Effect of strategic supplementation of phyto-feed additives on enteric methane emission and performance of river buffalo calves

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Abstract

A feeding trial of 180 days duration conducted on river buffalo calves following strategic feeding of phyto-feed additives (PFAs) to assess their ability to reduce enteric methane production. Twenty male buffalo calves (average body weight, 165±4 kg) divided into four groups using completely randomized design, were subjected to four treatments viz.; basal diet (T0, control), basal diet with PFAI (*Allium sativum* bulb, *Trachyspermum ammi* seeds, *Terminalia chebula* seed pulp and *Sapindus mukorossi* nut pulp, mixed in equal proportion) at the rate of 1% of dry matter intake (T1), basal diet with PFAII (*T. ammi* oil) at the rate of 1ml per kg DMI (T2) and basal diet with PFAs where PFAI was offered for 15 days thereafter PFAII for the next 15 days and the switching of the two PFAs continued throughout the experiment (T3). The treatments were formulated to compare the effects of different PFAs on growth, nutrient utilization and methane mitigation in buffalo calves. T3 was designed to examine the possible synergistic effect and reduction in microbial adaptation through alternate use of both additives. All the calves fed on a diet consisting of wheat straw and concentrate mixture in 50:50 ratios. Feeding of PFAI and II did not affect DMI, nutrients digestibility and growth performance of the animals. The methane production was reduced ($P<0.05$) by 20.2, 19.1 and 25.9 (l/kg DMI) and 24.5, 19.6 and 29.5% (l/kg digestible DMI) in T1, T2 and T3 group respectively, as compared to control. The methane energy loss as percent of gross energy intake was lesser ($P<0.07$) in PFA supplemented groups as compared to control group. All the animals were in positive nitrogen balance with no difference among the groups. The blood haematology and bio-chemicals were similar in all the groups. Interestingly, the anti-methanogenic characteristic of both the PFAs persisted up to six months of feeding but the alternate feeding of the PFAs did not have any additional advantage on the enteric methane reduction in buffaloes.

Key words: Methane; Phyto-feed additives; Buffalo calves; Nutrient utilization; Growth

Introduction

Reduction of enteric methane emission is a great challenge linked with livestock farming. Despite its contributions to food security and job creation for rural households, animal husbandry has environmental impact. Methane, a potent green house gas, has a global warming potential 28 times than that of carbon dioxide and expected to rise by 86 percent over the next two decades (Molina-Botero et al., 2019). Buffaloes are important source of milk and meat in developing and under developed countries (Gautam et al., 2021; Dhaware et al., 2024; Alshdaifat et al., 2024). Methane emissions from Indian livestock were estimated at around 12.74 Tg annually, with over 92% of these emissions coming from enteric fermentation, particularly in dairy cattle and buffalo. This makes India one of the largest methane emitters globally in terms of livestock agriculture. (Samal et al., 2024). Methane production in the rumen during carbohydrate fermentation represents inefficiency in energy utilization (loss of 2-12 percent of gross energy intake) that has a negative impact on the livestock owner's economy (Rooke et al., 2014). Therefore, it is imperative to develop strategies, maintaining a balance between minimizing environmental impacts and intensifying animal productivity to meet requirements of animal protein of the world population in the years to come (Richards et al., 2018). Among the existing methane mitigation strategies for ruminants include various feed additives like ionophores, fats, organic acids, antibiotics and anti-methanogens, phyto-feed additives is the most favoured one. The phyto-feed additives (PFAs) are the naturally occurring secondary compounds present in the plants and used in traditional medicine since long back worldwide. PFAs improve the breakdown of fiber in the rumen, enhancing nutrient absorption and feed efficiency. Essential oils stimulate the production of digestive enzymes and promote better fibre utilization (Kholif and Olafadehan, 2021). PFAs help reduce methane emissions by altering rumen microbes, improving feed efficiency and lowering livestock's environmental impact. Saponins from Yucca reduce methane production, while beta-glucans boost immune responses making animals more resistant to diseases (Karásková et al., 2015). Essential oils like thyme and star anise enhance growth rates and improve meat quality in livestock (Kholif et al., 2021). The plant parts like bulb of *A. sativum* rich in various organo-sulphur compounds is most commonly used remedy for various diseases due to its antibacterial, antifungal and immune enhancing properties (Kamra et al., 2012, Beshbishy et al., 2020) and because of these properties it is also explored for improving production performance and methane mitigation from ruminants (Sari et al., 2022, Khurana et. al., 2023). Harad, a part of *T. chebula* is rich in tannins and the anti-methane ability of tannins has been documented previously (Patra et. al., 2011, Magnani et al., 2023). Saponin, a popular anti-protozoal agent is also a very common component of phytogenic feed additives (Magnani et al., 2023). The berries of *S. mukurossi*, a rich source of saponins is a potent methane inhibitor (Agarwal et al., 2006). The third most common PFA, essential oils are widely used to improve animal productivity along with reduced methanogenesis and among them *T. ammi* seeds and oil are widely used (Kundan et al., 2021; Pawar et al., 2021,). The *T. ammi* seeds are rich source of macro and micronutrients, tannins, saponins, flavonoid etc and the essential extracted from seeds includes thymol, p-cymene, c-terpinene, and α- and β-pinene. Both seeds and oil are of medicinal value showing excellent health benefits (Asif and Hashmi 2020). The mode of action of different categories of PFAs is different, thereby combinations of these PFAs are strongly suggested (Magnani et al., 2023). The aim study aimed at investigation of the effect of PFAs on performance, digestibility and methane emission in river buffalo calves over a period of six month.

Material and methods

The study was conducted at ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Uttar Pradesh (India). All experimental protocols were approved (protocol number IAEC/26.07.2021/L4 dated 12.01.2022) as per the guidelines established by Institutional Animal Ethics Committee constituted (IAEC) under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India vide letter no. V-11011 (13)/19/2021- CPCSEA-DADF dated 07.01.2022.

