mangifera* leaves showed the highest antioxidant activity and flavonoid content respectively. Supplementation of TMR with leaves of *Acacia*, *Psidium* and *Cassia* resulted in higher ($P < 0.05$) DM digestibility, whereas NDF digestibility was highest in TMR supplemented with *Mangifera* and *Acacia* leaves. The VFAs concentration varied ($P < 0.05$) from 4.4 to 6.07 mM/dl in TMR supplemented with *Eucalyptus* and *Saraca* leaves. *Bauhinia* leaves supplementation resulted in the lowest ($P < 0.05$) methane production from TMR. It was concluded that TMR supplemented with leaves of *M. indica, A. nilotica, P. guajava, C. fistula, E. globules and P. dactylifera* at 1% has great potential to improve digestibility and or decrease methane production.

Keywords Methane · Metabolizable energy · Secondary metabolites · Tree leaves · Rumen fermentation

Introduction

Animal production is one of the main sources of CH_4 emissions and it has a warming potential of 21 CO_2-eq over a 100 year time horizon (UNFCCC 2014). Domesticated ruminants are estimated to produce about 80 teragram (Tg) of methane/annum, accounting for about 22% methane emission from human

P. Deuri
Regional A. I. Centre, Bokakhat, Assam 785612, India

N. Sood
Biotechnology, Punjab Agricultura University,
Ludhiana 141004, India

M. Wadhwa (✉) · M. P. S. Bakshi
Department of Animal Nutrition, Guru AngadDev
Veterinary and Animal Sciences University,
Ludhiana 141004, India
e-mail: mw_7in@yahoo.co.in

A. Z. M. Salem
Facultad de Medicina Veterinaria y Zootecnia,
Universidad Autónoma del Estado de México, Toluca,
Edo de México, Mexico

related activities. About 90% of CH_4 emission from enteric fermentation comes from ruminants. The amount of CH_4 that is released depends on number of factors and these include dietary factors such as type of carbohydrate in the diet, level of feed intake, level of production, rate of passage of digesta, presence of ionophores, degree of saturation of lipids in the diet, environmental factors such as temperature (McAllister et al. 1996) and genetic factors such as efficiency of feed conversion.

Chemicals like halogens (Ungerfeld et al. 2004), vaccines against methanogens (Williams et al. 2009), agents like bovicin HC50, a bacteriocin from *Strptococcus bovis* HC50 (Lee et al. 2002) and monensin (Hook et al. 2009) have given encouraging results, but their use has been limited due to environmental and human health concerns. Efforts are afoot throughout the world to find out the alternative agents to mitigate CH_4 emission. Therefore feed additives has been used because of their antimicrobial properties (Davidson and Naidu 2000) and because most of these are categorized under GRAS (Generally Recognized as Safe) for human consumption (FDA 2004). The PSMs have been suggested as effective alternatives to antibiotics. PSMs are group of chemicals present in plants that are not involved in primary biochemical processes of plant growth and reproduction but protect the plant from insect predation or grazing by herbivores (Greathead 2003; Patra and Saxena 2009). These PSM either pure or those present in herbal feed additives have been reported to reduce enteric CH_4 production (Jayanegara et al. 2012; Hundal et al. 2016a, b) reduce ammonia nitrogen concentration and ciliated protozoal population in in-vitro gas production test (Santra et al. 2012), improve nutrient utilization (Garcia-Gonzalez et al. 2006; Wadhwa and Bakshi 2006), but with or without affecting performance of the animals (Bakshi and Wadhwa 2004a; Neelam Rani et al. 2006). Besides herbal feed additives, tree leaves are also good source of PSMs, but only a few reports are available in the literature (Bakshi and Wadhwa 2004b; Rana et al. 2006). The efficacy of secondary metabolites depends on the source, type and level of intake. The present study was therefore planned to screen the tree leaves, widely distributed in Indian sub-continent and available in abundance for bioactive compounds, their impact on in-vitro rumen fermentation pattern and methane production from total mixed ration.

Materials and methods

The tree leaves [*Eucalyptus globules* (Safeda), *Populus tremula* (Poplar), *Ficus bengalensis* (Banyan), *Saraca asoca* (Ashoka), *Acacia nilotica* (Kikar), *Phoenix dactylifera* (Khajoor), *Aegle marmelos* (Bael), *Murray akoenigii* (Curry), *Cassia fistula* (Amaltas), *Bauhinia variegata* (Kachnar), *Mangifera indica* (Mango) and *Psidium guajava*(Guava)] were supplemented to complete feed individually at 0%, 1%, 2%, 3% levels on DM basis. All dried plant materials were then ground and passed through 1 mm sieve and stored in tightly sealed plastic bags for analysis. The ingredient and chemical composition of complete feed is presented in Table 1.

In vitro evaluation

Three male Murrah buffaloes (live weight 360 ± 5.02 kg) fitted with permanent rumen fistulae were used in this study. The animals were fed individually 2 kg concentrate mixture (Maize 32, barley 20, soybean meal 15, groundnut extraction 15, rice bran 15, mineral mixture 2 and common salt 1% each), 2 kg green fodder and wheat straw was offered ad libitum. The rumen contents were collected before feeding at 0900 in a thermos flask flushed with CO_2 and maintained at 39 °C. The rumen contents were blended for 2–3 min in a blender and strained through

Table 1 Composition of total mixed ration

Ingredient composition, % DM basis	
Maize	16.0
Barley	10.0
Wheat bran	7.5
Groundnut cake	15.0
Mineral mixture	1.0
Salt	0.5
Green fodder	50.0
Chemical composition, % DM basis	
Total ash	10.38
Organic matter	89.62
Crude protein	12.70
Ether extract	2.65
Neutral detergent fiber	59.20

DM dry matter

four-layers of muslin cloth. The solution, containing 960 ml distilled water, 0.16 ml micro-mineral solution, 660 ml bicarbonate buffer, 330 ml macro-mineral solution and 1.6 ml resazurine (0.1%) were mixed in a Woulff flask (3 L capacity) with magnetic stirrer in a water bath at 39 °C (Menke et al. 1979; Menke and Steingass 1988). The mixture was continuously flushed with CO_2. Then strained rumen liquor (SRL) was added to the buffer media in the ratio of 1:2. The tree leaves were supplemented at 0%, 1%, 2% and 3% of TMR in 100 ml calibrated glass syringes (Haberle Laborechink, Germany) containing 375 ± 5 mg TMR. 30 ml of buffered rumen fluid was added. Syringes were incubated in triplicate in a water bath at 39 °C and swirled every 60 min over a 24 h incubation period. If the volume of gas in the syringe exceeded 70 ml after 8 h the volume was recorded and the gas was expelled. After 24 h, the volume of gas produced in each syringe was recorded and the contents of syringes were transferred to spout-less beaker, boiled with neutral detergent solution for assessing the true OM and NDF digestibility. Each in-vitro gas production set was repeated thrice in order to check any variation in the net gas production and other parameters.

