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Effect of supplementation of saponin containing herbs on *in vitro* methane production under different feeding systems

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ABSTRACT

This study was taken up to assess the effect of herbal feed additives [HFAs; kulthi (*Dohichos biflorus*), patha (*Cissampelos pareria*), aritha (*Sapindus trifoliatus*)] supplemented at 0–3% on DM basis of total mixed rations (TMR) on the *in vitro* methane production and nutrient fermentation in a 3 × 4 factorial design. TMR with different roughage to concentrate ratio (R:C) of 80:20, 75:25, 70:30 and 65:35 on DM basis were formulated. The roughage portion was made up of wheat straw and maize green fodder in 70:30 ratio. The chemical analysis of HFAs revealed that aritha had the highest concentration of both water and methanol soluble saponins; and condensed tannins (Leucocyanidin). Patha followed by kulthi had the highest concentration of vitamin C, flavonoids, total phenols and true tannins. The digestion kinetic parameters revealed that with the increase in level of concentrate in the diet, irrespective of type and level of supplementation of HFAs, the lag phase for fermentation of diet decreased linearly. The data conclusively revealed that the best response with respect to net gas production (NGP), digestibility of nutrients, methane production, volatile fatty acid (VFA) production, ME availability and other fermentation parameters from TMRs with different R:C ratios was observed in kulthi and patha supplemented at the rate of 2% of TMR with R:C ratio of 65:35 on DM basis.

Key words: Herbs containing saponins, *In vitro* methane production, Roughage to concentrate ratio

Increasing attention has been placed on greenhouse gas (GHG) emissions in recent years with deteriorating scenario of global warming (O'Mara 2011). Approximately 7–18% of the global GHG emission originate from livestock sector (Hristov *et al.* 2013). Globally, about 80–115 Tg methane equivalent to 15–20% of total anthropogenic methane is produced/annum by domestic livestock (Houghton *et al.* 2001).

Number of studies has been conducted to assess the potential of plant secondary metabolites as natural manipulating agents for ruminal fermentation (Hundal *et al*. 2016a, b, Wallace *et al*. 2002). Saponins are phytochemicals commonly found in plants and are composed of steroids, triterpenoids, and steroid alkaloids (Wina *et al.* 2005a, Bakshi and Wadhwa 2010). Saponins as feed supplements may have the potential to modulate ruminal fermentation and improve nutrient utilization in ruminants (Patra and Saxena 2010, Wallace *et al.* 1994, Hirstov *et al.* 1999). Both *in vitro* (Hu *et al.* 2005, Lila *et al.* 2003) and *in vivo* (Yuan *et al.* 2007) studies revealed that saponins might reduce the pH and ammonia–N concentration in the rumen. Saponins have been reported to have mixed effect on feed intake decreasing (Lovett *et al.* 2006), or increasing (Holtshausen *et al.* 2009) or no effect on feed intake in ruminants (Mao *et al*. 2010). Supplementing the diet of sheep with saponins increased organic matter and fibre digestibility (Lu and Jorgensen 1987). Moreover, addition of saponin-rich plants, such as *Yucca schidigera* has been found to improve growth, feed efficiency and health in ruminants (Cheeke 1996). According to Wina *et al*. (2005b), many different factors affect the response of ruminants to saponins including sources, levels of supplementation, and diet composition. The present investigation was planned to assess the effect of herbal feed additives containing saponin as active component on the methane production potential using total mixed rations (TMRs) with different roughage to concentrate ratios, commonly used for different categories of animals.

MATERIALS AND METHODS

Bioactive components of HFAs: The HFAs [kulthi (*Dohichos biflorus*), patha (*Cissampelos pareria*) and aritha (*Sapindus trifoliatus*)] containing saponins were procured from Konark Herbals, Mumbai. Herbs (100 mg) in duplicate were extracted with 7.5 ml of distilled water. The contents were centrifuged at 3,000 g for 10 min. The extraction was repeated again and the extracts were pooled. The aqueous extract obtained was used for the estimation of phenolics/

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tannins (Makkar *et al.* 1993), condensed tannins (Porter *et al.* 1986), flavonoids (Balabaa *et al.* 1974), saponins (Baccou *et al.* 1977) and DPPH (Kumaran and Karakumaran 2007). Simultaneously 1 ml of 20% TCA was added to 1 ml of aqueous extract and kept overnight at 4°C. It was centrifuged and the supernatants were used for the estimation of vitamin-C (Jagota and Dani 1982). The herbs were extracted with 80% methanol following the same procedure as mentioned above. The saponins were estimated from the menthanolic extract as well.