Table 1. Experimental treatment details

Treatment	Description
T0	Control (basal diet without phytogenic feed additive)
T1	Basal diet + PFAI @ 1% of DMI
T2	Basal diet + PFAII @ 1 mL/kg DMI
T3	Basal diet + alternate supplementation of PFAI and PFAII at 15-day intervals throughout the experiment

T0, control; T1, PFAI (blend of garlic, ajwain, harad and soapnut in equal proportion) at the rate of 1% DMI; T2, PFAII (ajwain oil) at the rate of 1ml/kg DMI; T3, PFAI and PFAII alternatively for 15 days each

Experimental design and animal management

A feeding trial of 180 days was executed on twenty male river buffalo calves, 14±2-month-old, with average body weight of 165±4 kg using PFAs. The animals were divided into four groups of five animals each using completely randomized design, were assigned four treatments viz., T0, control; T1 with PFAI at the rate of 1% of DMI; T2 with PFAII at the rate of 1ml/kg DMI and T3 with PFAI and PFAII alternatively (PFAI was offered for 15 days thereafter PFAII for the next 15 days and the switching of the two PFAs continued throughout the experiment). Animals were fed individually as per ICAR (2013) feeding standards targeting the growth rate of 500 g/d. The concentrate mixture and wheat straw were fed in 1:1 ratio on DM basis. The concentrate mixture comprised of maize 35, soyabean meal 24, wheat bran 38, mineral mixture 2 and salt 1 part. The additives PFAI was a powder of *Allium sativum* bulb (garlic), *Trachyspermum ammi* seeds (ajwain), *Terminalia chebula* pulp (harad) and *Sapindus mukurossi* nut (soapnut), mixed in equal proportion and PFAII was *T. ammi* essential oil. The four plant parts used in PFAI were dried, powdered and then mixed in equal proportion. The PFAs were mixed well with the concentrate mixture daily for entire experimental period and ensured the complete consumption of conc. mix. Chopped wheat straw was offered after the concentrate mixture was completely consumed. To meet vitamin A (carotene) requirement, 5 kg chopped green maize fodder on fresh basis per animal was provided once a week. Fresh and clean drinking water made available *ad libitum*.

Feed intake and growth performance

Daily feed offered and orts were analysed for dry matter and dry matter intake was calculated by subtracting orts dry matter from dry matter of feed offered. All the animals weighed individually at every fortnight before feeding and watering using an electronic balance. The feed offered and the PFAs quantity was adjusted according to weight change. The body weight gain was calculated by subtracting final body weight (kg) from initial body weight.

Metabolism trial

At the end of the experimental feeding, all the animals were shifted in metabolic cages to conduct a metabolism trial. The metabolism trial was of 8 days duration including 2 days adaptation for the new environment followed by 6 days collection. The feed offered, orts, faeces and urine were collected discretely. Total faeces and urine excreted in 24 hours (h) by the particular animal were collected quantitatively in marked plastic bags. After mixing a suitable aliquot of fresh faeces was taken for the dry matter (DM) estimation. Another aliquot of faeces was taken in acidified sulphuric acid (25%) for the estimation of nitrogen. The container for urine collection contained 20% sulphuric acid to protect the loss of ammonia. The appropriate aliquots of feed offered, orts, faeces and urine were pooled animal wise and preserved for further analysis. The samples of feed and faeces were ground to pass through a 1 mm sieve and analysed for DM (method 930.15), ash (method 942.05), crude protein (CP) (N×6.25, method 954.01), and ether extract (EE) (ID number 920.39) as per AOAC (1995) and organic matter (OM) calculated. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) content were analysed as per Van Soest et al. (1991). Gross energy of feed, orts, urine and feaces was measured by Gallenkamp ballistic bomb calorimeter (Gallenkamp, C.B.370, UK) using benzoic acid as standard, according to the manufacturer's manual.

Methane production and energy partitioning

Methane emission was measured in an open circuit respiration chamber maintained at 25°C and 65% relative humidity. The chamber size of length x width x height 3.05x1.92x2.71m was locally fabricated under the guidance of experts from England under a collaborative project. The buffalo calves were shifted in the chambers and after two days adaptation, methane was evaluated for two consecutive days. Air flow rate of 250 l/min (measured by a flow meter, Thori Analytical and Instrumentation, Pune, India) was maintained and the amount of total volume of air passed through the chamber was measured. The concentration of methane in air going in and coming out of the chamber was measured by infrared methane analyser (Analytical Development Co. Ltd, Hoddesdon, England, Model 300). The chamber opened every 22 h to offer feed and to collect residues of feed and faeces. The energy intake and energy lost to feces, urine, and methane were used to calculate energy balance, following Agricultural Research Council.

Haemato-biochemical analysis

Blood collected at initial and final day of the feeding trial through jugular vein. The serum biochemical parameters (glucose, total protein, albumin, globulin, A:G ratio, urea, creatinine, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were estimated using commercial diagnostic kits (Coral Clinical Systems; Geno Biosciences Pvt. Ltd., India) as per manufacturer recommendations.

Statistical analysis

The experiment was conducted in completely randomized design. The data of feed intake, growth and metabolic trial were analysed by applying one way ANOVA. Data of blood indices were subjected to two-way analysis of variance using General Liner Model Multivariate (GLM) to find out the effect of treatment, period and their interaction using SPSS (2010) computer package applying the following model.

$$Yijk = i + T_i + P_j + (T*P)_{ij} + e_{ijk}$$

Yijk = The individual observation, i = the overall mean, T_i = the effect of treatment ($_i$ = 1, 2, 3 and 4), P_j = the effect of period ($_j$ = 1 and 2), $(T*P)_{ij}$ = effect of interaction between treatment and period ($_{ij}$ = 1......8), e_{ijk} = Random error associated with individual

The means were compared by the Duncan's test and the differences were declared significant at P<0.05 and considered as trend at 0.05<P<0.10.