Volatile fatty acids

After 24 h of incubation, a 5 ml aliquot of fluid from each syringe was mixed with 1 ml of 25% metaphosphoric and kept for 1 h at ambient temperature. Thereafter, it was centrifuged at 5500 rpm for 10 min and clear supernatant was collected and stored at − 20 °C until analyzed. The volatile fatty acids were estimated using Netchrom 9100 gas chromatograph equipped with glass column (packed with chromosorb 101) and flame ionization detector (Cottyn and Boucque 1968). Temperature of injection port, column and detector was set at 250 °C, 175 °C and 270 °C, respectively. The flow rate of carrier gas (N) through the column was 15 ml min^{-1}; and the flow rate of H2 and air through FID was 30 and 300 ml min^{-1}, respectively. Sample (2 µl) was injected through the injection port using a Hamilton syringe (10 µl). Individual VFA's of the samples were identified on the basis of their retention time and their concentration (mmol) and calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values.

Methane estimation

Methane produced during fermentation of the feeds in the culture bottles was estimated using the equation based on VFA proportions (Widiawati and Thalib 2009).

$$\text{Methane} = 0.5 \times (A) + 0.5 \times (B) - 0.25 \times (P) \tag{1}$$

The microbial mass was calculated from the values of ATP estimated using the equation based on VFA proportions.

$$\text{ATP}_{\text{pr}} = 2.5 \times (A) + 2.75 \times (P) + 3.5 \times (B) \tag{2}$$

$$\text{Microbial mass}(g) = 10 \times \text{ATP}_{\text{pr}} \tag{3}$$

where ATP_{pr} is the amount (mol) of ATP produced; A, B and P express the concentration of acetate, butyrate and propionate production in mmol.

Chemical analysis

The finely ground samples were analyzed for dry matter (DM), crude protein (CP), ammonia, ether extract (EE) and total ash (AOAC 2000) and neutral detergent fiber (NDF; Van Soest et al. 1991), total sugars (Dubois et al. 1956), proteins (Lowry et al. 1951), total phenols by using Folin-Ciocalteu reagent (Makkar et al. 1993), tannins (IAEA 2000), CT (Porter et al. 1986), flavanoids (Balabaa et al. 1974), saponins (Baccou et al. 1977), essential oils (steam distillation), DPPH activity (Kumaran and Karakumaran 2007), and vitamin C (Jagota and Dani 1982). The samples of TMR were defatted and then fractionated into water soluble (albumin), sodium chloride soluble (globulin), ethanol soluble (prolamin) and sodium hydroxide soluble (glutelins) proteins (Monteiro et al. 1982). The protein content of these fractions was estimated by Lowry's et al. (1951) method.

Hydrogen balance

$$\text{Hydrogen recovery}\ (\%) = (4\text{M} + 2\text{P} + 2\text{B})/(2\text{A} + \text{P} + 4\text{B}) \times 100 \quad (4)$$

$$\text{Hydrogen consumed via CH4}/\text{VFA} = 4\text{M}/(2\text{P} + 2\text{B}) \quad (5)$$

$$\text{Fermentation efficiency (FE; \%)} = (0.622\text{A} + 1.092\text{P} + 1.56\text{B})100/(\text{A} + \text{P} + 2\text{B}) \quad (6)$$

VFAs utilization index (VFA-UI) represents non-glucogenic VFAs to glucogenic VFAs ratio (NGGR) [Demeyer 1991; Ørskov 1975 and modified by Baran and Zitnan (2002)]

$$\text{VFA} - \text{UI or NGGR} = (\text{A} + 2\text{B} + \text{V})/(\text{P} + \text{V}) \quad (7)$$

where A, B, P, M and V express the concentration of acetate, butyrate, propionate, methane and valerate production in µmol/mmol.

Statistical analyses

The data on bioactive components were analyzed byone way analysis of variance and that of impact of different tree leaves at different levels on different parameters was analyzed by 12 × 4 factorial design (Snedecor and Cochran 1994) by using SPSS (2007) version 16.0. The means were tested for the significant difference by using Duncan's multiple range test. All possible combinations of tree leaves and levels were tested and interactions were worked out (Systat 1996). The statistical model used was:

$$Y_{ijk} = \mu + TL_i + L_j + TL_i * L_j + E_{ijk}$$

where Y_{ijk} = each individual observation for a given parameter (VFA production, CH_4 production etc.); µ = population mean; TL = effect of ith tree leaf (TL i.e. 1–12); L = effect of jth level of tree leaf (Levels i.e. 0%, 1%, 2%, 3%); $TL_i * L_j$ = effect of ith tree leaf at jth level; eijk = error.

Results and discussion

Chemical composition of complete feed and tree leaves

The CP, EE and NDF content in complete feed were as per the recommended NRC (1989) feeding standard (Table 1). The CP content was highest ($P < 0.05$) in *C*assia, while lowest ($P < 0.05$) was in that of *Mangifera* and *Psidium* leaves (Table 2). The leaves of *Phoenix* were highly fiberous (cell wall constituents, CWCs) confirming the earlier report (Bakshi et al. 2011), which also indicated that high CWCs were responsible for lowest effective and true degradability of DM and NDF.

The sugar content was the highest in *Eucalyptus* and lowest in *Bauhinia* leaves (Table 3). Misra et al. (2010) and Budzinski et al. (2016) also reported presence of reducing sugars in *Eucalyptus* leaves. Globulin constituted the major proportion of soluble proteins and prolamins constituted the insoluble proteins in almost all the TLs. *Eucalyptus* had the highest globulin content, while the lowest was in *Cassia* leaves. The albumin was highest ($P < 0.05$) in *Psidium* leaves and lowest in *Saraca* leaves. The prolamin was highest ($P < 0.05$) in ficus leaves and was the lowest in *Murraya* leaves. *Phoenix* leaves had the highest ($P < 0.05$) and the *Aegle* leaves had the lowest ($P < 0.05$) glutelin content.

