

Herbal feed additives containing tannins: impact on in vitro fermentation and methane mitigation from total mixed ration

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Abstract: Total mixed ration (TMR) containing roughage and concentrate mixture in a 65: 35 ratio on dry matter (DM) basis was supplemented with herbal feed additives [(HFAs); *Acacia catechu* (Katha), *Areca catechu* (Supari), and *Acacia nilotica* (Babul)] at 0–4% on DM basis to assess their impact on fermentation pattern and methane production by using in vitro gas production technique. *Areca catechu* had the highest ($P < 0.01$) concentration of condensed tannins (CTs), saponins, and vitamin C as compared to other HFAs. The net gas production (NGP), digestibility of NDF and true OM, and ME availability, partitioning factor (PF), volatile fatty acids (VFAs), and microbial biomass production were higher ($P < 0.01$) at 24 h as compared to t-half incubation, irrespective of type and level of HFAs supplemented. *Acacia nilotica* had an edge over *Acacia catechu* with respect to digestibility of nutrients and ME availability. VFAs production and efficiency of rumen fermentation was the highest from the *Areca catechu*-supplemented TMR. Irrespective of type of HFAs and incubation period, the digestibility of NDF and that of true OM were highest ($P < 0.01$) at the 1% level of supplementation, but depressed thereafter. Amongst HFAs-supplemented groups, VFA production and fermentation efficiency were highest at the 2% level. The results conclusively revealed that supplementing *Areca catechu* and *Acacia nilotica* at 2% of TMR (DM basis) inhibited the methane production, without affecting the fermentation pattern.

Key words: Bio-active compounds, herbal feed additives, hydrogen balance, in vitro, ME availability, methane emission

1. Introduction

The greenhouse gases emission intensity of milk production in developing dairy regions is much higher than in developed dairy regions (4.1–6.7 kg CO₂ eq./kg fat-and-protein corrected milk (FPCM) vs. 1.3–1.4 kg CO₂ eq./kg FPCM in 2015). Variation within the same region indicated that greenhouse gas emissions can be reduced by improving efficiency, capturing and sequestering carbon, and better linking dairy production to the circular bioeconomy [1]. Efforts are afoot to mitigate enteric methane emission through dietary manipulation and halogenated compounds, but in an era of antibiotic bans [2] and the beginning of an era of phytochemicals, the opportunity to exploit natural sources like plants, plant extracts, and herbal feed additives containing plant secondary metabolites as growth promoters, productivity enhancers, and as controllers of environment pollution has arisen [3]. Variable results have been reported in the literature due to variability in type and level of active components and nature of the diet [4,5,6,7]. Puchala et al. [8] suggested that feeding forage that contains tannins to ruminants generally effectively inhibits CH₄ produced during enteric fermentation. Tannin from different plants

may show a different response in gas production, true digestibility, and methane production, due to differences in tannin structure and concentration [9,10,11] and other chemical constituents and all the phyto-sources are not equally effective in achieving the methane reduction even when they possess similar tannin content. However, little information is available on the level of supplementation of herbal feed additives (HFAs) containing tannins. This study was therefore taken up on CH₄ mitigation using newer plant sources containing bioactive components like tannins.

2. Materials and methods

2.1. Procurement

HFAs; *Acacia nilotica* (Babul) bark, *Acacia catechu* (Katha), and *Areca catechu* (Supari) containing tannins were procured from Konark Herbals in Mumbai, India.

2.2. Bioactive components

The HFAs were analyzed for phenolics/tannins [12], condensed tannins [CTs; 13], flavonoids [14], saponins [15], 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) activity [16], and vitamin C [17].

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2.3. Preparation of total mixed ration

TMR was prepared with a roughage-to-concentrate ratio of 65:35 on the basis of percentage of dry matter. The roughage portion was made up of wheat straw and *Trifolium alexandrinum* (Berseem) at a 70:30 ratio, while the conventional concentrate mixture was made up of maize (15%), wheat (15%), deoiled mustard cake (15%), mustard cake (10%), soybean meal (10%), rice bran (15%), deoiled rice bran (16%), urea (1%), salt (1%), and mineral mixture (2%).

2.4. Chemical analysis

TMR was ground through a 1-mm sieve and analyzed for proximate principles [18], cellulose [19], and other cell wall constituents [20].

2.5. In vitro studies

Three rumen-fistulated male buffaloes (maintained on 2 kg conventional concentrate mixture, 2 kg green fodder, and ad libitum wheat straw) were used as donors for rumen liquor. The rumen contents were collected before feeding in a prewarmed double-walled (Thermos) flask maintained at 39 °C. Blended and strained rumen liquor was mixed with buffer at a 1:2 ratio. The TMR was supplemented with HFAs at 0–4% of TMR in 100-mL calibrated glass syringes (Haberle Labortechnik, Lonsee, Germany) containing 200 mg TMR (in triplicate) with buffered rumen fluid. Syringes were incubated in a water bath at 39 °C for 96 h. The gas produced was recorded at 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, and 96 h. The difference in the composition and activity of the rumen inoculum among the incubated samples was controlled by parallel incubation of reference standard feedstuffs as suggested by Menke and Steingass. [21]. The samples were run in triplicates and the set was repeated thrice to eliminate the differences if any. The data was subjected to a graph-pad prism programme to determine t½. The incubations were run again and were terminated at respective t½ [22]; the volume of gas was recorded. One hundred microliter of gas was collected from the headspace of each syringe by puncturing the silicon tube and methane was estimated using GLC (Netchrom 9100; Netal, New Delhi, India) equipped with stainless steel column packed with porapak-Q and flame ionization detector. Standard calibration gas (Sigma Gases, New Delhi, India) consisted of 50% methane and 50% carbon dioxide. The flow rates for nitrogen, hydrogen, and zero air were 30, 30, and 320 mL/min, respectively. Parallel sets were run using 375 ± 5 mg TMR with all the HFAs at all levels of supplementations to calculate net gas production, digestibility of nutrients, availability of metabolizable energy, and fermentation pattern [23]. The partitioning factor (PF) defined as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (mL) produced was calculated.