Results

Chemical composition of feeds

The chemical composition of feed and PFAs is presented in Table 2. The CP content of the concentrate mixture, wheat straw and PFA was 20.25, 3.61, and 10.72 percent, respectively. The concentrate mixture had sufficient nutrients to meet the requirement of targeted 500 g daily weight gain.

Table 2. Chemical composition of the diet offered to the animals

Attributes	Concentrate	Wheat straw	PFAI
Organic matter	92.5	92.8	92.3
Crude protein	20.2	3.61	10.7
Ether extract	3.03	1.18	3.71
Neutral detergent fibre	25.9	80.3	42.3
Acid detergent fibre	6.49	53.1	21.5
Total ash	7.45	7.11	7.64

Table 3. Effect of phyto-feed additives (PFAs) on growth and feed conversion efficiency of growing buffalo calves

Attributes	T0	T1	T2	T3	SEM	P value
Initial BW (kg)	164	164	165	166	3.71	0.99
Final BW (kg)	254	259	258	262	6.29	0.98
Net BW gain (kg)	90.3	95.1	92.6	95.9	3.57	0.95
ADG (g)	502	528	515	533	19.8	0.95
Average DMI (kg)	5.02	5.07	5.12	5.10	0.023	0.74
Concentrate DMI (kg)	2.20	2.20	2.20	2.20	-	-
WS DMI (kg)	2.82	2.88	2.90	2.89	0.03	0.64
Feed conversion ratio	10.03	9.72	9.85	9.98	0.39	0.99

T0, control; T1, PFAI (blend of garlic, ajwain, harad and soapnut in equal proportion) at the rate of 1% DMI; T2, PFAII (ajwain oil) at the rate of 1% DMI; T3, PFAI and PFAII alternatively for 15 days each; SEM., standard error of mean; BW, body weight; ADG, average daily gain

Body weight gain, feed intake and feed conversion efficiency

Changes due to supplementation of PFA in body weight gain, dry matter intake (DMI) and feed conversion ratio (FCR) are presented in Table 3. The DMI increased in all the groups with advancing age of the animals with no statistical difference among the groups. The average daily gain ranged between 502 to 533 g/d in all the four groups with no significant difference resulting in similar final body weight. The daily weight gain was as per expectation as the diet formulated for the feeding the animals was targeted for 500 g gain per day. Feeding of PFA did not affect FCR of the animals and ranged from 9.72 to 10.03 in the four groups.

Nutrient digestibility and plane of nutrition

During metabolic trial, the average body weights of the four groups were similar. The DMI and digestibility of DM, OM, EE, CP, NDF and ADF were comparable among the four groups (Table 4). The digestible crude protein (DCP) and total digestible nutrients (TDN) intake was also similar among the four groups indicating that during metabolic trial, the animals were in similar plane of nutrition.

Methane emission, energy and protein metabolism

Effects of supplementation PFAs on methane emission is given in Table 5. DMI was comparable among the four groups. The GE, DE and ME intake was similar in all the groups. The methane production in terms of l/d showed trend of lowering (P=0.088) and was reduced by 15, 12 and 18 percent in groups T1, T2 and T3 respectively, as compared to T0. The methane production (l/kg DMI) decreased by 20.2, 19.1 and 25.9 percent in T1, T2 and T3 group, respectively, whereas, when expressed as l/kg DDMI, the reduction (P<0.05) was 24.5 and 29.5 percent in T1 and T3 as compared to T0 (control). In T2 group the methane production (l/kg DDMI) was 19.6 percent less than the control but the difference was non-significant. The PFA did not influence energy partitioning, however, the methane energy loss as percent gross energy intake showed lowering trend (P=0.072) and was less by 16.3, 12.4 and 18.5 percent in T2, T3 and T4 as compared to control animals (Table 5). However, the animals of all the four groups were in positive energy balance resulting in similar growth rate in all the animals. The nitrogen intake and losses through feces and urine were similar in all the four groups. The animals were in positive nitrogen balance and was enough for supporting 500g/d body weight gain (Table 4).

Table 4. Effect of phyto-feed additives (PFAs) on apparent nutrients digestibility (%) and nitrogen balance in buffalo calves

Attributes	T0	T1	T2	T3	SEM	P value
DMI (kg/d)	5.68	5.77	5.71	5.76	0.17	0.243
Body weight (kg)	253.4	259.8	254.9	255.9	6.18	0.989
Nutrients digestibility (%)						
Dry matter	63.4	65.1	64.4	64.9	0.96	0.93
Organic matter	65.7	67.6	66.8	67.1	0.88	0.92
Crude protein	63.2	63.7	62.6	64.7	0.82	0.85
Ether extract	71.7	74.2	73.23	77.3	1.49	0.65
Neutral detergent fibre	61.1	63.5	60.8	62.9	3.40	0.85
Acid detergent fibre	47.0	49.3	49.4	49.8	1.44	0.92
Plane of nutrition						
DCP intake (g/d)	369	374	366	379	4.64	0.78
TDN intake (kg/d)	3.59	3.74	3.66	3.71	0.04	0.69
Nitrogen balance						
N intake (g/d)	93.5	93.9	93.6	93.9	0.10	0.29
N voided in faeces (g/day)	35.3	34.1	35.1	33.2	0.79	0.80
N loss in urine (g/d)	36.6	37.3	38.2	38.1	0.83	0.92
Total N loss (g/d)	71.9	71.4	73.2	71.3	1.07	0.93
N balance (g/d)	21.6	22.6	20.4	22.7	1.04	0.89
N intake (g/d)	93.5	93.9	93.7	93.9	0.10	0.29
N voided in faeces (g/day)	35.3	34.1	35.1	33.2	0.79	0.80

T0, control; T1, PFAI (blend of garlic, ajwain, harad and soapnut in equal proportion) at the rate of 1% DMI; T2, PFAII (ajwain oil) at the rate of 1ml/kg DMI; T3, PFAI and PFAII alternatively for 15 days each.