The soluble protein content was highest ($P < 0.05$) in *Eucalyptus* and the insoluble protein content was highest ($P < 0.05$) in *Ficus* leaves. The ratio of soluble to insoluble fraction was highest in *Murraya* ($P < 0.05$) leaves and lowest in *Cassia* leaves ($P < 0.05$). But, the susceptibility or resistance of a protein supplement to microbial degradation in the rumen may not always depend on the solubility or insolubility of protein supplements (Hancock et al. 1994; Wadhwa et al. 2010). There are other factors, which contribute towards microbial colonization e.g. disulphide bridges (Hancock et al. 1994), surface charge (Argos et al. 1982), hydrophobicity and folding of peptide chains.

The relative proportion of different protein fractions, irrespective of tree leaves, revealed that on an average the globulin constituted the major proportion (49.9%) followed by prolamin (28.4%), albumin (11.5%) and prolamin (10.2%) fractions. The soluble proteins as percent of total proteins were observed to

Table 2 Chemical composition of tree leaves (% DM basis)

Tree leaves		Proximate components				Cell wall components					
Botanical name	Local name	Ash	OM	CP	EE	NDF	ADF	HC	NDS	Cellulose	ADL
Populus tremula	Poplar	13.35^{h}	86.65^{a}	18.71^{g}	3.60^{bc}	40.40^{d}	31.85^{g}	8.55^{a}	59.60	13.00^{d}	9.05^{cd}
Saraca asoca	Ashoka	6.15^{b}	93.85^{g}	16.64^{f}	7.85^{i}	55.90^{h}	27.85^{e}	28.05^{g}	44.10	18.15^{g}	8.30^{c}
Aegle marmelos L.	Bael	13.0	87.00^{a}	13.11^{b}	2.70^{a}	21.40^{a}	13.30^{a}	8.10^{a}	78.60	7.80^{a}	4.05^{a}
Murraya koenigii	Curry patta	10.55^{g}	89.45^{b}	18.46^{g}	5.60^{g}	34.40^{b}	18.55^{b}	15.85^{cd}	65.60	9.80^{b}	6.30^{b}
Ficus bengalensis	Banyan	8.65^{f}	91.35^{c}	14.87^{d}	3.20^{ab}	52.00^{g}	41.15^{i}	10.85^{b}	48.00	17.60^{g}	13.35^{g}
Bauhinia variegata	Kachnar	8.68^{f}	91.32^{c}	13.75^{c}	4.60^{de}	49.40^{f}	30.35^{f}	19.05^{ef}	50.60	21.60^{h}	9.90d^{e}
Mangifera indica	Mango	8.12^{e}	91.88^{d}	11.08^{a}	4.30^{de}	58.75^{i}	37.75^{h}	21.00^{f}	41.25	22.40^{h}	9.85^{de}
Acacia nilotica	Kikar	6.92^{c}	93.08^{f}	18.74^{g}	4.05^{cd}	37.20^{c}	23.20^{d}	14.00^{c}	62.80	16.60^{f}	10.35^{ef}
Psidium guajava	Guava	7.68^{d}	92.32^{e}	11.18^{a}	3.10^{ab}	50.30^{f}	29.85^{fg}	20.45^{f}	49.70	11.80^{c}	10.95^{f}
Cassia fistula	Amaltas	6.13^{b}	93.88^{g}	24.29^{h}	4.90^{ef}	44.75^{e}	27.15^{e}	17.60^{de}	55.25	15.00^{e}	10.00^{def}
Eucalyptus globules	Safeda	5.62^{a}	94.38^{h}	15.86^{e}	6.55^{h}	56.40^{h}	20.25^{c}	36.15^{h}	43.60	12.60^{cd}	4.8^{a}
Phoenix dactylifera	Khajur	9.05^{f}	90.95^{c}	13.28^{bc}	5.30^{fg}	59.50^{i}	51.00^{j}	8.50^{a}	40.50	29.80^{i}	19.35^{h}
PSE		0.51	0.51	0.76	0.3	2.30	2.08	1.70	2.30	1.22	0.81

DM dry matter, *OM* organic matter, *CP* crude protein, *EE* ether extract, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *HC* hemicellulose, *NDS* neutral detergent soluble, *ADL* acid detergent lignin, *PSE* pooled standard error

Figures with different superscripts in a column differ significantly $P < 0.05$

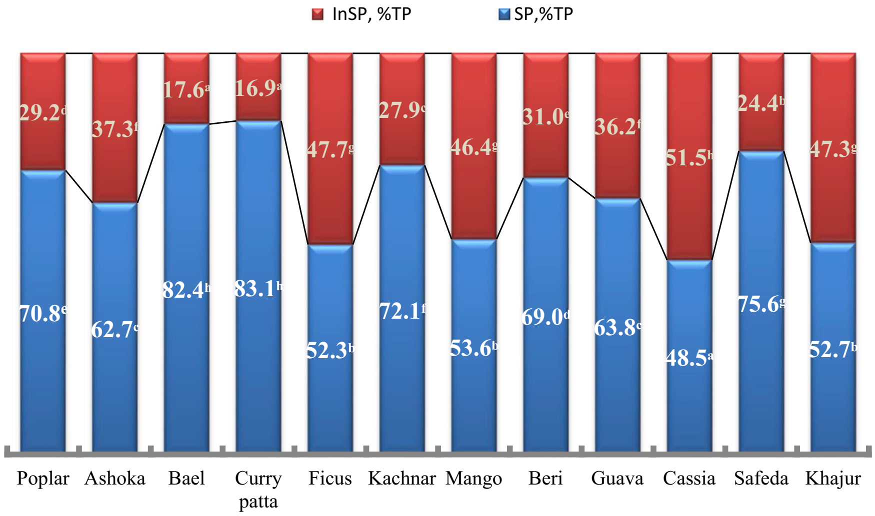
Table 3 Total sugars and protein fractions in tree leaves, % DM basis

