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Effects of dietary supplementation of *Mallotus philippensis* leaf powder on rumen fermentation pattern, enzyme profile and ciliate protozoal population in growing crossbred calves

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ABSTRACT

The aim of this study was to evaluate the effect of dietary supplementation of *Mallotus philippensis* (local name 'Kamela') leaf meal on rumen ciliate protozoal population, enzyme profile and fermentation characteristics in growing crossbred calves. Ten growing crossbred (Jersey × Tharparker) calves with an average body weight of 91.8±1.37 kg were divided in to two equal groups (G1 and G2) and fed individually under stall feeding for 140 days on a mixed ration containing paddy straw and concentrate mixture in 50:50 ratio. Two types (C1 and C2) of iso-nitrogenous concentrate mixtures were prepared. Wheat bran in concentrate mixture (C2) of test group (G2) was partially replaced (4 parts w/w) with sun dried ground *Mallotus philippensis* leaf meal. Experimental calves of test group (G2) were fed *Mallotus philippensis* leaf meal @ 2% of the diet. No significant differences were noticed between the two groups for daily dry matter intake while nutrient digestibility, i.e. organic matter and cellulose digestibility were higher in *Mallotus philippensis* leaf fed calves (G2). However, rumen pH, ammonia nitrogen concentration and total rumen protozoal as well as holotrich and spirotrich protozoal population decreased while ruminal TVFA and propionic acid production increased due to supplementation of *Mallotus philippensis* leaf. Activities of rumen fibre degrading enzymes e.g. carboxymethyl cellulase, xylanase and β-glucosidase enzyme activities were also higher in *Mallotus philippensis* leaf meal fed calves (G2). It is concluded that supplementation of *Mallotus philippensis* leaf have a potential for reducing rumen protozoal population and ammonia nitrogen concentration with improving rumen fibre degrading enzyme activities and nutrient digestibility in growing crossbred calves.

Keywords: Ciliate protozoa, Crossbred calves, Enzyme profile, *Mallotus philippensis* leaf, Rumen fermentation

The symbiosis of protozoa and methanogenic archae in the rumen is well established and therefore, suppression of protozoa has been suggested to be a promising approach to reduce methane production and more microbial protein flow to the duodenum which leads to better utilization of dietary energy and protein (Santra *et al*. 2020). Feed additives such as ionopheres, defaunating agents, antibiotics were proved very effective in reducing dietary energy and protein losses; however, contemporary biosecurity threats have restricted their use in the diet in many European countries (Malik *et al*. 2019, e Silva *et al*. 2021). Very often residue of the chemical feed additive was detected in animal products like meat and milk leading to unfit or harmful for human consumption (Liu *et al*. 2019). Recently, there is increasing interest in the use of tree leaves/plant secondary metabolites to mitigate ruminal methane emission as well as to reduce rumen protozoal population for efficient utilization of dietary energy and protein (Jafari *et al*. 2019, Wann *et al.* 2019, Santra *et al*. 2020, de Silva *et al.* 2021). It has been reported that several tropical plants containing secondary metabolites have been shown to suppress or eliminate protozoa from the rumen and reduced methane and ammonia production (Lakhani *et al*. 2019, Santra and Karim 2019, Bhatt *et al*. 2021). However, effectiveness of plant to manipulate rumen fermentation varied depending upon the source and type of secondary metabolites present in these plants. The screening of these plants is an important step for the discovery of new compounds and their development as feed additives to mitigate rumen methanogenesis and nitrogen turnover as well as to remove rumen protozoa (Santra *et al*. 2020). Therefore, this experiment was conducted with the objective to study the efficiency of dietary supplementation of *Mallotus philippensis* leaf to suppress the rumen ciliate protozoal population and to measure the effect on rumen fermentation pattern and enzyme profile in growing crossbred calves fed on paddy straw based diet.