2.6. Volatile fatty acids estimation

After incubations (24h or t½), 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 the Netchrom 9100 gas chromatograph equipped with glass column (packed with Chromosorb 101) and flame ionization detector [24]. A sample (2 µL) was injected through the injection port using a Hamilton syringe (10 µL). Individual VFAs of the samples were identified on the basis of their retention time and their concentration (mmol).

2.7. Methane estimation from VFAs:

Methane produced during fermentation of the feeds in the culture bottles was estimated using the equation based on VFA proportions.

$$\text{Methane} = 0.5 \times (A) + 0.5 \times (B) - 0.25 \times (P) \quad [25] \text{ (Eq. 1)}$$

2.8. Microbial biomass

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

$$\text{ATP}_{pr} = 2.5 \times (A) + 2.75 \times (P) + 3.5 \times (B) \quad [25] \text{ (Eq. 2)}$$

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

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.

2.9. Hydrogen balance

Hydrogen recovery (%) = $(4M + 2P + 2B) / (2A + P + 4B) \times 100$ (Eq. 4)

Hydrogen consumed via CH_4 / VFA = $4M / (2P + 2B)$ [26] (Eq. 5)

2.10. Fermentation efficiency (FE)

FE (%) = $(0.622A + 1.092P + 1.56B) / (A + P + 2B) \times 100$ [27, 28] (Eq. 6)

2.11. VFAs utilization index (VFA-UI) represents non-glucogenic VFAs to glucogenic VFAs ratio

$$\text{VFA-UI} = (A + 2B + V) / (P + V) \text{ (Eq. 7)}$$

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

2.12. Efficiency of fermented hexose energy to VFA energy (E_1)

$$(E_1) = (62 + 0.47(P + 2B + 2V) / (100 + B + V)) \times 100 \quad [29] \text{ (Eq. 8)}$$

2.13. Methane energy (E_2)

$$(E_2) = (28 - 0.47(P + V) / (100 + B + V)) \times 100 \quad [30] \text{ (Eq. 9)}$$

A, P, B, and V are the molar % of acetic, propionic, butyric and valeric acids.

2.14. Statistical analysis

The data of bioactive components was analyzed using one-way ANOVA. The impact of different HFAs and level of HFAs on methane mitigation was analyzed at t half by 3 × 5 factorial design or 2-way ANOVA [31] using SPSS [32] version 16, and the differences in means were tested with Duncan's multiple range test. The interactions were worked out between type of HFAs and their level of incorporation in all possible combinations [33]. The model used is given below

$$Y_{ijk} = \mu + H_i + L_j + H_i \times L_j + E_{ijk}$$

where

Y_{ijk} = Each individual observation for a given variable (VFA production, CH_4 production etc.)

μ = Overall mean

H_i = Effect of i^{th} herbal feed additive (3HFAs)

L_j = Effect of j^{th} level of herbal feed additive (HFAs at 0%, 1%, 2%, 3%, 4% of TMR on DM basis)

$H_i \times L_j$ = Effect of i^{th} herbal feed additive at j^{th} level

E_{ijk} = Residual error

The data of incubation time (t-half and 24h), different types and levels of HFAs on nutrient digestibility, VFA concentration, hydrogen balance etc. was analyzed by 2 × 3 × 5 factorial design or 3 way ANOVA [31] using SPSS [32] version 16, and the differences in means were tested with Duncan's multiple range test. The interactions were worked out between incubation time, type of HFAs, and their level of incorporation in all possible combinations [33]. The model used is given below

$$Y_{ijkl} = \mu + T_i + H_j + L_k + T_i \times H_j + T_i \times L_k + H_j \times L_k + T_i \times H_j \times L_k + E_{ijkl}$$

where

Y_{ijkl} = Each individual observation for a given variable (VFA production, CH_4 production etc.)