Tree leaves		Total sugars	Protein fractions						
Botanical name	Local name		Globulin	Albumin	Prolamin	Glutelin	Soluble (SP)	Insoluble (InSP)	S: I
Populus tremula	Poplar	13.80^{c}	14.99^{f}	2.44^{c}	4.68^{c}	2.50^{d}	17.43^{e}	7.18^{d}	2.43^{e}
Saraca asoca	Ashoka	10.53^{b}	9.73^{b}	1.35^{a}	4.46^{c}	2.12^{c}	11.07^{a}	6.58^{c}	1.68^{c}
Aegle marmelos L.	Bael	10.56^{b}	25.03^{j}	1.47^{ab}	4.41^{c}	1.27^{a}	26.49^{j}	5.68^{b}	4.67^{h}
Murraya koenigii	Curry patta	9.29^{b}	22.29^{i}	1.54^{b}	3.14^{a}	1.69^{b}	23.82^{i}	4.83^{a}	4.93^{i}
Ficus bengalensis	Banyan	16.14^{d}	19.46^{h}	3.85^{f}	17.32^{j}	3.92^{f}	23.31^{h}	21.24^{h}	1.10^{b}
Bauhinia variegata	Kachnar	2.92^{a}	11.76^{d}	1.53^{b}	3.70^{b}	1.45^{a}	13.29^{c}	5.15^{a}	2.58^{f}
Mangifera indica	Mango	3.94^{a}	14.14^{e}	3.85^{f}	12.50^{i}	3.06^{e}	18.00^{f}	15.56^{g}	1.16^{b}
Acacia nilotica	Kikar	17.95^{d}	16.05^{g}	3.29^{e}	5.53^{d}	3.17^{e}	19.34^{g}	8.70^{e}	2.22^{d}
Psidium guajava	Guava	16.44^{d}	19.36^{h}	8.00^{h}	11.75^{h}	3.77^{f}	27.36^{k}	15.53^{g}	1.76^{c}
Cassia fistula	Amaltas	3.81^{a}	9.08^{a}	2.85^{d}	10.24^{f}	2.42^{d}	11.92^{b}	12.65^{f}	0.94^{a}
Eucalyptus globules	Safeda	18.02^{d}	35.5^{k}	4.94^{g}	10.78^{g}	2.25^{cd}	40.45^{j}	13.03^{f}	3.10^{g}
Phoenix dactylifera	Khajur	12.66^{c}	11.0^{c}	3.36^{e}	6.24^{e}	6.50^{g}	14.33^{d}	12.84^{f}	1.12^{b}
PSE		1.1	1.52	0.92	2.19	0.7	1.67	1.04	0.65

PSE pooled standard error, *InSP* insoluble proteins, *SP* soluble proteins, *TP* true proteins

Figures with different superscripts in a column differ significantly $P < 0.05$

be highest ($P < 0.05$) in *Murraya* leaves followed by that in *Aegle* leaves and lowest in *Cassia* leaves (Fig. 1). The relative proportion of soluble and insoluble proteins as per cent of total proteins revealed that leaves of *Ficus, Mangifera, Cassia and Phoenix* had almost half of each fraction. Only *Cassia* leaves has significantly higher ($P < 0.05$) proportion of insoluble proteins as per cent of total proteins amongst the leaves evaluated.



InSP-Insoluble proteins; SP-Soluble proteins; TP-True proteins; Figures with different superscripts in a column differ significantly p<0.05

Fig. 1 Proportion of soluble and insoluble protein as per cent of total protein content

Table 4 Bio-active compounds in tree leaves (% DM basis)

Tree leaves		Phenols					Antioxidants			Saponins	EOs
Botanical name	Local name	TP	NTP	TT	CT	HT	DPPH	Vit C	Flavonoids		
Populus tremula	Poplar	8.23^{d}	0.97^{f}	7.26^{e}	1.67^{c}	5.59^{d}	13.96^{c}	1.58^{cd}	1.33^{e}	0.27^{b}	ND
Saraca asoca	Ashoka	6.22^{c}	0.47^{bc}	5.75^{d}	2.62^{d}	3.13^{b}	11.81^{b}	0.53^{a}	1.92^{g}	0.32^{a}	0.2
Aegle marmelos L.	Bael	5.51^{b}	0.58^{c}	4.93^{bc}	0.38^{ab}	4.55^{c}	9.66^{b}	1.25^{bcd}	1.65^{f}	0.26^{ab}	ND
Murraya koenigii	Curry patta	5.30^{b}	0.77^{d}	4.53^{b}	0.23^{a}	4.31^{c}	7.09^{a}	0.89^{ab}	0.64^{b}	0.38^{d}	ND
Ficus bengalensis	Banyan	15.90^{h}	2.16^{h}	13.74^{h}	12.23^{h}	1.51^{a}	66.56^{f}	2.64^{e}	1.63^{f}	0.53^{g}	ND
Bauhinia variegata	Kachnar	5.90^{bc}	0.38^{b}	5.52^{cd}	0.85^{e}	1.67^{a}	9.81^{b}	0.85^{ab}	0.65^{b}	0.24^{a}	ND
Mangifera indica	Mango	13.32^{f}	1.02^{f}	12.30^{g}	9.33^{f}	2.97^{b}	67.60^{f}	1.49^{cd}	5.65^{h}	0.28^{ab}	ND
Acacia nilotica	Kikar	11.02^{e}	0.82^{d}	10.19^{f}	8.84^{f}	1.35^{a}	42.81^{d}	1.20^{bc}	1.14^{d}	0.48^{f}	ND
Psidium guajava	Guava	15.11^{g}	1.87^{g}	13.24^{h}	10.68^{g}	2.56^{b}	82.87^{g}	3.91^{f}	1.71^{f}	0.42^{e}	ND
Cassia fistula	Amaltas	8.07^{d}	0.43^{b}	7.64^{e}	3.49^{e}	4.15^{c}	5.34^{a}	0.96^{ab}	0.43^{a}	0.48^{f}	0.2
Eucalyptus globules	Safeda	20.17^{i}	3.04^{i}	17.12^{i}	0.89^{b}	16.23^{f}	86.41^{h}	4.14^{f}	0.79^{c}	0.32^{c}	1.0
Phoenix dactylifera	Khajur	3.48^{a}	0.15^{a}	3.33^{a}	2.30^{d}	1.03^{a}	46.99^{e}	1.72^{d}	1.36^{e}	1.18^{h}	ND
PSE		1.1	–	1.02	0.99	–	6.32	–	–	–	–

TP total phenols, *NTP* non- tannin phenols, *TT* total tannins, *CT* condensed tannins, *HT* hydrolysable tannins, *DPPH* 2,2-diphenyl-1-picryl-hydrazyl-hydrate, *Vit C* vitamin C, *EOs* essential oils, *PSE* pooled standard error