Preparation of total mixed rations: Four TMRs with different R:C ratios (80:20, 75:25, 70:30 and 65:35) were prepared. The roughage portion was made up of wheat straw and maize green fodder in 70:30 ratio, while the concentrate mixture was made up of 15% maize, 15% wheat, 15% deoiled mustard cake, 10% mustard cake, 10% soybean meal, 15% rice bran, 16% deoiled rice bran, 1% urea, 1% salt and 2% mineral mixture.

Chemical analysis: The samples were ground to pass through 1 mm sieve and were analyzed for proximate and cell wall constituents. The protein contents were analyzed using standard method (AOAC 2000). The samples of TMR were analyzed for neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL; Robertson and Van Soest 1981) and cellulose (Crampton and Maynard 1938).

In vitro *studies:* Rumen fistulated male buffaloes (maintained on 2 kg conventional concentrate mixture, 2 kg green and *ad lib.* wheat straw) were used as a donor for rumen liquor. The rumen contents were collected at 0 h in double walled (Thermos) flask flushed with CO_2 and maintained at 39°C. The rumen contents were blended for 2–3 min in a blender, maintained at 39°C and then strained through 4 layered muslin cloth. The HFAs containing saponins were supplemented @ 1–3% of TMRs in 100 ml calibrated glass syringes (Haberle Labortechnik, Germany) containing 200 mg TMR with buffered rumen fluid. Syringes were incubated in a water bath at 39ºC for 96 h to determine the rate of degradation of the substrate, with recording of the gas volumes after 2, 4, 6, 8, 10, 12, 24, 36, 48, 72 and 96 h and swirled every 60 min for initial 8 h. The data were subjected to graph-pad prism programme to determine Y min (minimum amount of gas production), Y max (maximum potential of gas production), t½ and k (rate of gas production). These sets were repeated three times.

The incubations were conducted in triplicate for each sample on 2 successive days and these incubations were repeated three times, with an interval of 1 week. The difference in the composition and activity of the rumen inoculum among incubations was controlled by parallel incubation of reference standard feedstuffs as suggested by Menke *et al.* (1979). The incubations were terminated at respective t½ and the volume of gas was recorded and true substrate degradability was measured (Blummel and Lebzien 2001). The partitioning factor (PF) defined as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (ml) produced by it (France *et al.* 1993).

Volatile fatty acids estimation: Volatile fatty acids were estimated using Netchrom 9100 gas chromatograph (Netal, New Delhi, India) equipped with flame ionization detector as per method described by Cottoyn and Boucque (1968). The gas column (6 ft length and 1/8 inch diameter) packed with chromosorb 101 was used for the estimation of VFA. The gas flows for nitrogen, hydrogen and zero air were 15, 30 and 300 ml/min, respectively. Temperature of injector oven, column even and detector were 250°C, 175°C and 270°C respectively. Samples were prepared by adding 0.2 ml of 25% metaphosphoric acid per ml of rumen liquor/ contents of *in vitro* syringes, allowing it to stand for 2 h followed by centrifugation at 4,000 rpm for 7 min. Supernatant was used for estimation of VFA. Standard VFA mixture was prepared by mixing stock solutions (each of 25 mg/ml concentration) of standard VFAs and distilled water in the proportion of acetic acid 1.68 ml, propionic acid 0.48 ml, butyric acid 0.24 ml, distilled acid 7.24 ml to obtain final concentration of acetic acid, propionic acid, valeric acid as 7, 1.62 and 0.68 mM/100 ml.

Methane estimation: Methane was estimated by using GLC (Netchrom 9100) equipped with stainless steel column packed with porapak-Q and flame ionization detector. Standard calibration gas (Sigma Gases, New Delhi) consisted of 50% methane and 50% carbon dioxide. The flow rates for nitrogen, hydrogen and zero air were 30, 30, 320 ml/min respectively. From the headspace of each syringe, 100 ml gas was collected by puncturing the silicon tube and injected in gas chromatograph for the estimation of methane.