μ = Overall mean

T_i = Effect of i^{th} incubation time (t-half and 24h)

H_j = Effect of j^{th} herbal feed additive (3HFAs)

L_k = Effect of k^{th} level of herbal feed additive (HFAs at 0%, 1%, 2%, 3%, 4% of TMR on DM basis)

$T_i \times H_j$ = Effect of i^{th} Incubation time at j^{th} type herbal feed additive

$T_i \times L_k$ = Effect of i^{th} Incubation time at k^{th} level herbal feed additive

$H_j \times L_k$ = Effect of j^{th} type herbal feed additive at k^{th} level herbal feed additive

$T_i \times H_j \times L_k$ = Effect of i^{th} Incubation time at j^{th} type herbal feed additive and k^{th} level herbal feed additive

E_{ijkl} = Residual error

3. Results and discussion

3.1. Screening of herbs for bioactive compounds

The data revealed that selected HFAs are rich in phenolics, tannins, and CTs, and have great antioxidant activity as is evident from the content of flavonoids, vitamin C, and DPPH activity (Table 1). The total phenolic content varied ($P < 0.01$) from 1.8 (*Acacia nilotica*) to 18.53 (*Acacia catechu*). True tannins were observed to be highest ($P < 0.01$) in *Acacia catechu*, while CTs were observed to be highest ($P < 0.01$) in *Areca catechu*. *Acacia nilotica* had a significantly ($P < 0.01$) higher amount of flavonoids than the other herbal feed additives evaluated. The higher antioxidant activity of *Acacia nilotica* could be due to the higher amount of flavonoids. In addition to a higher amount of condensed tannins, *Areca catechu* had the highest ($P < 0.01$) concentration of saponins and vitamin C. *Acacia catechu* has been shown to contain 2–12% catechins, 25–33% phlobatannin, 20–30% gummy matter, quercitrin, quercetin, alkaloids, flavonoids, and toxifolin [34]. *Areca catechu* nut contains several alkaloids of the pyridine group, β - sitosterol, catechin, gallic acid, and leucocyanidins [35]. *A. nilotica* and *A. catechu* contain a variety of bioactive components having antimutagenic,

Table 1. Bioactive compounds in herbal feed additives, % DM basis.

Active component	<i>Acacia catechu</i>	<i>Areca catechu</i>	<i>Acacia nilotica</i>	PSE	P-value
Total phenolics	18.53 ^c	3.73 ^b	1.80 ^a	3.34	<0.001
No tannin phenols	3.95 ^b	0.87 ^a	0.63 ^a	0.83	<0.001
True tannin phenols	14.58 ^c	2.86 ^b	1.18 ^a	2.67	<0.001
Leucocyanidin	4.12 ^b	45.97 ^c	0.27 ^a	9.26	<0.001
Saponins	2.15 ^a	7.14 ^c	5.26 ^b	1.12	<0.001
Vitamin C	2.70 ^b	5.7 ^c	0.33 ^a	1.2	<0.001
DPPH	1.95 ^{ab}	1.83 ^a	2.12 ^b	-	0.043
Flavonoids	0.89 ^a	0.65 ^a	9.85 ^b	1.91	<0.001

DPPH: 2, 2-diphenyl-1-picryl-hydrazyl-hydrate; PSE: Pooled standard error. Means with different superscripts (a, b, c) in a row differ significantly.

antihypertensive, antiinflammatory, antioxidant, antispasmodic, and antiplatelet aggregatory properties [36]. The contribution of condensed tannins and true tannins by the HFA at different levels of supplementation is presented in Table 2.

3.2. Effect of supplementing HFAs on NGP, digestibility of nutrients and availability of ME from TMR

TMR contained 91.2%, 13.80%, 2.41%, 51.4%, 26.1%, and 26.1% organic matter, crude protein, ether extract, neutral detergent fiber, acid detergent fiber, and cellulose content, respectively on a DM basis.

The t-half of TMR supplemented with different HFAs and levels of supplementation varied from 13.33 to 15.11 h indicating that diet supplemented with *A. nilotica* at the 2% level took less time, while the one supplemented with *Areca catechu* took a longer time to ferment. The results revealed that NGP, digestibility of NDF and true OM, and ME availability were lower ($P < 0.01$) at t-half (time to reach half asymptote) in comparison to that observed at 24 h incubation (Tables 3A and 3B), irrespective of type and level of HFAs supplemented. The high NGP, digestibility of nutrients, and ME availability at 24 h might be due to the increased exposure to microbes. PF, an index of the substrate dependent variation in the ratio of substrate degraded to gas volume produced by it at different incubations, differed significantly and was observed to be higher at 24 h. The PF for a given feedstuff can vary with the incubation time partly because of the dynamics of microbial growth. However, ammonia nitrogen was observed to be higher at t-half in comparison to that observed at 24 h incubation. Sahoo et al. [37] concluded that halfway time to maximum gas volume is positively correlated with speed of microbial attachment and rate of degradation, which ultimately decides substrate degradability.

The effect of supplementation of HFAs containing tannins to the TMR on fermentability, irrespective of incubation time and level of supplementation, revealed that NGP was not affected ($P > 0.05$), but the digestibility of nutrients was higher ($P < 0.05$) in TMR supplemented with *Acacia catechu* and *Acacia nilotica* compared to that supplemented with *Areca catechu* (Tables 3A and 3B). The availability of ME from the diet supplemented with *Areca catechu* was observed to be lowest ($P < 0.01$), while the highest was observed from the TMR supplemented with *Acacia nilotica*. Ammonia-N was observed to be lower ($P < 0.01$) in the diets supplemented with *Areca catechu* and *Acacia catechu*. The available data indicated that *Acacia nilotica* had an edge over *Acacia catechu* with respect to digestibility of nutrients and ME availability. The condensed tannins have been reported to be negatively correlated with the digestibility of DM, NDF, and CP with the correlation coefficient of -0.71 , -0.79 , and -0.64 , respectively, whereas hydrolysable tannins showed no

Table 2. Contribution of condensed and true tannins by the respective HFA at different levels of supplementation, mg %.