Figures with different superscripts in a column differ significantly $P < 0.05$

Screening of tree leaves for bioactive compounds

The total phenols, non-tannin phenols (NTP) and true tannins were highest in *Eucalyptus* and lowest in *Phoenix* leaves (Table 4). The CT content was highest in *Ficus* (12.23%) and lowest in *Murraya* leaves (0.23%). Reed et al. (1990) showed that tannins could exhibit both positive and negative effects on nutritive value depending upon their net tannin content in the forages. Moderate levels of tannins (less than 4%) can have beneficial responses in ruminants, such as making tannin-protein complex which bypass rumen fermentation, decrease CH_4 production, prevent bloat and increase conjugated linoleic acid content in ruminant derived foods, higher growth rate, wool growth, milk yield and reproductive performance (Barry and Manley 1984; Patra and Saxena 2011). However, even in ruminants, tannins exceeding 6% of the diet resulted in negative effect on growth rate and milk yield (Makkar 2003). Bakshi and Wadhwa (2004b) reported the adverse effects of CT on the digestibility of nutrients (correlation coefficient for DM, NDF and CP was − 0.71, − 0.79 and − 0.64, respectively), whereas hydrolysable tannins showed no such adverse effect (correlation coefficient for DM, NDF and CP were, 0.56, 0.46 and 0.4, respectively).The hydrolysable tannin content was observed to be highest in *Eucalyptus* and lowest in *Phoenix* leaves.

It is well known that the antioxidant activities of plant extracts, which contain various phenolic compounds, are due to their abilities to donate hydrogen atoms or electrons and to capture free radicals (Shon et al. 2003; Mohamed et al. 2009). The data revealed that leaves of *Eucalyptus* exhibited the highest ($P < 0.05$) DPPH activity followed by leaves of *Psidium* and the lowest in *Cassia* leaves. The highest DPPH activity in *Eucalyptus* leaves could be attributed to presence of essential oils (1%). The vitamin C content varied ($P < 0.05$) from 0.53% in *Saraca* to 4.14% in *Eucalyptus* leaves. Flavonoid content was highest ($P < 0.05$) in *Mangifera* leaves and lowest was in *Cassia* leaves. The leaves of *Ficus* and *Psidium* showed the presence of high concentration of water soluble saponins which may provide antioxidant activity to these leaves. The level of 0.46% has been observed to be safe. The preliminary screening of TLs revealed that they had great potential to act as antioxidants. Therefore, these were supplemented to TMR to assess their effect on the digestibility and/or enteric methane production.

Impact on digestibility of nutrients and methane mitigation of TMR

The NGP was the highest ($P < 0.05$) in TMR supplemented with *Aegle* leaves and the lowest in *Mangifera* leaves, which could be attributed to high concentration of total phenols, CT and flavonoids in *Mangifera* leaves (Table 5). The true OM digestibility was the highest ($P < 0.05$) in the diet supplemented with *Populus* leaves and lowest ($P < 0.05$) in TMR supplemented with *Bauhinia,* while digestibility of NDF was the highest ($P < 0.05$) in the diet supplemented with *Acacia* and lowest ($P < 0.05$) in diet supplemented with *Saraca* leaves. Blümmel et al. (1997) advocated that gas production should be accompanied by measuring 'Partitioning factor' (PF), which indicates the efficiency of nutrient (OM) utilization and is positively correlated with microbial biomass (Blümmel and Becker 1997) and feed intake (Blümmel and Becker 1997; Blümmel et al. 2005). The PF values of the diet supplemented with *Mangifera* and *Acacia* leaves were comparable and highest, while that supplemented with *Aegle* leaves had the lowest PF value. The availability of metabolizable energy (ME) was not affected by supplementation of TMR with TLs, irrespective of the level of supplementation.

Chandaramoni et al. (2002) and Aregheore and Abdulrazak (2005) indicated that VFAs are waste for rumen microbe but valuable for the host as the major source of energy and contribute about 70% ME requirement of ruminants (Dung et al. 2011). The total VFAs production from the TMR varied ($P < 0.05$) from 4.4 mm/dl (*Eucalyptus* leaves) to 6.07 mm/dl in that supplemented with *Saraca* leaves (Table 6). The low total VFAs production from the TMR supplemented with in *Eucalyptus* leaves could be due to high phenolics content. The individual VFAs and acetate to propionate ratio followed the similar trend.

The molar proportions of acetate from TMR varied between 67% (*Cassia*) to 69.5% (*Mangifera* and *Acacia),* propionate between 16.3% (*Cassia* and *Eucalyptus*) to 17.5% (*Bauhinia*) and that of butyrate between 10.4% (*Murraya* and *Ficus*) to 13.10% (*Cassia*) (Fig. 2). The proportion of iso-butyrate and that of iso-valerate varied ($P < 0.05$) from 1.8 to 2.8% in TMR supplemented with *Acacia* and *Murraya* leaves, respectively. The high level of these BCFAs may act as precursors for synthesis of branched chain amino acids required for synthesis of microbial protein.

Metabolic hydrogen in the form of protons can be used during the synthesis of VFAs or incorporated into microbial biomass. Acetate and butyrate promote methane production, while propionate formation can be considered as a competitive pathway for hydrogen use in the rumen (Moss et al. 2000; Hegarty and Nolan

Table 5 Effect of supplementation of tree leaves on net gas production, digestibility and availability of metabolizable energy from TMR, irrespective of level of supplementation of tree leaves

Tree leaves		NGP	Digestibility (%)		NH_3 (mg/dl)	PF (mg/ml)	ME
Botanical name	Local name		TOM	NDF			
Populus tremula	Poplar	194.56^{g}	68.45^{b}	40.78^{ab}	0.031^{f}	1.55^{ab}	8.37
Saraca asoca	Ashoka	193.56^{fg}	67.70^{ab}	39.54^{a}	0.032^{g}	1.56^{b}	8.35
Aegle marmelos L.	Bael	196.44^{h}	67.69^{ab}	39.82^{a}	0.033^{h}	1.54^{a}	8.42
Murraya koenigii	Curry patta	188.78^{d}	64.06^{ab}	43.49^{bcd}	0.029^{d}	1.92^{d}	8.35
Ficus bengalensis	Banyan	185.56^{c}	64.76^{ab}	44.56^{cde}	0.028^{c}	1.95^{e}	8.27
Bauhinia variegata	Kachnar	188.44^{d}	63.44^{a}	42.36^{abc}	0.029^{d}	1.92^{d}	8.34
Mangifera indica	Mango	182.22^{a}	65.09^{ab}	46.14^{de}	0.026^{a}	2.02^{g}	8.2
Acacia nilotica	Kikar	183.44^{b}	65.65^{b}	47.04^{e}	0.027^{b}	2.01^{g}	8.24
Psidium guajava	Guava	185.89^{c}	65.37^{ab}	46.48^{de}	0.026^{a}	1.98^{f}	8.29
Cassia fistula	Amaltas	191.22^{e}	65.75^{b}	46.42^{de}	0.031^{f}	1.90^{c}	8.42
Eucalyptus globules	Safeda	191.33^{e}	64.93^{ab}	45.18^{cde}	0.030^{e}	1.90^{cd}	8.42
Phoenix dactylifera	Khajur	192.22^{ef}	64.95^{ab}	45.12^{cde}	0.031^{f}	1.89^{c}	8.44
PSE		0.30	0.51	0.71	–	0.004	0.06