Table 5. Effect of phyto-feed additives (PFAs) on methane emission, energy partitioning and nitrogen metabolism in buffalo calves

Attributes	T0	T1	T2	T3	SEM.	P-value
Body weight (kg)	208	228	228	197	11.7	0.79
DMI (kg/d)	4.10	4.23	4.41	4.53	0.10	0.67
Methane emission						
Methane (l/d)	149	126	131	123	9.81	0.08
Methane (l/kg DMI)	36.7^{a}	29.3^{b}	29.7^{b}	27.2^{b}	1.30	0.04
Methane (l/kg DDMI)	62.9^{a}	47.5^{b}	50.5^{ab}	44.3^{b}	2.70	0.02
Energy metabolism						
GE intake (MJ/d)	94.8	95.9	95.3	95.9	0.21	0.53
FE loss (MJ/d)	35.9	29.6	31.7	34.5	1.88	0.25
DE (MJ/d)	58.1	66.1	63.7	61.4	1.88	0.19
UE loss (MJ/d)	5.02	5.48	5.02	4.14	0.29	0.47
CH_4 GE loss (MJ/d)	5.90	4.98	5.19	4.85	0.39	0.09
CH_4 loss as % of GEI	6.20	5.19	5.43	5.05	0.39	0.07
ME (MJ/d)	47.2	55.6	53.5	52.4	4.70	0.15
HP (MJ/d)	33.9	39.4	36.9	36.9	1.97	0.12
Net energy (MJ/d)	13.3	16.2	16.6	15.4	1.31	0.47

T0, control; T1, PFAI (blend of garlic, ajwain, harad and soapnut in equal proportion) at the rate of 1% DMI; T2, PFAII (ajwain oil) at the rate of 1ml/kg DMI; T3, PFAI and PFAII alternatively for 15 days each; SEM., standard error of mean; DDMI, digestible dry matter intake; GEI, gross energy intake; FE: fecal energy; DE: digestible energy; UE: urinary energy; ME: metabolizable energy; HP: heat production; N, nitrogen

Haemato-biochemicals

The results of blood indices are presented in Table 6. The values of blood albumin, total protein, globulin, and blood urea nitrogen were similar in all the four groups. However, haemoglobin concentration increased with the age of the animals but the values were within the normal physiological range. The lipid profile, such as total cholesterol, HDL, LDL was also not affected ($P > 0.05$) by the PFAs supplementation. Likewise, the activities of enzymes namely LDH, ALP, ALT and AST were also not different in control and PFA supplemented groups.

Table 6. Effect of phyto-feed additives (PFAs) on hemato-biochemicals in growing buffalo calves

Attributes	T0	T1	T2	T3	SEM	T	P	T*P
Haemoglobin (g/dL)	11.7	11.5	11.5	11.4	0.21	0.97	0.04	0.64
PCV (%)	32.9	32.8	32.9	33.0	0.33	0.99	0.09	0.80
Glucose (mg/dL)	61.4	61.9	60.0	60.0	0.84	0.82	0.18	0.12
Total protein (g/dL)	7.37	8.16	7.77	7.89	0.15	0.33	0.75	0.28
Albumin (g/dL)	3.37	3.51	3.45	3.59	0.09	0.86	0.65	0.84
Globulin (g/dL)	4.00	4.66	4.32	4.30	0.146	0.48	0.54	0.48
Urea (mg/dL)	37.1	37.2	37.4	38.6	0.76	0.88	0.93	0.47
Cholesterol (mg/dL)	104	99.0	107	107	2.76	0.68	0.74	0.62
HDL (mg/dL)	34.6	35.0	35.6	36.5	0.96	0.91	0.20	0.92
LDL (mg/dL)	66.7	61.1	68.7	68.0	2.82	0.77	0.45	0.62
LDH(IU/L)	595	597	598	604	9.21	0.98	0.68	0.50
ALP(IU/L)	41.4	45.3	42.5	46.3	3.06	0.93	0.82	0.90
AST (IU/L)	103	98.7	100	98.8	2.00	0.86	0.70	0.98
ALT(IU/L)	27.8	28.6	28.2	28.0	0.65	0.97	0.87	0.91

T0, control; T1, PFAI (blend of garlic, ajwain, harad and soapnut in equal proportion) at the rate of 1% of DMI; T2, PFAII (ajwain oil) at the rate of 1ml/kg DMI; T3, PFAI and PFAII alternatively for 15 days each; T, treatment; P, period; T*P, treatment period interaction; SEM., standard error of mean; PCV, packed cell volume; HDL, high density lipid; LDL, low density lipid; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; AST, alanine amino transferase; ALT, aspartate amino transferase

Discussion

In the past, plant secondary metabolites (PSM) or phytochemicals like tannins, saponins, essential oils, flavonoids etc were considered toxic to the animals hence were categorized as anti-nutritional factors (Sarwar et al., 2012). But since last few decades, their beneficial effects on rumen fermentation have been recognized, and therefore, made them one of the most preferred choices as feed additives for altering rumen fermentation and improving feed utilization and health of the animals (Morsy et al., 2018). Because of specific antimicrobial characteristics, the plant parts either in the form of extract or powder, rich in tannins, saponins or essential oils also have ability to reduce enteric methane production (Patra et al., 2011; Jayanegara et al., 2020; Ahmad et al., 2021, Magnani et al 2023). However, most of these PSM have inconsistent efficacy and have detrimental effects on feed intake, digestibility and rumen fermentation when fed at higher doses (Patra et al., 2017). It was hypothesized that a PFA with combination of PSMs will reduce the level of a specific PSM or completely overcome their harmful effects and can maintain efficacy to improve livestock performance. Therefore, a combination of PFA prepared by mixing equal proportion of A. *sativum* (rich in organo-sulphur compounds) and *T. ammi* (rich in essential oils), *T. chebula* (rich in tannin) and *S. mukorossi* (rich in saponin) was evaluated for its impact on animal performance. Also, a third group was added where the two PFAs were fed alternately just to check whether such strategic feeding may give more beneficial impact on methane inhibition.