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MATERIALS AND METHODS

Collection and preparation of Mallotus philippensis *leaf meal:* Kamela (*Mallotus philippensis*) tree leaves were collected from Agartala, Tripura during the month of April. Both old and newly emerge type of leaves from trees were harvested and sun dried. The sun dried leaves were grounded (2 mm size) in a hammer mill prior to the determination of chemical composition and subsequent use in concentrate mixture preparation.

Experimental animals and feeding schedule: Ten Jersey male crossbred (Jersey × Tharparker) calves (about 4 months of age) having an average body weight of 91.8±1.37 kg were selected from institute herd for conducting the experiment. Calves were randomly divided into two equal groups (G1 and G2) of five animals each on the basis of body weight so that average body weight of the two experimental groups becomes similar. All the calves were fed individually under stall feeding on a paddy straw based total mixed ration with roughage to concentrate ratio of 50:50 for 140 days to meet out maintenance and growth (600 g average daily gain) requirement (NRC 2001). Two types (C1 and C2) of iso-nitrogenous concentrate mixtures were prepared (Table 1). Wheat bran in concentrate mixture (C2) of test group (G2) was partially replaced (4 parts w/w) with *Mallotus philippensis* leaf meal. Concentrate mixture was offered once daily at 9:00 AM after discarding previous day's residue if any. Calves of test group (G2) were fed *Mallotus philippensis* leaf meal @ 2% of total diet as herbal feed additive. Calves of G1 group were not supplemented with *Mallotus philippensis* leaf meal and remained as control. Measured quantity of paddy straw was offered twice daily at 10:00 AM and 18:00 PM for an excess of 10% after discarding residue. Record of daily feed intake was maintained throughout the experimental period. Animals were dewormed once at the beginning of the experiment using 'Albendazole' at 10 mg/kg body weight.

Digestion trial: A digestion trial was conducted after 120 days of experimental feeding in all experimental calves. The digestion trial lasted for 10 days, i.e. 3 days adaptation followed by 7 days of sample collection. During this period, daily feed offered, feed refused and faeces voided by individual calf were collected and recorded. 24 h faecal output of each calf was quantified and 25% of the voided faecal sample, offered and refusal feed samples were dried to a constant weight in forced draught hot air oven at 60°C. Dried samples for each day from individual animals covering 7 days collection period were pooled, sampled and ground in a hammer mill to passed through 1 mm sieve. The ground samples were preserved in air tight containers until required for chemical analysis.

Collection of rumen liquor samples: Rumen liquor was collected from all experimental calves on 01, 02, 04, 08, 15, 30, 45, 60, 75, 90, 105 and 120 days of experimental feeding at 0 h post feeding by stomach tube for counting the rumen protozoal numbers. Rumen liquor was also collected for consecutive four days from all experimental calves after 135 days of experimental feeding at 0, 3, 6, 9 and 12 h post feeding by stomach tube for studying the rumen fermentation pattern, protozoal population and enzyme profile. The collected rumen fluid was strained through two layer of muslin cloth having 100 µm pores to remove feed particles. After collection of the rumen liquor, *p*H was recorded immediately and a sub samples were preserved at –25°C for further chemical analysis.

Chemical analysis of biological samples: Samples were analyzed for DM, OM and CP as per AOAC (2005). NDF, ADF and ADL were estimated following the method of Van Soest *et al.* (1991). TVFA estimation of rumen liquor was carried out as described by Barnett and Reid (1957) and VFA fractionations were estimated in a gas chromatograph (Netel Ultima 2100) as described by Cottyn and Boueque (1968). Total and differential count of rumen protozoa were done as described by Kamra *et al*. (1991). Ammonia nitrogen concentration in the rumen liquor was estimated as per method of Weatherburn (1967). For estimation of