NGP net gas production (ml/g DM/24 h), *TOM* true organic matter, *NDF* neutral detergent fiber, *NH_3* ammonia, *PF* partitioning factor, *ME* metabolizable energy (MJ/kg DM), *PSE* pooled standard error

Figures with different superscripts in a row differ significantly $P < 0.05$

Table 6 Effect of supplementation of tree leaves on volatile fatty acid production (mm/dl) from TMR, irrespective of level of supplementation of tree leaves

Tree leaves		TVFAs	Volatile fatty acids						A:P
Botanical name	Local name		Acetate	Propionate	Iso butyrate	Butyrate	Iso valerate	Valerate	
Populus tremula	Poplar	5.71^{j}	3.88^{h}	0.95^{f}	0.054^{fg}	0.658^{f}	0.100^{e}	0.073^{d}	4.07^{bc}
Saraca asoca	Ashoka	6.07^{l}	4.10^{j}	1.03^{g}	0.058^{g}	0.692^{h}	0.107^{f}	0.076^{d}	3.99^{a}
Aegle marmelos L.	Bael	5.85^{k}	4.00^{i}	0.96^{f}	0.045^{ef}	0.672^{g}	0.101^{e}	0.074^{d}	4.20^{de}
Murraya koenigii	Curry patta	4.75^{e}	3.25^{c}	0.81^{c}	0.038^{de}	0.495^{a}	0.096^{d}	0.059^{abc}	4.00^{b}
Ficus bengalensis	Banyan	5.11^{i}	3.55^{g}	0.84^{e}	0.030^{cd}	0.529^{d}	0.106^{f}	0.064^{c}	4.22^{e}
Bauhinia variegata	Kachnar	4.58^{c}	3.07^{b}	0.80^{c}	0.029^{cd}	0.518^{c}	0.096^{d}	0.063^{bc}	3.84^{a}
Mangifera indica	Mango	4.68^{d}	3.24^{c}	0.81^{c}	0.017^{ab}	0.494^{a}	0.068^{a}	0.053^{a}	4.04^{b}
Acacia nilotica	Kikar	4.90^{g}	3.39^{f}	0.84^{e}	0.012^{a}	0.522^{c}	0.076^{b}	0.057^{abc}	4.03^{b}
Psidium guajava	Guava	4.84^{f}	3.34^{d}	0.83^{de}	0.023^{bc}	0.511^{b}	0.074^{b}	0.060^{abc}	4.01^{b}
Cassia fistula	Amaltas	5.03^{h}	3.37^{e}	0.82^{cd}	0.021^{abc}	0.660^{f}	0.096^{d}	0.064^{bc}	4.15^{de}
Eucalyptus globules	Safeda	4.41^{a}	3.01^{a}	0.72^{a}	0.017^{ab}	0.531^{d}	0.085^{c}	0.053^{a}	4.20^{de}
Phoenix dactylifera	Khajur	4.54^{b}	3.09^{b}	0.75^{b}	0.021^{abc}	0.539^{e}	0.086^{c}	0.057^{ab}	4.13^{cd}
PSE		0.006	0.006	0.004	0.002	0.002	0.001	0.002	0.017

TVFAs total volatile fatty acids, *PSE* pooled standard error

Figures with different superscripts in a row differ significantly $P < 0.05$

2007). Therefore, the proportions of acetate, butyrate and propionate determine the amounts of available H_2 in the rumen to be used by methanogens. By this relation, CH_4 emission was calculated stoichiometrically from the respective VFA. The data revealed that supplementation of diet with *Bauhinia* leaves resulted

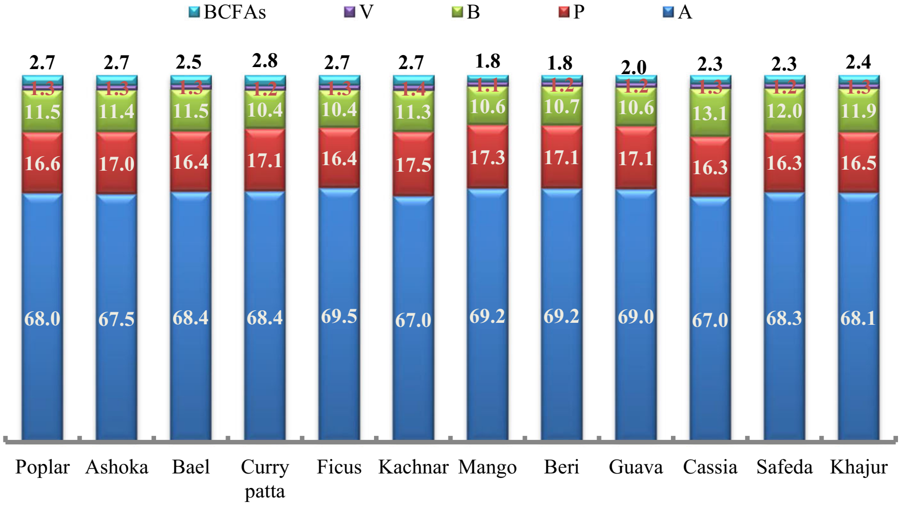


Fig. 2 Effect of supplementation of tree leaves on relative proportion of volatile fatty acid production from complete feed, Irrespective of level of tree leaves. *BCFA* branched chain fatty acids, *V* valerate, *B* butyrate, *P* propionate, *A* acetate

Table 7 Effect of supplementation of tree leaves on fermentability, methane and microbial protein production from complete feed, irrespective of level of supplementation of tree leaves