Hydrogen balance: Hydrogen recovery (%) was estimated as (4M+2P+2B) / (2A+P+4B) × 100, the ratio of hydrogen consumed *via* CH4/VFA was estimated as 4M/ (2P+2B), where acetate (A), propionate (P), butyrate (B) and methane (M) production was expressed in mmol (Demeyer 1991).

Fermentation efficiency: This was calculated on the basis of the equation worked out by Rskov (1975) and modified by Baran and Zitnan (2002).

$$FE = (0.622a + 1.092p + 1.56b)\ 100/\ (a + p + 2b)$$

where a, p and b express the concentrations (µmol) of acetic, propionic and butyric acids respectively in the total concentration of VFAs produced. The final result of this equation is expressed in percentage and shows an amount of energy stored in VFAs as a percentage participation of the initial energy.

VFAs utilization index: This was expressed by non-glucogenic VFAs/glucogenic VFAs ratio (NGGR) according to Rskov (1975).

$$NGGR = (A + 2B + V) / (P+V)$$

where A, P, B and V express the concentrations (µmol) of acetic, propionic, butyric and valeric acids respectively. Valeric acid is classified as both glucogenic and non-glucogenic VFA because, its oxidation creates 1 mole of acetic acid and 1 mole of propionic acid. Too high NGGR indicates high loss of energy in the form of gases.

Statistical analysis: Data were analyzed by 3 × 4 factorial

design (Snedecor and Cochran 1994), by using SPSS (2007) version 12 and the differences in means were tested by Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Chemical composition of TMRs: The chemical composition of TMRs varying in R:C ratios revealed that with increase in level of concentrate in TMRs, the OM, EE and CP content increased, while cellulose, NDF, ADF and ADL content decreased (Table 1).

Screening of herbs for bioactive compounds: The data revealed that water soluble saponin content was highest (P<0.01) in aritha, followed by kulthi and lowest in patha, whereas saponin content in methanol extract was highest (P<0.01) in aritha and lowest in kulthi (Table 2). Beside saponins, these were rich in antioxidant activity due to presence of vitamic C and flavonoids. Patha had the highest (P<0.01) concentration of vitamin C and that of flavonoids and aritha had the lowest concentration of these antioxidants. Among plant secondary metabolites, flavonoids have gained importance because of their wide range of biological activities and in particular antimicrobial properties. These natural compounds are believed to have direct effects against methanogens (Bodas *et al.* 2012) and to be an alternative agent to suppress methane production and improve animal health and productivity. Addition of flavonoid substances enhances fermentation efficiency by improving propionate in detriment of acetate production and clearly depressed hydrogenotrophic methanogenic archaea communities (Seradj *et al.* 2014). The data revealed that aritha exhibited the highest (P<0.01) DPPH activity followed by patha.

Table 1. Chemical composition of TMRs

Parameter	Roughage: Concentrate ratio			
	80:20	75:25	70:30	65:35
Total ash	12.22	11.22	10.95	10.78
Organic matter	87.78	88.78	89.05	89.22
Crude protein	14.65	17.9	18.55	21.45
Ether extract	2.05	2.25	2.85	3.35
Cellulose	37.6	32.9	30.3	26.1
Neutral detergent fibre	69.2	64.9	60.7	57.4
Acid detergent fibre	37.6	32.9	30.3	26.1
Hemicellulose	31.6	32.0	30.4	31.3
Acid detergent lignin	8.10	7.55	6.60	5.60

Table 2. Bio-active components in herbal feed additives (% DWB)