Level of HFA, %	<i>Acacia catechu</i>	<i>Areca catechu</i>	<i>Acacia nilotica</i>
Condensed tannins (CTs)			
1	0.155	1.724	0.010
2	0.309	3.448	0.020
3	0.464	5.172	0.030
4	0.618	6.896	0.041
True tannin phenols			
1	0.55	0.11	0.04
2	1.09	0.21	0.09
3	1.64	0.32	0.13
4	2.19	0.43	0.18

such adverse affect on the digestibility of these nutrients [38]. Bhatta et al. [39] mentioned that negative effect of tannins on fermentation and digestion could be related to the formation of tannin-carbohydrate and tannin-protein complexes that are less degradable [40] or are toxic to rumen microbes, especially the methanogens. Lowry and Kennedy [41] observed an inhibition of rumen microbial activity with catechin, though it is closely related to quercetin, indicating that these behave differently and show different effects—namely, positive effects for quercetin and negative effects for catechin on rumen microbial activities. These observations strengthen the belief that different herbal feed additives containing different/similar concentrations of bioactive compounds would behave differently in the system.

The NGP and available ME from TMR were not affected by the level of supplementation of HFAs, irrespective of type of HFAs and incubation period. The digestibility of NDF and that of true OM were observed to be highest ($P < 0.01$) at the 1% level of supplementation, but depressed thereafter. The data clearly indicated the potential of HFAs containing tannins in manipulating rumen fermentation. The higher concentration of tannins and other secondary metabolites in tropical legumes affected NDF digestibility, reduced methane production, and provided a positive fermentation pattern with better acetate:propionate and nonglucogenic:glucogenic VFA ratios [42]. No significant interaction was observed between time of incubation and level of HFA or type of HFA and level of supplementation for NGP, digestibility, and ME availability from the TMR. Time of incubation and type of HFA had significant interaction for these parameters. No significant interaction was observed between time of incubation, type and level of HFA for NGP, PF and ME availability from TMR.

Table 3A. Effect of herbal feed additives, their level of supplementation in TMR, and incubation period on in vitro net gas production and digestibility of nutrients.

Parameter	Incubation time, h ¹		PSE	Type of herbal feed additives (HFAs) ²			PSE	Level of HFAs ³ , % DM basis					PSE
	24	t ^{1/2}		<i>Areca catechu</i>	<i>Acacia catechu</i>	<i>Acacia nilotica</i>		0	1	2	3	4	
t-half, h	-	-	-	15.11 ^c	13.92 ^b	13.33 ^a	0.045	14.21 ^b	14.40 ^c	13.97 ^a	13.97 ^a	14.05 ^{ab}	0.058
NGP	160.40 ^b	105.22 ^a	0.95	132.51	131.02	134.91	1.17	133.94	132.56	132.14	131.86	133.56	1.50
NDFD, %	34.93 ^b	32.23 ^a	0.27	30.45 ^a	35.30 ^b	34.98 ^b	0.33	34.07 ^b	35.68 ^c	32.78 ^{ab}	33.47 ^{ab}	31.88 ^a	0.42
TOMD, %	59.89 ^b	57.78 ^a	0.16	57.04 ^a	59.99 ^b	59.49 ^b	0.19	59.15 ^{bc}	59.97 ^c	58.45 ^{ab}	58.80 ^b	57.81 ^a	0.25
PF, mg/mL	2.21 ^b	1.84 ^a	0.04	1.96	2.08	2.02	0.043	2.05	2.04	2.06	2.04	1.92	0.06
ME	8.23 ^b	7.53 ^a	0.09	7.60 ^a	7.88 ^{ab}	8.17 ^b	0.11	7.87	7.93	7.91	8.00	7.71	0.15
NH ₃ -N, mg/dL	0.022 ^a	0.024 ^b	0.01	0.022 ^a	0.022 ^a	0.026 ^b	0.02	0.024 ^b	0.023 ^a	0.023 ^a	0.023 ^a	0.023 ^a	0.003

NGP: Net gas production mL/g DM/24 h; NDFD: Neutral detergent fiber digestibility; TOMD: True organic matter digestibility; PF: Partitioning factor; ME: Metabolizable energy MJ/kg DM; ¹: irrespective of type and level of supplementation; ²: irrespective of level of supplementation and incubation period; ³: irrespective of type of herb and incubation period; PSE: Pooled standard error; Means with different superscripts (a, b, c) in a row differ significantly.

Table 3B. P-values of incubation time, type and level of herbal feed, and their interactions.