Intake, digestibility and plane of nutrition

The present findings revealed that the supplementation of PFAs to buffalo calves have not shown any effect on feed intake, nutrient digestibility and body weight gain in any of the treatment groups. This is an indication that both the PFAs at the dose levels, fed to the animals did not disrupt nutrient utilization and the animals performed in a normal manner. A PFAs prepared by mixing herbs comprising equal proportions of root of *Withania somnifera*, whole plant of *Boerhavia diffusa* and bark of *Holarrhena antidysenterica* when fed to the goat kids did not affect intake and digestibility of nutrients (Ingale et al., 2017). In addition, dietary supplementation of pods and legume foliage rich in condensed tannins and saponins had no effect on feed intake in crossbred heifers (Valencia et al., 2018). Kumar et al. (2011) reported no impact on nutrients intake and digestibility by feeding a combination of *Foeniculum vulgare* (fennel) seed (a source of essential oils), *T. chebula* seed pulp (a source of tannins), and leaves (source of tannins) of *Eugenia jambolana* (jamun), *Psidium guajava* (guava) and *Mangifera indica* (mango) at the rate of 40g/100 kg body weight) to buffaloes. Yatoo et al. (2018) also did not found any change in nutrients intake and digestibility in buffalo calves supplemented with blend of essential oils at the rate of 0.15 and 0.30 ml/kg DMI. The bioactive ingredients, including some essential oils, flavonoids and tannins could restrict DMI due to unpleasant or bitter taste, (Oliveira et al., 2010). But when these PFAs are used as combination, the level of individual bioactive ingredients reduces to minimum and also, they overshadow each other thereby neutralizing the harmful effects. The lack of consistency in the results in various studies might be due to the variability in the animal diet, type of phyto-feed additive, dose of feeding etc (Salem et al., 2014).

Methane emission, energy and protein metabolism

The current study showed that as compared to control, the methane production (l/kg DMI and l/kg DDMI) was significantly reduced in all the treated groups fed either PFAI or PFAII or the two PFAs fed alternately, however, PFAI was more effective than PFAII. The ME intake in all the four groups was as per the recommendation (about 50 MJ/d for the calf of 200 kg BW) of ICAR (2013). The results indicated that both the feed additives were effective in reducing methane production even after long term (180 days) continuous feeding. The percent inhibition (28% in T3) in methane production was higher by alternate feeding of both the PFA each at every 15 days as compared to daily individual PFA feeding (24.5% in T1 and 19.6% in T2) but the difference was not significant. The alternate supplementation strategy in T3 was designed to minimize possible rumen microbial adaptation by periodically changing the source of phytochemicals. However, the comparable methane reduction observed in T1 and T2 and the absence of significant difference between T1 and T3 suggest that microbial adaptation to these PFAs during long-term feeding was minimal. The non-significant difference despite numerically higher reduction in T3 may also be attributed to biological variability among animals. Patra et al. (2011) observed about 24% reduction in methane production (l/kg DDMI) in sheep fed on the diet supplemented with *T. chebula* (a rich source of tannins) or a mixture of *T. chebula* and *A. sativum* (a rich source of essential oils) and demonstrated no additional advantage in nutrient digestibility and methane reduction by mixing the two. However, Magnani et al (2023) advocated combination of tannins and saponin for methane mitigation in Nellore bulls. Tannins commonly found PSM in plants, are known to inhibit enteric methane production by their deleterious effect on methanogens, hydrogen producing bacteria and protozoa (Cardoso-Gluierrez et al., 2021) Likewise saponins are toxic to rumen protozoa and similarly, essential oils inhibit methane production through modifying rumen microbiome as reduction in abundance of protozoa and changes in the diversity of methanogen archea have been demonstrated (Ku-Vera et al., 2020). Kumar et al. (2020) reported 18% reduction in methane production in buffaloes by dietary supplementation of *T. ammi* seeds at the rate of 2% of DMI which is very similar to the present study where *T. ammi* oil was supplemented. Albores-Moreno et al. (2017) reported that the feeding of ground pods of *E. cyclocarpum* (saponins) resulted in 36 percent reduction in methane emission in Pelibuey sheep. Deuri et al. (2020) found that TMR supplemented with leaves of *M. indica, A. nilotica, P. guajava, C. fistula, E. globules* and *P. dactylifera* at 1% has good potential to decrease methane production. In the present study, methane was reduced in both T1 and T2 groups where PFAI and PFAII were fed continuously for 6 months, which indicate that there was no animal adaptation to these two PFAs and this might be the reason that by alternate feeding of these two PFAs, no additional advantage could be obtained though the methane reduction was numerically more.

Feeding the PFAs had no effect on faecal energy, urine energy or methane energy losses. There was no discernible difference in energy balance between the four groups. Similar results were reported by Samal et al. (2016) where, faecal energy, urinary energy and methane energy loss were not affected by feeding feed additives containing PSM in male buffalo calves. Pawar et al. (2014) also did not find any effect on percent methane energy loss by dietary supplementation of 2ml/d ajwain oil in buffalo calves. However, in present study PFA supplementation resulted in trend (P=0.072), in less methane loss as percent of gross energy intake in all the groups showing which indicates that the energy metabolism was not affected by PFAs but due to less methane production some amount of dietary gross energy was conserved. Patra et al. (2011) observed a trend (P<0.08) of decrease in methane energy loss as percent of digestible energy intake in sheep fed on diet supplemented with the mixture of *T. chebula* and *A. sativum.* Though in the present experiment, methane inhibition reached to 28% but the energy saved was not reflected in any improvement in animal performance. Beauchemin et al. (2020) suggested the animal performance can be improved only by more than 50% reduction in methane production, otherwise the sparing smaller amount of digestible energy is not sufficient enough to affect energy partition. The values of nitrogen intake, outgo and balance were in normal range and no difference among the groups indicates that the feeding PFAs did not alter nitrogen metabolism in buffalo calves.