Table 1. Composition of experimental ration

Attribute	Concentrate mixture		Kamela (*Mallotus philippensis*) leaf	Paddy straw
	C1	C2		
Feed ingredients (% as fresh basis)				
Kamela (*Mallotus philippensis*) leaf	00.00	04.00	–	–
Maize grain	45.00	45.00	–	–
Wheat bran	25.00	21.00	–	–
Mustard cake	23.00	23.00	–	–
Mineral mixture	2.00	2.00	–	–
Common salt	1.00	1.00	–	–
Vitablend® AD_3 @ 20 g/100 kg concentrate mixture				
Chemical composition (% DM)				
Organic matter (OM)	93.70	92.90	94.80	86.60
Crude protein (CP)	18.20	17.90	14.20	3.40
Ether extract (EE)	4.70	4.50	2.90	1.90
Total carbohydrate (TCHO)	70.80	71.50	77.80	81.30
Neutral detergent fibre (NDF)	34.80	35.70	49.10	71.50
Acid detergent fibre (ADF)	11.80	12.10	24.50	41.10
Cellulose	7.60	7.80	18.90	34.30
Acid detergent lignin (ADL)	3.90	4.10	5.20	6.10

enzyme activity, rumen liquor was processed as described by Patra *et al*. (2006) and enzyme activity was estimated by the dinitrosalicyclic acid method (Miller 1959).

Statistical analysis: Data on feed intake, nutrient utilization, rumen metabolites, protozoal number and enzyme profile were subjected to analysis of variance (ANOVA) and treatment means were ranked using Duncan's multiple range tests according to Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Chemical composition of feeds and fodder: The chemical composition of concentrate mixture, *Mallotus philippensis* leaf and paddy straw is presented in Table 1. The overall nutrient composition of concentrate mixture (C1) for feeding to the control group (G1) and concentrate mixture (C2) for feeding to the test group (G2) was similar. On an average, concentrate mixture contained 93.3% OM, 18.1% CP, 4.6% EE, 35.2% NDF and 7.7% cellulose. The chemical composition of paddy straw used in the present experiment was comparable to the values reported earlier by Santra *et al.* (2013a) and Cherdthong *et al*. (2019). In the present experiment, *Mallotus philippensis* leaf contained crude protein higher than earlier reported value for tree leaves from North-Eastern state (Taku *et al*. 2019). Variation in protein and fibre content of tree leaves for different studies may be attributed to growth stage or age of the plant, season of harvesting, variety, fertilization, location (Yusuf *et al.* 2020).

Nutrient intake, nutrient digestibility and nutritive value of ration: The actual intake roughage and concentrate ratio by the calves in G1 and G2 experimental groups was 47.8:52.2 and 48.9:51.1 respectively. Dietary supplementation of *Mallotus philippensis* leaf meal as herbal feed additive did not have any effect on voluntary feed intake (Table 2). Daily dry matter intake in terms of per kg body weight as well as per kg metabolic body size was similar among the calves of two experimental groups. It was earlier reported that supplementation of *Sapindus mukorosssi* leaves @ 3% in diet as herbal feed additive had no effect on daily feed intake in Rathi calves (Meel *et al*. 2015). Olafadehan *et al.* (2016) observed that inclusion of tannin-containing *Ficus polita* foliage up to 700 g/kg of DM in a total mixed ration for goat did not compromise voluntary feed intake. Similarly, a non-significant effect of tropical tree leaves supplementation as herbal feed additive on dry matter intake (DMI, g/d) was reported in sheep between control and test group (Malik *et al.* 2017).

The digestibility of organic matter, neutral detergent fibre and cellulose were improved ($P<0.01$) due to dietary supplementation of *Mallotus philippensis* leaf meal as feed additive. However, digestibility of crude protein was not influenced by dietary supplementation of *Mallotus philippensis* leaf meal. In the present experiment, organic matter digestibility improved by 2% due to dietary supplementation of *Mallotus philippensis* leaf meal as feed additive. This result could be attributed to protozoa, which are capable of ingesting fibrolytic bacteria; therefore

Table 2. Effect of dietary Kamela (*Mallotus philippensis*) leaf meal supplementation as herbal feed additive on nutrient utilization in growing crossbred calves