Parameter	Herbal feed additive			PSE	P value
	Patha	Kulthi	Aritha		
Aqueous saponin**	5.06^{a}	5.55^{b}	5.81^{c}	-	0.00
Methanol saponin**	3.75^{b}	2.00^{a}	6.75^{c}	0.75	0.00
Antioxidants					
Vitamin C**	5.68^{c}	1.95^{b}	0.48^{a}	-	0.00
Flavonoids**	11.37^{c}	6.62^{b}	0.02^{a}	1.16	0.00
DPPH AA%**	403.96^{ab}	400.14^{a}	446.02^{c}	11.80	0.00
DPPH mg%**	2.02^{ab}	2.00^{a}	2.23^{c}	0.10	0.00
Tannins					
Total phenolics**	37.48^{c}	11.19^{a}	14.41^{b}	4.18	0.00
Non tannin phenols**	5.93^{c}	0.78^{a}	1.63^{b}	1.35	0.00
True tannins**	31.56^{c}	10.41^{a}	12.78^{b}	3.40	0.00
CT, Leucocyanidin**	0.07^{a}	0.40^{b}	2.51^{c}	-	0.00

Figures with different superscripts in a row differ significantly (**P<0.01).

Total phenols and true tannins were highest (P<0.01) in patha followed by aritha and lowest in kulthi. Phenolic compounds, including tannins have been recognized as modulators of rumen fermentation with potential to inhibit methanogenesis (Bhatta *et al.* 2014, Mangwe *et al.* 2016). Phenolic compounds play an important role as antimicrobials by interacting with the extracellular microbial enzymes, as well as depriving microbe substrates required for growth (Patra and Saxana 2011). In addition to direct inhibition on methanogenic archaea and protozoa (Patra *et al.* 2012), it is possible that phenolic compounds could indirectly affect H_2 production (Patra *et al.* 2017) or form insoluble complexes with protein resulting in suppression of overall microbial degradation and methane emissions (Patra and Saxena 2011). Feeding plants containing total phenols and tannins has been demonstrated to reduce methane and total gas production (Pal *et al.* 2015).

Condensed tannins (CTs) were highest (P<0.01) in aritha and lowest in patha (Table 2). The molecular weight and chemical structures are primary factors determining the influence of CTs on CH_4 production in ruminants (Spencer *et al.* 1988, Osborne and McNeill 2001, Liang and Khamseekhiew 2006). Moderate levels of CT (less than 4.0%) in forage legumes can have beneficial responses in ruminants, resulting in higher CP utilization, growth rates and milk yield, but levels of CT exceeding 6% of the diet resulted in negative effect on growth rate and milk yield (Makkar 2003).

Digestion kinetic parameters of gas production in vitro*:* The gas production profiles indicated that the diet supplemented with kulthi showed the lowest lag phase, highest y max (maximum potential of gas production), highest rate of degradation (k) and lowest t½ (time taken for reaching half asymptote) as compared to other HFAs supplemented groups, irrespective of level of supplementation and type of diet (Table 3). With the increase in level of HFA supplementation, irrespective of type of herb and diet, the lag phase decreased and y max increased (P<0.01) linearly. However, rate of degradation (k) and t½ values were not affected by level of supplementation of herb. With increase in level of concentrate in the diet, irrespective of type and level of supplementation of herbs, the lag period for fermentation of diet decreased (P<0.01) linearly, degradation rate (k) and y max increased linearly (P<0.01), but y min (minimum potential of gas production) and t½ followed the reverse trend (P<0.01).

In vitro *screening of optimum level of HFAs containing saponins using TMRs varying in R:C ratios at t-half:*

Table 3. Effect of herbs containing saponins on the fermentation kinetics of diet.

Parameter	Herbal feed additives[1]			PSE	Levels of HFA[2] (%)				PSE	Roughage to Concentrate ratio[3]				PSE
	Kulthi	Patha	Aritha		0	1	2	3		80:20	75:25	70:30	65:35	
Lag time (h)**	-0.54^{a}	-0.95^{c}	-0.83^{b}	0.11	-1.87^{a}	-1.16^{b}	-1.27^{b}	-1.18^{b}	0.09	-1.9^{a}	-1.7^{a}	-1.2^{b}	-0.7^{c}	0.09
Ymax (ml)**	45.36^{b}	45.28^{b}	43.56^{a}	0.12	42.46^{a}	43.65^{b}	44.11^{c}	45.09^{d}	0.1	42.9^{a}	43.5^{b}	44.9^{d}	43.9^{c}	0.1
Rate of degradation (k)**	0.059^{c}	0.058^{b}	0.057^{a}	-	0.058	0.058	0.058	0.058	-	0.056^{a}	0.057^{b}	0.059^{c}	0.061^{d}	-
Ymin (ml)**	17.00^{a}	17.29^{a}	17.61^{b}	0.08	17.24	17.38	17.28	17.22	0.06	18.0^{d}	17.7^{c}	17.0^{b}	16.3^{a}	0.06
t½ (h)**	11.78^{a}	11.99^{a}	12.21^{b}	0.05	11.95	12.05	11.98	11.94	0.04	12.5^{d}	12.3^{c}	11.8^{b}	11.3^{a}	0.04