	Incubation Time (T)	Type HFA (H)	Level HFA (L)	T×H	T×L	H×L	T×H×L
t-half, h	-	<0.001	<0.001	-	-	<0.001	-
NGP	<0.001	0.069	0.838	0.001	0.815	0.361	0.391
NDFD, %	<0.001	<0.001	<0.001	<0.001	0.069	0.567	<0.001
TOMD, %	<0.001	<0.001	<0.001	<0.001	0.046	0.477	<0.001
PF, mg/mL	<0.001	0.144	0.357	0.824	0.387	0.443	0.253
NH ₃ -N, mg/dL	<0.001	<0.001	0.001	0.001	<0.001	<0.001	<0.001
ME, MJ/kg DM	<0.001	0.005	0.728	0.004	0.238	0.433	0.529

3.3. Effect of supplementing HFAs on fermentation pattern of TMR

The perusal of data revealed that total and individual VFA production was higher ($P < 0.01$) at 24 h in comparison to that observed at t-half incubations (Tables 4A and 4B). However, the acetate to propionate ratio followed a reverse trend and was observed to be significantly higher ($P < 0.01$) at t-half than that observed at 24 h. The 24 h findings can be explained by a distortion of PF measurements through secondary fermentation of lysed microbial cells into SCFA and, consequently, of the gases after microbial peak yield. Van Nevel and Demeyer [43] reported that hydrogen accumulation hinders the pathway for C₂ synthesis and favors C₃ production resulting in characteristic lower C₂:C₃ ratios, which were observed at 24 h of incubation in comparison to those at t^{1/2}. The shift of VFA products from C₂ to C₃ can probably be explained by the reduction of the protozoa population.

The fermentation pattern revealed that production of total and individual VFAs, irrespective of incubation time and level of supplementation of HFAs was highest ($P < 0.01$) in TMR supplemented with *Areca catechu* and the lowest was observed with *Acacia nilotica* (Tables 4A and 4B). Valerate and branched chain fatty acids were comparable in TMR supplemented with *Acacia catechu* and *Acacia nilotica*, but lower ($P < 0.01$) than in the *Areca catechu*-supplemented diet. BCFAs are produced during ruminal fermentation of branched-chain amino acids such as valine and leucine and hence may reflect the degradation of protein. The highest ($P < 0.01$) acetate-to-propionate ratio was observed in the diet supplemented with *Acacia catechu*. This shift of the acetic-to-propionic acid ratio, in favor of propionic acid, may reduce the energy losses that occur in metabolism at the cellular level. In agreement with the present findings, Patra and Saxena [44] also suggested that the presence of tannins reduced acetate and butyrate

Table 4A. Total volatile fatty acids (TVFAs) production from fermentation of TMRs supplemented with HFAs containing tannins.

Parameter	Incubation time ¹ , h		PSE	Type of herbal feed additives (HFAs) ²			PSE	Level of HFA ³ , %DMB					PSE
	24	t½		<i>Areca catechu</i>	<i>Acacia catechu</i>	<i>Acacia nilotica</i>		0	1	2	3	4	
TVFA	4.94 ^b	3.75 ^a	0.015	4.96 ^c	4.16 ^b	3.92 ^a	0.02	5.07 ^d	3.95 ^a	4.11 ^b	4.16 ^b	4.45 ^c	0.024
Acetate	3.24 ^b	2.50 ^a	0.015	3.25 ^c	2.78 ^b	2.57 ^a	0.02	3.36 ^d	2.58 ^a	2.70 ^b	2.74 ^b	2.96 ^c	0.023
Propionate	1.13 ^b	0.80 ^a	0.001	1.12 ^c	0.90 ^b	0.88 ^a	0.001	1.12 ^c	0.88 ^a	0.91 ^b	0.92 ^c	0.98 ^d	0.002
Butyrate	0.462 ^b	0.377 ^a	0.000	0.485 ^c	0.391 ^b	0.384 ^a	0.005	0.48 ^c	0.39 ^a	0.40 ^b	0.41 ^c	0.42 ^d	0.000
Valerate	0.042 ^b	0.027 ^a	0.000	0.039 ^b	0.032 ^a	0.032 ^a	0.004	0.040 ^c	0.033 ^{ab}	0.033 ^a	0.033 ^a	0.034 ^b	0.000
BCFAs	0.073 ^b	0.054 ^a	0.000	0.074 ^b	0.058 ^a	0.057 ^a	0.002	0.074 ^c	0.063 ^b	0.06 ^a	0.059 ^a	0.06 ^a	0.000
A:P	2.86 ^a	3.14 ^b	0.01	2.95 ^a	3.11 ^b	2.95 ^a	0.01	3.04 ^{bc}	2.96 ^a	2.97 ^a	3.00 ^{ab}	3.06 ^c	0.150

BCFAs: branched chain fatty acids; ¹: irrespective of type and level of supplementation; ²: irrespective of level of supplementation and incubation period; ³: irrespective of type of herb and incubation period; PSE: Pooled standard error; Means with different superscripts (a, b, c) in a row differ significantly.

Table 4B. P-values of incubation time, type and level of herbal feed, and their interactions.

	Incubation time (T)	Type HFA (H)	Level HFA (L)	T×H	T×L	H×L	T×H×L
TVFA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Acetate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Propionate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Butyrate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Valerate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
BCFAs	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
A:P	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.010

production (i.e. reduced fiber degradation) or the organic matter (OM) digestion.