Hemato-biochemicals

In veterinary medicine, serum metabolic profiles are widely applied for clinical health assessment of the animals. The data pertaining to haemoglobin (Hb), packed cell volume (PCV), glucose, total protein, albumin, globulin, or albumin: globulin ratio revealed no appreciable (P>0.05) differences among the four groups and all the values were in normal physiological range. Similarly, Pawar et al (2019) also did not find any change in blood indices in buffalo calves fed ajwain seed oil (2 ml/ d). The serum lipid profile was also similar in all the groups (P>0.05) and were within the normal range (Lakhani et al., 2019). Manju et al (2019) reported no effect on hemato-biochemicals by feeding 4% *Asparagus racemosus* or 3% *Bacopa monnieri* or 3% *Eclipta alba* and 3% *Leptadenia reticulate* to Marwari rams. Blood biochemical parameters were not affected with PFA supplementation in previous studies also (Toprak, 2020; Castro Filho et al. 2021), however, Hassan et al. (2013) reported the improvement in serum total protein, globulin and albumen/globulin ratio and the lower serum total cholesterol in buffalo calves fed *caraway* seed powder (2 g/kg diet), garlic powder (2g/kg diet) and combination of both (2 g/kg diet each). The lower serum cholesterol levels may be attributed to the pharmacological properties of Garlic and its bioactive organosulfur compounds, which inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key regulatory enzyme involved in cholesterol biosynthesis (Liang et al., 2025).

ALP, AST, ALT and LDH are serum enzymes that are commonly measured as biomarkers for organs disorder specifically liver disorder because their activity is highest in liver cells. The activity of serum enzymes was similar (P>0.05) among the groups and were within the normal range for bovines (Lakhani et al., 2019). Hossoda et al. (2019) by feeding peppermint or lemongrass or clove to steers, observed no change in blood biochemicals and the activities of ALT and AST, however, the level of total cholesterol, HDL and LDL increased in clove fed animals. This increase was attributed to some unknown components of clove, which stimulated the circulating concentration of total cholesterol.

Conclusion

The results are concluded, the dietary supplementation of PFAI (a blend of *Allium sativum* bulb, *Trachyspermum ammi* seeds, *Terminalia chebula* bulb and *Sapindus mukorossi*) at the level of 1% per kg DMI and PFAII (*Trachyspermum ammi* seeds oil) at the level of 1 ml per kg DMI have ability to reduce enteric methane emissions without affecting nutrient utilization and performance of buffalo calves.

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References

1) Alshdaifat, M.M., Al-Lataifeh, F., Al-Yacoub, A.N., Al-Barakeh, F., Al-Najjar, K. 2024. Multivariate of Physicochemical Properties of Daily Milk throughout Milking Seasons on Syrian Buffaloes (Bubalus Bubalis). Journal of Livestock Science 15: 46-52 doi.10.33259/JLivestSci.2024.46-52

2) Dhaware, S.A., Vaidya, M.S., Dongre, V.B., Jadhav, P.V. 2024. Polymorphism of receptor genes of Follicle Stimulating Hormone (FSHR), Luteinizing Hormone (LHR) and Estrogen receptor alfa in Marathwadi buffaloes. Journal of Livestock Science 15: 307-313 doi. 10.33259/JLivestSci.2024.307-313

3) Ahmed, E., Batbekh, B., Fukuma, N., Kand, D., Hanada, M. Nishida, T. 2021. A garlic and citrus extract: impacts on behaviour, feed intake, rumen fermentation, and digestibility in sheep. Animal Feed Science Technology, 278, 115007. https://doi.org/10.1016/j.anifeedsci.2021.115007

4) Albores-Moreno, S., Alayón-Gamboa, J.A., Ayala-Burgos, A.J., Solorio-Sánchez, F.J., Aguilar-Pérez, C.F., Olivera-Castillo, L. and Ku-Vera, J.C., 2017. Effects of feeding ground pods of *Enterolobium cyclocarpum* Jacq. Griseb on dry matter intake, rumen fermentation, and enteric methane production by Pelibuey sheep fed tropical grass. Tropical animal health and production, 49(4): 857-866.

5) AOAC. 1995. Method 991.42 & 993.19. Official methods of analysis (16th ed.) Washington, DC: Association of Official Analytical Chemists.

6) Asif, H.M. and Hashmi, H.A.S. 2020. Bioactive Compounds of Ajwain (*Trachyspermum ammi* [L.] Sprague). In: Bioactive Compounds in Underutilized Vegetables and Legumes (eds Murthy, H.N. and Paek, K.Y.), Cham: Springer International Publishing, 2021. 257-273 DOI: https://doi.org/10.1007/978-3-030-44578-2.

7) Beshbishy, M., Elewa, Y.H.A., Abd El-Hack, M.E. and Taha, A.E., 2020. Chemical Constituents and Pharmacological Activities of Garlic (Allium sativum L.): A Review. Nutrients, 12(3): E872-E872. https://doi.org/10.3390/nu12030872

8) Beauchemin, K. A., E. M. Ungerfeld, R. J. Eckard, and M. Wang. 2020. Review: fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. Animal. 14:S2‑S16. doi:10.1017/S1751731119003100

9) Cardoso-Gutierreza, E., Aranda-Aguirrea, E., Robles-Jimeneza, L.E., Castelán-Ortegaa, O.A., Chay-Canulb, A.J.,Foggic, G., Angeles-Hernandezd, J.C., Vargas-Bello-Péreze, E., and González-Ronquilloa, M. 2021. Effect of tannins from tropical plants on methane production from ruminants: A systematic review. Veterinary and Animal Science, 14:100214. ***https://doi.org/10.1016/j.vas.2021.100214***