Figures with different superscripts in a row differ significantly (**P<0.01); [1]Irrespective of level of supplementation and type of diet; [2]Irrespective of type of herb and diet, of supplementation; [3]Irrespective of level of supplementation and type of herb.

Amongst the herbs evaluated, supplementation of patha, irrespective of level of supplementation and type of diet resulted in higher (P<0.01) NGP, digestibility of nutrients and availability of ME as compared to other HFAs supplemented groups (Table 4). The partitioning factor varied (P<0.01) from 1.91 mg/ml (patha supplemented diet) to 2.31 mg/ml (diet supplemented with aritha). However, the PF for a given feedstuff can vary with the incubation time partly because of the dynamics of microbial growth. The differences amongst herbs can be attributed to nature of saponin in different herbs.

With the increase in level of supplementation of herbal feed additive, the NGP and availability of ME from diet increased linearly (P<0.01). The digestibility of NDF and that of the true OM also increased (P<0.01) linearly up to 2% level of supplementation, clearly indicating the potential of saponin containing herbs at optimum level in manipulating rumen fermentation.

The type of feed offered to a ruminant can have a major effect on rumen fermentation. The types of diet are potential modifiers of ruminal fermentation and may offer a strategy to reduce protozoal and methanogen populations, thus improving the efficiency of feed utilization in the ruminants (Anantasook and Wanapat 2012). With the increase in level of concentrate in TMRs, the NGP, digestibility of OM and availability of ME increased linearly (P<0.01). However, the digestibility of NDF decreased (P<0.01) with increase in level of concentrate in TMRs. The partitioning factor was not affected by increase in level of concentrate in the TMRs.

The end-product of anaerobic microbial fermentation of carbohydrates in the rumen of ruminants has been reported to be VFAs, carbon dioxide and methane (Camero and Franco 2001). The total VFAs, propionate, acetate, butyrate and that of isobutyrate concentration was highest (P<0.01) from the diet supplemented with kuthi (Table 5), irrespective of the level of HFA and the R:C ratio of diet. The perusal of data on fermentation pattern revealed that total and individual VFAs were highest (P<0.01) when the diet was supplemented at 2% level, irrespective of the type of herb and the type of diet used. The A:P ratio was also the lowest (P<0.01) in the diet supplemented with HFAs at 1%, comparable with that supplemented at 2% level, but lower (P<0.01) than control and the diet supplemented with HFA at 3% level. Indeed, total and individual VFAs increased with high-concentrate level as compared with high-forage level. Unlike many studies, the total VFAs, acetate, propionate concentration decreased (P<0.01) linearly, while that of butyrate increased (P<0.01) linearly with the increase in concentrate mixture in the TMR, irrespective of type and level of HFA supplemented.

The molar proportions of acetate was higher (P<0.01) in diet supplemented with kulthi (70.1%), while that of propionate butyrate and isobutyrate was higher (P<0.01) in diet supplemented with aritha (Table 6) as compared to other HFA supplemented TMRs, irrespective of the level of supplementation and type of TMR. This agrees with the report of Dung *et al.* (2011) who reported that, even though volatile fatty acids contribute about 70% of the caloric

Table 4. Effect of herbs, their level of supplementation and type of TMRs on *in vitro* gas production and digestibility of nutrients at t½