Tree leaves		Ferm CH_4	Hydrogen balance				MB (g)
Botanical name	Local name	mmoles	HR (%)	HC	FE (%)	VFA UI	
Populus tremula	Poplar	35.54^{cd}	84.43^{c}	1.96^{b}	72.87^{cd}	5.14^{d}	113.40^{a}
Saraca asoca	Ashoka	35.27^{b}	81.19^{a}	1.82^{a}	73.00^{e}	5.05^{c}	116.59^{b}
Aegle marmelos L.	Bael	35.88^{gh}	83.06^{b}	1.97^{b}	72.70^{b}	5.27^{e}	116.96^{b}
Murraya koenigii	Curry patta	35.14^{b}	96.02^{h}	2.40^{f}	72.86^{cd}	4.93^{b}	124.13^{d}
Ficus bengalensis	Banyan	35.71^{def}	91.26^{b}	2.33^{de}	72.53^{a}	5.17^{d}	120.91^{c}
Bauhinia variegata	Kachnar	34.82^{a}	98.46^{j}	2.36^{e}	73.21^{f}	4.84^{a}	120.58^{c}
Mangifera indica	Mango	35.62^{cde}	96.64^{i}	2.44^{g}	72.81^{cd}	5.00^{bc}	149.74^{h}
Acacia nilotica	Kikar	35.64^{cde}	93.72^{f}	2.33^{d}	72.82^{cd}	5.00^{bc}	155.26^{i}
Psidium guajava	Guava	35.47^{c}	94.71^{g}	2.35^{de}	72.85^{cd}	4.95^{b}	146.12^{g}
Cassia fistula	Amaltas	36.02^{h}	92.58^{e}	2.20^{c}	72.94^{de}	5.42^{f}	126.24^{e}
Eucalyptus globules	Safeda	36.05^{h}	100.98^{l}	2.57^{i}	72.75^{bc}	5.36^{f}	129.81^{f}
Phoenix dactylifera	Khajur	35.84^{fgh}	99.53^{k}	2.50^{h}	72.82^{cd}	5.25^{e}	130.20^{f}
PSE		0.05	0.08	0.01	0.03	0.02	0.16

FCH_4 fermentative methane, *HR* hydrogen recovery, *HC* hydrogen consumed, *FE* fermentation efficiency, *VFA UI* VFA utilization index, *MBM* microbial biomass, *PSE* pooled standard error

Figures with different superscripts in a column differ significantly $P < 0.05$

in lowest ($P < 0.05$) methane production (Table 7), which could be due to presence of high concentration of CT (Tan et al. 2011; Soltan et al. 2012).

The supplementation of diet with TLs, irrespective of their levels, influenced H recovery significantly, which was highest ($P < 0.05$) when TMR was supplemented with *Eucalyptus* leaves, while it was lowest from diet supplemented with *Saraca* leaves (Table 7). The ratio of HC via methane to HC via VFA followed a similar trend being lowest in TMR supplemented with *Saraca* leaves and highest in that supplemented with *Eucalyptus* leaves. The

Table 8 Effect of supplementation levels of tree leaves on net gas production, digestibility and availability of metabolizable energy from complete feed, irrespective of nature of tree leaves

Parameter	Level of tree leaves (%)				PSE
	0	1	2	3	
NGP, ml/24 h/g DM	181.04^{a}	183.75^{b}	185.35^{c}	185.76^{c}	0.22
TOMD (%)	71.10^{b}	64.13^{a}	63.96^{a}	63.42^{a}	0.28
NDFD (%)	45.52^{b}	43.79^{a}	43.60^{a}	42.73^{a}	0.42
NH_3 (mg/dl)	0.029^{b}	0.029^{a}	0.030^{bc}	0.030^{c}	–
PF (mg/ml)	1.69^{a}	1.91^{d}	1.90^{c}	1.88^{b}	0.002
ME (MJ/kg DM)	8.22^{a}	8.34^{b}	8.39^{b}	8.41^{b}	0.03

NGP net gas production, *TOMD* true organic matter digestibility, *NDFD* neutral detergent fiber digestibility, *NH_3* ammonia, *PF* partitioning factor, *ME* metabolizable energy (MJ/kg DM), *PSE* pooled standard error

Figures with different superscripts in a row differ significantly $P < 0.05$

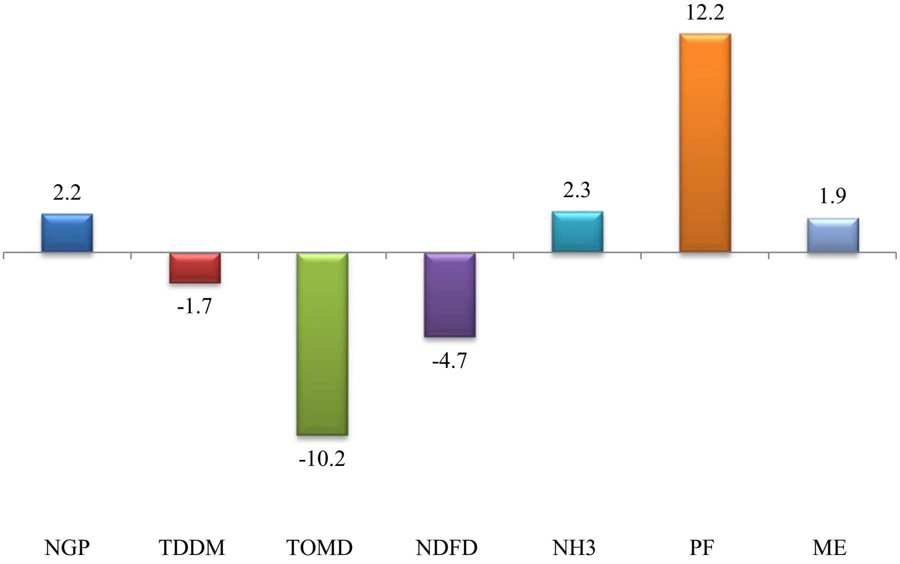


Fig. 3 Percent change over control in-vitro gas production parameters, irrespective of level and nature of tree leaves. Footnote as in Table 8. *TDDM* true DM digestibility

fermentation efficiency varied from 72.5 to 73.2% when the TMR was supplemented with *Ficus* and *Bauhinia* leaves, respectively. The increased fermentation efficiency achieved by supplementation of TMR with *Bauhinia* leaves is actually the end result of its decrement effect on methane production and its ability to increase propionate at the expense of acetate and butyrate. The VFAs utilization index/NGGR is reported to be optimum around 3.5 and higher values indicate the worse use of VFA (Czerkawski 1986). In contrast to fermentation efficiency, the lowest value of NGGR, which indicates the best utilization of VFA, was achieved when diet was supplemented with *Bauhinia* leaves, irrespective of the level of supplementation. The microbial biomass synthesized was highest ($P < 0.05$) from TMR supplemented with *Acacia* leaves followed by *Mangifera* leaves which had high concentration of tannins, phenolics and or flavanoids, making the protein resistant to microbial degradation and in return resulting in higher microbial biomass production.