The perusal of data on fermentation pattern revealed that total and individual VFAs were observed to be highest ($P < 0.01$) in the unsupplemented diet (Tables 4A and 4B) compared to TMR supplemented with HFAs at different levels, irrespective of the type of HFAs and incubation time. Among different levels of supplementation total and individual VFAs were observed to be the lowest at 1% and increased thereafter, except that of branched chain fatty acids which decreased; supplementation of herbal feed beyond 2% had no additional benefits. Dung et al. [45] reported that, even though VFAs contribute about 70% of the caloric requirement of ruminants, the nutrients in the diets (supplemented or unsupplemented) undoubtedly affect the amount of VFAs production in the rumen. There was a significant ($P < 0.01$) interaction in all possible combinations for total and individual VFAs production and acetate-to-

propionate ratio and the best was observed in *Areca catechu* supplemented at 4% of TMR incubated for 24 h.

The relative proportion of acetate and butyrate was observed to be higher, while that of propionate and branched chain fatty acids were observed to be lower at t-half than that at 24 h incubations (Tables 5A and 5B). TMR supplemented with *Acacia catechu* showed higher ($P < 0.01$) proportions of acetate compared to diet supplemented with other HFAs (Tables 5A and 5B). Propionate and butyrate were higher ($P < 0.01$) in the diet supplemented with *Areca catechu* and *Acacia nilotica* compared with diet supplemented with *Acacia catechu*, irrespective of the level of supplementation. The proportion of BCFAs was observed to be higher ($P < 0.01$) in the diet supplemented with *Acacia nilotica*, which could help in high microbial biomass synthesis; alternatively, the tannin is able to reduce the number of protozoa that are predators of rumen bacteria [46]

Table 5A. Relative proportion of volatile fatty acids from TMR supplemented with HFAs containing tannins.

Parameter	Incubation time ¹ , h		PSE	Type of herbal feed additives (HFAs) ²			PSE	Level of HFA ³ , % DM basis					PSE
	24	t½		<i>Areca catechu</i>	<i>Acacia catechu</i>	<i>Acacia nilotica</i>		0	1	2	3	4	
Acetate	65.40 ^a	66.62 ^b	0.077	65.6 ^a	66.9 ^b	65.5 ^a	0.10	66.32 ^{cd}	65.39 ^a	65.72 ^{ab}	65.95 ^{bc}	66.67 ^d	0.12
Propionate	22.87 ^b	21.19 ^a	0.051	22.3 ^b	21.5 ^a	22.3 ^b	0.063	21.86 ^a	22.21 ^b	22.21 ^b	22.07 ^{ab}	21.83 ^a	0.081
Butyrate	9.38 ^a	10.06 ^b	0.021	9.9 ^b	9.4 ^a	9.9 ^b	0.026	9.61 ^b	9.99 ^d	9.82 ^c	9.77 ^c	9.41 ^a	0.034
Valerate	0.86 ^b	0.71 ^a	0.004	0.77 ^a	0.76 ^a	0.83 ^b	0.005	0.771 ^b	0.84 ^d	0.796 ^c	0.783 ^{bc}	0.749 ^a	0.006
BCFAs	1.49 ^b	1.42 ^a	0.004	1.46 ^b	1.40 ^a	1.49 ^c	0.005	1.45 ^b	1.57 ^c	1.46 ^b	1.42 ^b	1.35 ^a	0.06

BCFAs: branched chain fatty acids, ¹: irrespective of type and level of supplementation, ²: irrespective of level of supplementation and incubation period; ³: irrespective of type of herb and incubation period; PSE: Pooled standard error; Means with different superscripts (a, b, c) in a row differ significantly.

Table 5B. P-values of incubation time, type and level of herbal feed, and their interactions.

	Incubation time (T)	Type HFA (H)	Level HFA (L)	T×H	T×L	H×L	T×H×L
Acetate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Propionate	<0.001	<0.001	0.003	<0.001	0.001	0.002	0.060
Butyrate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Valerate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
BCFAs	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.060

The relative proportion of VFAs was observed to be highest ($P < 0.01$) in diet supplemented with HFAs containing tannins at the rate of 1% on a DM basis, except for acetate which was observed to be highest at the highest level of supplementation (4% on a DM basis), irrespective of type of HFA and incubation time (Tables 5A and 5B). With the increase in the level of supplementation, the total VFAs, acetate, propionate, butyrate, valerate, and branched chain fatty acids decreased significantly, but the negative impact reduced. On an average supplementation of tannins containing herbal feed additives resulted in a 16–18% decrease in VFAs production.

3.4. Effect of supplementation if diet with HFAs on hydrogen balance and microbial biomass production

Metabolic hydrogen produced in the form of reduced protons is used for the synthesis of VFAs. Acetate and butyrate promote methane production while propionate formation may be considered a competitive pathway for hydrogen use in the rumen. 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. Methane production, hydrogen recovery, and microbial biomass were higher ($P < 0.01$) at

24 h incubation than that observed at t-half (Tables 6A and 6B). The pattern of hydrogen consumed via CH_4 or VFA and VFAs utilization index was higher at t-half ($P < 0.01$) than that observed at 24 h, irrespective of type and level of supplementation of HFA. Higher fermentation efficiency (E) and efficiency of fermented hexose energy to VFA energy (E_1) were observed when the diet was incubated for 24 h compared to that at t-half, resulting in lower values for efficiency of fermented hexose to methane (E_2).