10) Castro Filho, E.S., Júnior, L.C.R., Ezequiel, J.M.B., Salles, M.S.V., Almeida, M.T.C., Perez, H.L., Suguino, E., van Cleef, E.H.C.B., 2021. Effect of thyme essential oil supplementation on feed intake, apparent digestibility, rumen fermentation, blood parameters and *in vitro* methane yield of Nellore cattle. Livestock Science, 244: 104349. https://doi.org/10.1016/j.livsci.2020.104349

11) Gautam, S., Neupane, N., Dhital, B., Neupane, H., Bhatta, S.P. 2021. Status of cattle and buffalo farming in Banepa, Panchkal, Panauti of Kavrepalanchock district, Nepal. Journal of Livestock Science 12: 125-131. doi. 10.33259/JLivestSci.2021.125-131
12) Hosoda, K., Kuramoto, K., Eruden, B., Nishida, T., Shioya S. 2019. The effects of three herbs as feed supplements on blood metabolites, hormones, antioxidant activity, IgG concentration, and ruminal fermentation in holstein steers. Asian-Australasian Journal of Animal Sciences, 19: 35-41.
13) Hundal, J.S., Singh, I., Wadhwa, M., Singh, C., Uppal, C., Kaur, G., 2019. Effect of *Punica granatum* and *Tecomella undulata* supplementation on nutrient utilization enteric methane emission and growth performance of murrah male buffaloes. Journal of Animal and Feed Sciences, 28:110–119. https://doi.org/10.22358/jafs/109237/2019
14) ICAR. 2013. Nutrient requirements of cattle and buffalo. 3rd ed. Indian Council of Agricultural Research, New Delhi.
15) Ingale, S.L., Pattanaik, A.K., Baliyan, S., Kankoriya, S., Dutta, N., Sharma, K., 2017. Long-term effects of feeding a novel phytoadditive on nutrient utilization, growth performance, metabolic profile and antioxidant status of goats. Agricultural research, 6: 82-90.
16) Jayanegara, A., Yogianto, Y., Wina, E., Sudarman, A., Kondo, M., Obitsu, T., Kreuzer, M. 2020. Combination effects of plant extracts rich in tannins and saponins as feed additives for mitigating *in vitro* ruminal methane and ammonia formation. Animals, 10: 1531. https://doi.org/10.3390/ani10091531
17) Kamra D.N., Agarwal Neeta, Sakthivel P.C. and Chaudhary L.C. 2012. Garlic as a rumen modifier for eco-friendly and economic livestock production. Journal of Applied Animal Research, 40: 90-96. https://doi.org/10.1080/09712119.2011.607764
18) Karásková, K. Suchý, P. and Straková, E. 2015. Current use of phytogenic feed additives in animal nutrition: a review. Czech Journal of Animal Science, 60: 521-530. doi: 10.17221/8594-CJAS
19) Kholif, A.E. and Olafadehan, O.A. 2021. Essential oils and phytogenic feed additives in ruminant diet: Chemistry, ruminal microbiota and fermentation, feed utilization and productive performance. Phytochemistry Reviews, 20: 1087-1108.
20) Kholif, A.E. Anele, U.Y. Patra, A.K. and Varadyova, Z. 2021. The use of phytogenic feed additives to enhance productivity and health in ruminants. Frontiers in Veterinary Science, 8: 685262. https://doi.org/10.3389/fvets.2021.685262
21) Khurana, R., Brand, T., Tapio, L., Bayat, A-R. 2023. 2023. Effect of a garlic and citrus extract supplement on performance, rumen fermentation, methane production, and rumen microbiome of dairy cows. Journal of dairy science, 106: 4608-4621. https://doi.org/10.3168/jds.2022-22838
22) Kumar R., Kamra D.N., Agarwal N. and Chaudhary L.C. 2011. Effect of feeding plant part mixture and peppermint oil on rumen fermentation, microbial profile and nutrient digestibility in fistulated buffaloes. Indian Journal of Animal Sciences, 81: 488–92.
23) Kumar, K., Chaturvedi, V.B., Kala, A.,Chaudhary, L.C., 2020. Effect of feeding ajwain (*Trachyspermum ammi*) seed as a feed additive on enteric methane production and rumen ecosystem in Murrah buffaloes. Animal Nutrition and Feed Technology, 20: 257-266. 10.5958/0974-181X.2020.00023.2
24) Ku-Vera, J.C., Jiménez-Ocampo, R., Valencia-Salazar, S.S., Montoya-Flores, M.D., Molina-Botero, I.C., Arango, J., Gómez-Bravo, C.A., Aguilar-Pérez, C.F. and Solorio-Sánchez, F.J., 2020. Role of secondary plant metabolites on enteric methane mitigation in ruminants. Frontiers in Veterinary Science, 7:584. https://doi.org/10.3389/fvets.2020.00584
25) Lakhani, N., Kamra, D.N., Lakhani, P. and Alhussien, M.N., 2019. Immune status and haemato-biochemical profile of buffalo calves supplemented with phytogenic feed additives rich in tannins, saponins and essential oils. Tropical Animal Health and Production, 51(3): 565-573.
26) Liang, N., Liu, J.H., Li, Q., He, Z., Huang, X. and Chen, Z.Y., 2025. Roles of garlic and its bioactive compounds in modulating cholesterol metabolism. Phytomedicine, 157543. https://doi.org/10.1016/j.phymed.2025.157543
27) Magnani, E., Silva, T.H., Sakamoto, L., Manella, M.Q., Dias, F.M.G.N., Mercadante, M.E., Henry, D., Marcatto, J.O.S., Paula, E.M. and Branco, R.H. 2023. Tannin-based product in feedlot diet as a strategy to reduce enteric methane emissions of Nellore cattle finished under tropical conditions. Translational Animal Science, 7. https://doi.org/10.1093/tas/txad048
28) Manju, R.K. Dhuria, R.K. Khinchi, Padma M., Meel, M.S. 2019. Effect of herbs as feed additive on rumen fermentation patterns and haemato-biochemical parameters in marwari rams fed wheat straw based complete feed. International Journal of Livestock Research, 9(2): 32-40. Doi10.5455/ijlr.2018050707463.
29) Molina-Botero, I.C., Arroyave-Jaramillo, J., Valencia-Salazar, S., Barahona-Rosales, R., Aguilar-Pérez, C.F., Burgos, A.A., Arango, J., Ku-Vera, J.C., 2019. Effects of tannins and saponins contained in foliage of *Gliricidia sepium* and pods of *Enterolobium cyclocarpum* on fermentation, methane emissions and rumen