Parameter	Herbal feed additives[1]			PSE	Levels of HFA[2] (%)				PSE	Roughage to Concentrate ratio[3]				PSE
	Kulthi	Patha	Aritha		Control	1	2	3		80:20	75:25	70:30	65:35	
NGP (ml/g DM)**	97.7^{b}	101.7^{c}	81.8^{a}	0.56	90.8^{a}	91.4^{a}	95.1^{b}	97.6^{c}	0.64	91.2^{a}	94.1^{b}	95.7^{b}	93.89^{b}	0.64
NDFD (%)**	28.1^{a}	31.7^{c}	28.9^{b}	0.08	27.2^{a}	30.1^{b}	30.7^{c}	30.2^{b}	0.09	32.0^{d}	30.2^{c}	29.5^{b}	26.44^{a}	0.09
TOMD (%)**	54.7^{a}	56.9^{c}	55.3^{b}	0.05	54.1^{a}	56.0^{b}	56.4^{c}	56.0^{b}	0.06	52.9^{a}	54.7^{b}	57.2^{c}	57.78^{d}	0.06
PF** (mg/ml)	1.97^{a}	1.91^{a}	2.31^{b}	0.04	2.11	2.10	2.07	1.97	0.04	2.12	2.03	2.06	2.04	0.04
ME** (MJ/kg DM)	6.5^{b}	6.6^{c}	6.1^{a}	0.02	6.4^{a}	6.4^{ab}	6.5^{bc}	6.5^{c}	0.03	6.0^{a}	6.4^{b}	6.6^{c}	6.8^{d}	0.03

Figures with different superscripts in row differ significantly (**P<0.01); [1]Irrespective of level of supplementation and type of diet; [2]Irrespective of type of herb and diet, of supplementation; [3]Irrespective of level of supplementation and type of herb.

Table 5. Total volatile fatty acids (TVFAs) production from fermentation of TMRs

Parameter	Herbal feed additives[1]			PSE	Levels of HFA[2] (%)				PSE	Roughage to Concentrate ratio[3]				PSE
	Kulthi	Patha	Aritha		0	1	2	3		80:20	75:25	70:30	65:35	
TVFA**	4.5^{c}	4.01^{b}	3.75^{a}	0.01	4.13^{c}	3.96^{a}	4.20^{d}	4.08^{b}	0.01	4.13^{b}	4.13^{b}	4.05^{a}	4.07^{a}	0.01
Acetate**	3.2^{c}	2.78^{b}	2.52^{a}	0.003	2.85^{c}	2.74^{a}	2.91^{d}	2.80^{b}	0.003	2.86^{c}	2.84^{b}	2.80^{a}	2.81^{a}	0.003
Propionate**	0.91^{c}	0.82^{a}	0.83^{b}	0.001	0.85^{b}	0.82^{a}	0.88^{d}	0.87^{c}	0.001	0.87^{c}	0.86^{b}	0.85^{a}	0.85^{a}	0.001
Isobutyrate**	0.022^{b}	0.014^{a}	0.017^{a}	0.001	0.016	0.017	0.018	0.019	0.002	0.017	0.021	0.017	0.015	0.002
Butyrate**	0.36^{c}	0.32^{b}	0.32^{a}	0.001	0.334^{b}	0.319^{a}	0.342^{c}	0.335^{b}	0.001	0.330^{ab}	0.332^{b}	0.328^{a}	0.340^{c}	0.001
Isovalerate	0.041	0.039	0.038	0.007	0.057	0.032	0.035	0.033	0.008	0.032	0.056	0.034	0.036	0.008
Valerate	0.025	0.028	0.023	0.004	0.023	0.030	0.027	0.023	0.004	0.025	0.030	0.023	0.025	0.004
A:P**	3.49	3.38	3.02	0.002	3.33^{c}	3.31^{b}	3.31^{b}	3.22^{a}	0.002	3.29^{a}	3.31^{b}	3.29^{a}	3.29^{a}	.002

Figures with different superscripts in row differ significantly (**P<0.01), [1]Irrespective of level of supplementation and type of diet; [2]Irrespective of type of herb and diet, of supplementation, [3]Irrespective of level of supplementation and type of herb.

requirement of ruminants, the nutrients in the diets (supplemented or un-supplemented) undoubtedly affects the amount of volatile fatty production in the rumen. The relative proportion of acetate, valerate and that of BCFAs was not affected by level of supplementation of HFAs (Table 6). However, the proportion of propionate and that of butyrate increased, with increase in level of supplementation HFAs, irrespective of type of herb and type of TMR.