Herbs containing tannins were evaluated for their antimethanogenic properties and the data (Tables 6A and 6B) revealed that supplementation of diet with *Acacia nilotica* showed the lowest ($P < 0.05$) methane production in comparison to other HFAs. Hydrogen balance parameters revealed that hydrogen recovery was higher ($P < 0.01$) in the diet supplemented with *Acacia nilotica* and *Areca catechu*, while the hydrogen consumed via CH_4 or VFA was observed to be lowest when diet was supplemented with these HFAs. The type and source of tannin present in herb could be the reason for different responses. The data clearly revealed that *Acacia nilotica* and *Areca catechu* had an edge over *Acacia catechu*, in terms of lowering methane production without affecting the digestibility of nutrients. *Acacia nilotica* had significantly

Table 6A. Fermentation parameter, hydrogen balance of TMRs supplemented with HFAs containing tannins.

Parameter	Incubation time ¹ , h		PSE	Type of herbal feed additives (HFAs) ²			PSE	Level of HFA ³ , % DM basis					PSE
	24	t½		<i>Areca catechu</i>	<i>Acacia catechu</i>	<i>Acacia nilotica</i>		0	1	2	3	4	
CH ₄ , mmol	1.57 ^b	1.24 ^a	0.07	1.59 ^c	1.36 ^b	1.26 ^a	0.09	1.64 ^d	1.26 ^a	1.32 ^b	1.34 ^b	1.44 ^c	0.01
HR,%	36.67 ^b	35.14 ^a	0.08	36.30 ^b	35.10 ^a	36.36 ^b	0.09	35.63 ^a	36.36 ^c	36.19 ^{bc}	35.99 ^b	35.42 ^a	0.12
HC, %	0.088 ^a	0.094 ^b	0.003	0.089 ^a	0.094 ^b	0.089 ^a	0.004	0.092 ^c	0.089 ^a	0.090 ^{ab}	0.090 ^b	0.093 ^c	0.005
E, %	75.01 ^b	74.38 ^a	0.027	74.82 ^b	74.41 ^a	74.85 ^b	0.033	74.59 ^a	74.84 ^b	74.79 ^b	74.72 ^b	74.53 ^a	0.04
E ₁ , %	74.71 ^b	74.10 ^a	0.026	74.54 ^b	74.14 ^a	74.56 ^b	.03	74.3 ^a	74.54 ^b	75.51 ^b	74.44 ^b	74.26 ^a	0.041
E ₂ , %	15.29 ^a	15.98 ^b	0.026	15.52 ^a	15.91 ^b	15.48 ^a	0.03	15.73 ^b	15.49 ^a	15.54 ^a	15.61 ^a	15.79 ^b	0.04
VFAUI	3.59 ^a	4.00 ^b	0.009	3.75 ^a	3.89 ^b	3.74 ^a	0.01	3.82 ^b	3.76 ^a	3.75 ^a	3.79 ^{ab}	3.83 ^b	0.015
MB, g	128.18 ^b	97.56 ^a	0.38	128.88 ^c	107.99 ^b	107.74 ^a	0.46	131.60 ^d	102.54 ^a	106.58 ^b	108.15 ^b	115.48 ^c	0.59

HR: Hydrogen recovery; HC: Hydrogen consumed; E: Efficiency of rumen fermentation; E₁: Energetic efficiency of conversion of fermented hexose energy to VFA energy; E₂: Efficiency of conversion of fermented hexose to methane; VFA Utilization index; MB: Microbial biomass; ¹: irrespective of type and level of supplementation; ²: irrespective of level of supplementation and incubation period; ³: irrespective of type of herb and incubation period; PSE: Pooled standard error; Means with different superscripts (a, b, c) in a row differ significantly.

Table 6B. P-values of incubation time, type and level of herbal feed, and their interactions.

	Incubation time (T)	Type HFA (H)	Level HFA (L)	T×H	T×L	H×L	T×H×L
FCH ₄ , mmol	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.010
HR,%	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.008
HC, %	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.005
E, %	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.011
E ₁ , %	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.018
E ₂ , %	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.014
VFAUI	<0.001	<0.001	0.002	<0.001	0.001	0.001	0.027
MB, g	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

higher concentration of flavonoids (Table 1) which are believed to have direct effects against methanogens and to be an alternative agent to suppress methane production and improve animal health and productivity.

Supplementation of diet with herbs containing tannins, irrespective of the level of supplementation of herbs and incubation period, revealed lowest (P < 0.05) fermentation efficiency in the diet supplemented with *Acacia catechu* in comparison to *Acacia nilotica* and *Areca catechu*. Despite the different HFAs, VFAUI changed this coefficient from 3.74 (diet supplemented with *Acacia nilotica*) to 3.89 (diet supplemented with *Acacia catechu*). Czerkawski [47] reported optimum VFA utilization index as 3.5. Microbial mass was higher in the diet supplemented with *Areca catechu* while it was

low in *Acacia nilotica*-supplemented diet. Broudisou et al. [42] reported that the *A. millefolium*, *A. chamissonis*, and *L. angustifolia* leaf extracts, which contained flavonoids increased synthesis of microbial biomass without affecting the efficiency of microbial protein synthesis (EMPS), respectively. The variations in the results may relate to the type and concentration of the flavonoids present in the plant extract. In the case of high concentrations of flavonoids, the EMPS may decrease as observed in this study.