Table 9 Effect of supplementation levels of tree leaves on volatile fatty acid production (mm/dl) from TMR, irrespective of nature of tree leaves

Parameter	Level of tree leaves (%)				PSE
	0	1	2	3	
TVFAs	4.91^{a}	5.06^{b}	5.09^{c}	5.09^{c}	0.004
Acetate (A)	3.34^{a}	3.46^{b}	3.48^{c}	3.48^{c}	0.003
Propionate (P)	0.82^{a}	0.86^{c}	0.85^{b}	0.86^{bc}	0.002
Butyrate	0.551^{a}	0.559^{b}	0.585^{d}	0.579^{c}	0.001
Valerate	0.061^{a}	0.060^{a}	0.065^{b}	0.065^{b}	0.001
A:P	4.06^{a}	4.06^{a}	4.11^{b}	4.07^{a}	0.010

TVFAs total volatile fatty acids, *PSE* pooled standard error

Figures with different superscripts in a row differ significantly $P < 0.05$

The results revealed that with increase in level of supplementation, irrespective of nature of tree leaves, the NGP and ME availability increased ($P < 0.05$), whereas the digestibility of OM and NDF decreased ($P < 0.05$) by 10.2 and 4.7% as compared to un-supplemented control TMR (Table 8). The lowest levels of ammonia at 1% levels of supplementation indicated efficient utilization of ammonia in presence of VFAs for production of microbial biomass. The PF was observed to highest when diet was supplemented @1% on DM basis and the PF was improved by 12% over control un-supplemented diet (Fig. 3).

With the increase in level of supplementation with tree leaves, irrespective of their nature, the fermentation of TMR was observed to increase as indicated by increase in TVFAs and acetate production (Table 9). However, the level of propionate was observed to be highest when TMR was supplemented with tree leaves at 1% of DM, further increase in level of supplementation did not show any beneficial effect on propionate production. The level of butyrate was observed to be highest at 2% level of supplementation, irrespective of nature of tree leaves. The acetate to propionate ratio was observed to be highest at 2% level of supplementation. The increase over control un-supplemented diet was 3.5, 3.9, 4.5 and 4.2% in TVFAs, acetate, propionate and butyrate concentration, resulting in mild increase in A: P ratio (Fig. 4), clearly indicating the tree leaves have positive effect on fermentation of the diet.

The estimated methane values (Table 10) varied ($P < 0.05$) from 35.5 to 35.7 mmol in un-supplemented control diet and diet supplemented with tree leaves at 2% on DM basis. The hydrogen recovery percent was observed to be the lowest ($P < 0.05$) when diet supplemented with tree leaves at 2% on DM basis. The ratio of HC via methane or HC via VFA decreased with increase in level of supplementation of tree leaves to the diet, indicating that addition of tree leaves was more effective in driving H into the VFA

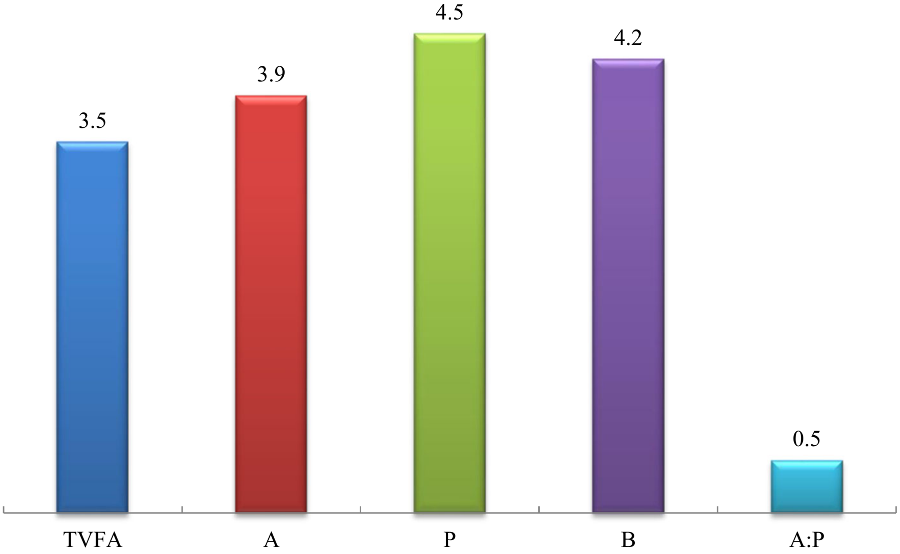


Fig. 4 Percent change over control in volatile fatty acid production profile, on an average, irrespective of level. *TVFAs* total volatile fatty acids, *A* acetate, *P* propionate, *B* butyrate

Table 10 Effect of supplementation of different level tree leaves on fermentability, methane and microbial protein production from complete feed, irrespective of nature of tree leaves

Parameter	Level of tree leaves (%)				PSE
	0	1	2	3	
Fermentative CH_4 (mmol)	35.49^{a}	35.53^{a}	35.68^{b}	35.63^{b}	0.03
H recovery (%)	94.61^{d}	92.51^{c}	91.94^{a}	91.79^{b}	0.04
HC via CH_4/VFA	2.33^{c}	2.27^{b}	2.24^{a}	2.24^{a}	–
FE (%)	72.86	72.84	72.83	72.85	0.02
VFA UI	5.09^{a}	5.09^{a}	5.17^{b}	5.11^{a}	0.01
Microbial biomass (g)	130.68^{c}	130.69^{c}	125.56^{a}	129.71^{b}	1.09

HR hydrogen recovery, *HC* hydrogen consumed; *VFA UI* VFA utilization index, *MBM* microbial biomass, *PSE* pooled standard error

Figures with different superscripts in a row differ significantly $P < 0.05$

synthesis and not into methanogenesis. The fermentation efficiency differed non significantly amongst the levels supplementation and un-supplemented TMR. In contrast, VFAs utilization index/NGGR in diet supplemented with tree leaves at 2% on DM basis was higher than other levels of supplementations and un-supplemented TMR. The production of microbial biomass decreased with increase in level of supplementation of diet with tree leaves, irrespective of their nature. Low microbial biomass production in rumen points out towards the capability of tree leaves to provide rumen undegradable protein.

Conclusion

The results clearly indicated the potential of tree leaves like *Mangifera indica, Acacia nilotica, Psidium guajava, Cassia fistula, Eucalyptus globules* and *Phoenix dactylifera* at 1% level of supplementation has great potential to improve digestibility and or reduce methane production.

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