Hydrogen recovery was highest (P<0.01) when diet was supplemented with aritha, but hydrogen consumed via methane/VFA was highest when diet was supplemented with patha (Table 7). In this study, supplementation of diet with different saponin containing HFAs, irrespective of the nature of diet and level of supplementation of herbs, the fermentation efficiency was highest (P<0.01) in diet supplemented with aritha and lowest from diet supplemented with kulthi. VFA utilization index (NGGR) varied (P<0.01) from 3.71 (diet supplemented with aritha) to 4.18 (diet supplemented with kulthi). The non-glucogenic to glucogenic VFA ratio is associated with effects on methane production, milk composition, and energy balance (Morvay *et al.* 2011). Glucogenic propionate contributes to energy deposition in body tissues, whereas nonglucogenic acetate and butyrate are sources for long-chain fatty acid synthesis. Higher NGR was related to a higher milk fat content (Abrahamse 2009). Too high NGR indicates a high loss of energy in the form of gases (Ørskov 1975). The type of saponin present in herb could be the reason for different response.

The supplementation of diet with saponin containing herbs at different levels, irrespective of type of herb and that of diet, H recovery, ratio of H consumed via methane to H via VFA increased (P<0.01) with increase in level of supplementation and these parameters were highest at 3% level of supplementation. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved when diet was supplemented at 3% on DM basis, irrespective of type of herb used and the substrate used. Fermentation efficiency was also highest when diet was supplemented at 3% on DM basis.

Hydrogen consumption via methane or via VFA and fermentation efficiency was lowest in high concentrate diet. The VFA utilization index decreased with increase in level of concentrate in the diet (Table 7).

Herbs containing saponins were evaluated for their anti-methanogenic properties also and the data revealed that the methane production (as % NGP, ml/100 mg DM/DMD/OMD) was lowest (P<0.05) in diet supplemented with patha in comparison to diet supplemented with other HFAs (Table 8). The type of saponin present in herb could be the reason for different response. The presence of different substituents in the sapogenin such as hydroxyl, hydroxymethyl, carboxyl, and acyl groups, as well as differences in the composition, linkage and number of sugar chains accounts for significant structural variation and thus their bioactivity (Patra and Saxena 2009, Podolak *et al.*

Table 6. Relative proportion of volatile fatty acids as affected by supplementation of saponin containing herbs, level of supplementation and type of diet

Parameter	Herbal feed additives[1]			PSE	Levels of HFA[2] (%)				PSE	Roughage to Concentrate ratio[3]				PSE
	Kulthi	Patha	Aritha		0	1	2	3		80:20	75:25	70:30	65:35	
Acetate**	70.07^{c}	69.43^{b}	67.10^{a}	0.14	68.89	68.91	69.05	68.61	0.16	69.12	68.61	69.00	68.72	0.16
Propionate**	20.14^{a}	20.54^{b}	22.26^{c}	0.04	20.77^{a}	20.91^{a}	20.91^{a}	21.33^{b}	0.05	21.08^{b}	20.84^{a}	21.06^{b}	20.94^{ab}	0.05
Isobutyrate**	0.44^{b}	0.31^{a}	0.46^{b}	0.03	0.34	0.43	0.38	0.46	0.04	0.41	0.42	0.39	0.39	0.04
Butyrate**	7.88^{a}	8.06^{b}	8.54^{c}	0.02	8.10^{a}	8.15^{ab}	8.18^{b}	8.22^{b}	0.02	8.02^{a}	8.07^{ab}	8.12^{b}	8.44^{c}	0.02
Isovalerate	0.92	0.95	1.03	0.16	1.36	0.84	0.84	0.82	0.18	0.78	1.33	0.84	0.91	0.18
Valerate	0.56	0.71	0.62	0.09	0.55	0.76	0.64	0.57	0.10	0.59	0.74	0.58	0.62	0.10

Figures with different superscripts in row differ significantly (**P<0.01), [1]Irrespective of level of supplementation and type of diet; [2]Irrespective of type of herb and diet, of supplementation; [3]Irrespective of level of supplementation and type of herb.