microbial population in crossbred heifers. Animal Feed Science and Technology, 251: 1-11. https://doi.org/10.1016/j.anifeedsci.2019.01.011
30) Morsy, T.A., Kholif, A.E., Matloup, O.H., 2018. Mustard and cumin seeds improve feed utilisation, milk production and milk fatty acids of Damascus goats. Journal of Dairy Research, 85: 142–151.
31) Oliveira, R.A., Narciso, C.D., Bisinotto, R.S., Perdomo, M.C., Ballou, M.A., Dreher, M., Santos, J.E.P. 2010. Effects of feeding polyphenols from pomegranate extract on health, growth, nutrient digestion, and immunocompetence of calves. Journal of Dairy Science, 93: 4280-4291. https://doi.org/10.3168/jds.2010-3314
32) Patra, A.K., Kamra, D.N., Bhar, R., Kumar, R. and Agarwal, N. 2011. Effect of *Terminalia chebula* and *Allium sativum* on in vivo methane emission by sheep. Journal of Animal physiology and Animal nutrition, 95: 187–191. https://doi.org/10.1111/j.1439-0396.2010.01039.x
33) Pawar, M.M., Kamra, D.N. Chaudhary L.C., Agarwal, N., Chaturvedi, V. B. 2019. Nutrients utilization, methane emission, immune function, blood metabolites and performance of buffalo calves fed *Trachyspermum copticum* seed oil. Indian Journal of Animal Science, 89: 63–67. https://doi.org/10.56093/ijans.v89i1.86385
34) Pawar, M.M., Kamra. D.N., Agarwal, N., Chaudhary, L.C., 2014. Effects of essential oils on in vitro methanogenesis and fermentation of feed with buffalo rumen liquor. Agricultural Research, 3: 67-74.
35) Pawar. M.M., Kamra D.N., Agarwal N., Chaudhary L.C. and V. B. Chaturvedi. 2021. Use of essential oils as feed additive for modulation of rumen fermentation and methanogenesis in buffalo. Animal Nutrition and Feed Technology 21: 49-60. https://doi.org/10.5958/0974-181X.2021.00004.4
36) Richards, M.B., Wollenberg, E., van Vuuren, D., 2018. National contributions to climate change mitigation from agriculture: allocating a global target Climate Policy, 18: 1271-1285. https://doi.org/10.1080/14693062.2018.1430018
37) Rooke, J.A., Wallace, R.J., Duthie, C.A., McKain, N., de Souza, S.M., Hyslop, J.J., Ross, D.W., Waterhouse, T., Roehe, R., 2014. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. British Journal of Nutrition,112: 398-407. https://doi.org/10.1017/S0007114514000932
38) Salem, A.Z., Kholif, A.E., Elghandour, M.M., Hernandez, S.R., Domínguez Vara, I.A., Mellado, M. 2014. Effect of increasing levels of seven tree species extracts added to a high concentrate diet on *in vitro* rumen gas output. Animal Science Journal, 85: 853-860. https://doi.org/10.1111/asj.12218
39) Samal, A. Sahu, S.K. Mishra, A. Mangaraj, P. Pan, S.K. and Beig, G. 2024. Assessment and quantification of methane emission from Indian livestock and manure management. Aerosol and Air Quality Research, 230204.
40) Samal, L., Chaudhary, L.C., Agarwal, N., Kamra, D.N., 2016. Effects of plants containing secondary metabolites as feed additives on rumen metabolites and methanogen diversity of buffaloes. Animal Production Science, 56: 472-48. https://doi.org/10.1071/AN15596
41) Sari, N.F., Ray, P., Rymer, C., Kliem, K.E. and Stergiadis, S. 2022. Garlic and its bioactive compounds: Implications for methane emissions and ruminant nutrition. Animals (Basel), 12: 2998. doi: 10.3390/ani12212998
42) Sarwar Gilani G, Wu Xiao, C., Cockell K.A. 2012. Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. British Journal of Nutrition, 108S, 315–32. https://doi.org/10.1017/S0007114512002371
43) SPSS. 2010. Statistical Packages for Social Sciences, Version 16, SPSS Inc., Illinois, USA
44) Toprak, N.N., 2020. Effects of Macleaya cordata extract supplementation in milk on growth performance, some biochemical parameters and a number of selected bacterial groups of the recto-anal microbiota of calves. Medycyna Weterynaryjna-Veterinary Medicine-Science And Practice, 76(8). http://doi.org/10.21521/mw.6438
45) Valencia, S., Piñeiro, A.T., Molina, I.C., Lazos, F.J., Uuh J.J., Segura M., Ramírez L., Solorio, F.J., Ku Vera, J.C., 2018. Potential of *Samanea saman* pod meal for enteric methane mitigation in crossbred heifers fed low-quality tropical grass. Agricultural and Forest Meteorology, 258: 108-116.
46) Van Soest, P.V., Robertson, J.B.,Lewis, B. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of dairy science, 74: 3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2
47) Yatoo, M.A., Chaudhary, L.C., Agarwal, N., Chaturvedi, V.B., Kamra, D.N., 2018. Effect of feeding blend of essential oils on methane production, growth, and nutrient utilization in growing buffaloes. Asian-Australasian journal of animal sciences, 31: 672-676. https://doi.org/10.5713/ajas.16.0508