The fermentative methane on an average decreased by 18% on average when the diet was supplemented with HFAs containing tannins, irrespective of level of supplementation and nature; however, the reduction was observed to be 23% when the diet was supplemented

Table 7A. Methane production at 1/2 from TMRs supplemented with HFAs containing tannins.

Parameter	Type of herbal feed additives (HFAs) ¹			PSE	Level of HFA ² , % DM basis					PSE
	<i>Areca Catechu</i>	<i>Acacia catechu</i>	<i>Acacia nilotica</i>		0	1	2	3	4	
CH ₄ , %	21.67	23.46	23.27	0.68	22.89	23.52	22.78	21.32	23.51	0.87
CH ₄ , mL/100 mg DM	2.36	2.40	2.44	0.07	2.48	2.48	2.36	2.23	2.44	0.08
CH ₄ , mL/100 mg DDM	4.28	4.05	4.30	0.12	4.35	4.25	4.18	4.03	4.24	0.16
CH ₄ , mL/100 mg DOM	2.44	2.40	2.48	0.08	2.54	2.54	2.39	2.29	2.43	0.10

CH₄: methane; DM: dry matter; DDM: digestible dry matter; DOM: digestible organic matter; ¹: irrespective of level of supplementation; ²: irrespective of type of herb; PSE: Pooled standard error.

with HFAs at 1% on a DM basis, irrespective of their nature (Tables 6A and 6B). Further increase in level of supplementation did not reduce the fermentative methane production. Hydrogen recovery from a diet supplemented with HFAs was also observed to be highest, and hydrogen consumed via CH₄/VFA was observed to be lowest at the 1% level. Fermentation efficiency (E) and efficiency of fermented hexose energy to VFA energy (E₁) increased when the diet was supplemented with HFAs up to the 3% level, resulting in lower values of efficiency of fermented hexose to methane (E₂). VFAUI was lower when the diet was supplemented with HFAs up to the 2% level; further increase improved the VFAUI. Supplementation of diet with HFAs depressed the microbial biomass synthesis. The differences amongst HFAs can be attributed to tannin content and tannin structural composition and also to the presence of other secondary metabolites which might or might not have additional effects. Addition of flavonoid substances has been known to enhance fermentation efficiency by improving propionate in detriment to acetate production, which clearly depressed hydrogenotrophic methanogenic archaea communities [48]. There was a significant (P<0.01) interaction in all possible combinations for rumen fermentation efficiency, efficiency of conversion of hexose energy to CH₄ energy, and VFAUI; and the best was observed in *Areca catechu* and *Acacia nilotica* supplemented at 2% of TMR on a DM basis incubated for 24 h.

3.5. Effect of supplementation of diet with HFAs on methane production

The methane production expressed as either percentage of NGP or mL/100 g DM/DDM/DOM was comparable amongst the different herbal feed additives used irrespective of the levels of supplementation as well as at different levels of supplementation, irrespective of HFAs used (Tables 7A and B). VFA synthesis would be responsible for 19–33% of the hydrogen (H₂) uptake and

Table 7B. P-values of incubation time, type and level of herbal feed, and their interactions.

	Type HFA (H)	Level HFA (L)	H×L
CH ₄ , %	0.138	0.394	0.026
CH ₄ , mL/100 mg DM	0.676	0.211	0.004
CH ₄ , mL/100 mg DDM	0.304	0.708	0.005
CH ₄ , mL/100 mg DOM	0.771	0.386	0.006

only propionate and valerate formation uses H₂, with one mole H₂ required per mole produced propionate or valerate [49]. Increase in propionate production is a competitive pathway for methane and this could lower methane production. Sinz et al. [50] investigated polyphenols, like flavonoids in extracts or when present in intact plants as methane mitigating dietary supplements in ruminants and reported that luteolin-7-glucoside seems to have a similar potential as tannic acid in mitigating methane and ammonia formation during ruminal fermentation in vitro. The management of H₂ production, in the rumen should be considered the most important factor, while developing strategies to control ruminant CH₄ emissions, indicating that CH₄ production can be reduced by inhibiting H₂-liberating reactions or by promoting alternative H₂-using reactions or routes for disposing of H₂ during fermentation [50].

Earlier reports [4, 5] and many others have indicated that CH₄ production decreased with the inclusion of tannin in ruminant diets, unlike the present report, which could be attributed to the fact that the impact of active components (tannins, saponins, and/or essential oils) on methane production varies with chemical structure (plant origin) as well as with their concentration and copresence of these components. Oskoueian et al. [51] stated that

flavonoids (naringin and quercetin) at the concentration of 4.5% of the substrate (on a dry matter basis) could suppress methane production without any negative effects on rumen microbial fermentation.

4. Conclusion

The results have conclusively revealed that supplementing 2% (on a DM basis) *Areca catechu* (providing 3.66, 0.54, and 0.61 mg % total tannins, saponins and total antioxidants, respectively) or *Acacia nilotica* (providing 0.11, 0.40, and 0.92 mg % total tannins, saponins, and total antioxidants, respectively) mitigated the methane production, without affecting the fermentation pattern of the total mixed ration (roughage-to-concentrate ratio of 65:35 on a DM

basis). Further in vivo studies are required to establish these HFAs as methane-suppressant phytosources in the ruminant ration.

Conflict of interest

The authors declare that they have no conflict of interest.

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