Table 7. Fermentation parameter, hydrogen balance of TMRs supplemented with saponin containing herbs, level of supplementation and type of diet

Parameter	Herbal feed additives[1]			PSE	Levels of HFA[2] (%)				PSE	Roughage to Concentrate ratio[3]				PSE
	Kulthi	Patha	Aritha		0	1	2	3		80:20	75:25	70:30	65:35	
HR (%)**	99.7^{a}	109.5^{b}	114.7^{c}	0.18	106.6^{a}	107.7^{b}	107.6^{b}	110.0^{c}	0.20	106.3^{a}	112.8^{b}	106.0^{a}	106.7^{a}	0.20
HC, *via* CH_4/VFA**	2.40^{a}	2.65^{c}	2.54^{b}	0.006	2.49^{a}	2.53^{b}	2.52^{b}	2.58^{c}	0.007	2.51^{b}	2.69^{c}	2.48^{b}	2.45^{a}	0.007
FE (%)**	73.48^{a}	73.70^{b}	74.56^{c}	0.004	73.91^{b}	73.89^{a}	73.92^{c}	73.94^{c}	0.005	73.84^{a}	73.88^{b}	73.87^{b}	74.06^{c}	0.005
VFAUI**	4.18^{c}	4.07^{b}	3.71^{a}	0.01	3.97	3.98	3.98	4.01	0.01	4.03^{c}	3.99^{b}	4.00^{bc}	3.92^{a}	0.01

H, Hydrogen; R, Recovery; C, Consumed; F, Fermentation; E, Efficiency; U, Utilization; I, Index; Figures with different superscripts in a row differ significantly (**P<0.01); [1]Irrespective of level of supplementation and type of diet; [2]Irrespective of type of herb and diet, of supplementation; [3]Irrespective of level of supplementation and type of herb.

Table 8. Methane production from TMRs supplemented with saponin containing herbs

Parameter	Herbal feed additives[1]			PSE	Levels of HFA[2] (%)				PSE	Roughage to Concentrate ratio[3]				PSE
	Kulthi	Patha	Aritha		0	1	2	3		80:20	75:25	70:30	65:35	
CH_4 (% of NGP) **	46.1^{b}	35.1^{a}	47.6^{b}	0.68	44.7^{b}	40.9^{a}	40.9^{a}	45.2^{b}	0.79	41.5^{a}	47.4^{b}	42.4^{a}	40.4^{a}	0.79
CH_4 (ml/100 mg DMD)**	8.82^{c}	6.84^{a}	7.46^{b}	0.06	8.00^{b}	7.27^{a}	7.29^{a}	8.28^{c}	0.08	7.48^{b}	8.76^{c}	7.61^{b}	6.98^{a}	0.08
CH_4 (ml/100 mg OMD)**	4.72^{c}	3.70^{a}	4.12^{b}	0.07	4.22^{a}	3.98^{a}	3.94^{a}	4.58^{b}	0.08	3.86^{a}	4.73^{c}	4.16^{b}	3.98^{ab}	0.08
CH_4 (ml/100 mg DM)**	4.80^{c}	3.89^{a}	4.12^{b}	0.04	4.29^{b}	4.07^{a}	4.11^{a}	4.60^{c}	0.04	3.95^{a}	4.76^{c}	4.35^{b}	4.01^{a}	0.04

Figures with different superscripts in row differ significantly (**P<0.01); [1]Irrespective of level of supplementation and type of diet; [2]Irrespective of type of herb and diet, of supplementation, [3]Irrespective of level of supplementation and type of herb.

2010). The CH_4 production was lowest when diet was supplemented with 1 or 2% HFA on dry matter basis. It was also lowest in diet containing high amount of concentrate, irrespective of level of supplementation and type of saponin containing HFA. Yan *et al.* (2000) reported the negative correlation between proportion of concentrate in diet and methane emissions. Goel *et al.* (2008) also reported that methane suppressing effects of saponins from *Sesbenia sesban* and fenugreek were profound in concentrate-based diets compared to roughage based diets.

It can be concluded that saponin containing herbal feed additives like kulthi and patha has great potential in mitigating enteric methane production when supplemented @ 2% on dry matter basis in high concentrate based diet (roughage to concentrate ratio of 65:35).

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