Attribute	Level of kamela leaf (%)		SEM	Level of significance
	0 (G1)	2 (G2)		
DM intake				
Roughage (g/d)	2.20	2.30	0.03	NS
Concentrate (g/d)	2.40	2.40	0.05	NS
Total (g/d)	4.60	4.70	0.11	NS
DMI (% b.wt.)	3.30	3.20	0.09	NS
DMI (g/kg $^{0.75}$/d)	112.70	111.60	5.13	NS
Nutrient intake and its digestibility				
Organic matter				
Intake (kg/d)	4.10	4.20	0.09	NS
Digestibility (%)	62.30	64.30	0.57	P<0.01
Crude protein				
Intake (kg/d)	0.51	0.49	0.04	NS
Digestibility (%)	65.80	66.50	0.51	NS
Neutral detergent fibre				
Intake (kg/d)	2.40	2.50	0.07	NS
Digestibility (%)	52.70	54.80	0.43	P<0.01
Cellulose				
Intake (kg/d)	0.80	0.90	0.06	NS
Digestibility (%)	49.70	52.10	0.45	P<0.01
Nutritive value of experimental diet				
Digestible crude protein (g/kg DM)	73.40	73.90	1.31	NS
TDN (g/kg DM)	601.80	634.50	13.58	P<0.01

NS, Non-significant; SEM, Standard error of mean.

suppressing these protozoa is expected to increase number of fibrolytic bacteria in the rumen, which can enhance feed digestibility in animals (Cherdthong *et al*. 2019). However, the present study could not confirm whether fibrolytic bacterial number enhances or improve feed digestion. Previous *in vitro* studies in our laboratory confirmed that inclusion of *Mallotus philippensis* leaf meal in incubation media reduced rumen protozoal population (Lotha 2015). Meel *et al*. (2015) reported that incorporation of *Sapindus mukorossi* (Reetha) leaf @ 3% in the diet as herbal feed additive improved the digestibility of nutrients in Rathi calves. Similarly, supplementation of *Moringa oleifera* leaves, improved nutrient digestibility as well as rumen fermentation in dairy cows (Li *et al.* 2019). Protozoa represents about half of the rumen microbial biomass and play an important role in rumen digestion and the digestion of plant cell wall increases by 15% in the presence of protozoa (Jouany and Ushida 1999). However, Malik *et al*. (2017), did not find any change in apparent dry matter as well as organic matter digestibility in sheep when fed tanniniferous tropical tree leaves as feed additive to manipulate rumen fermention to reduce ruminal methanogenesis and protozoal population.

Digestible crude protein (DCP) content of ration was similar in G1 and G2 group. However, total digestible nutrients (TDN) content of the ration was higher ($P<0.01$) for *Mallotus philippensis* leaf meal fed calves (G2). Higher TDN content in the ration of G2 group might be due to higher nutrient (OM, NDF and cellulose) digestibility.

Rumen ciliate protozoa: The ciliate protozoal population present in the rumen of the experimental calves in both the experimental groups were B type due to presence of *Epidinium* sp, and the absence of *Polyplastron multivesiculatum* (Coleman 1980, Santra and Karim 2020). Rumen protozoal number decreased drastically up to 18 days of experimental feeding in the calves of G2 group due to dietary supplementation of *Mallotus philippensis* leaf powder as herbal feed additive and thereafter the number of rumen protozoa remains almost static during rest of the experimental period (Fig. 1). Earlier it was also reported that due to dietary supplementation of *Piper betle* leaf as herbal feed additive in lactating cow drastically reduced the rumen protozoal population up to 16 days of experimental feeding and thereafter rumen protozoal number remained almost static during rest of the experimental period (Santra *et al.* 2013b).

Numerically spirotrich protozoa comprised more than 90% of total protozoal population in the calves of both the experimental groups (Table 3) in the present experiment which is also similar to the earlier findings. Santra and Karim (2002 and 2020) also reported that spirotrich protozoa comprised more than 80% of total rumen protozoal population under Indian condition. The lowest number of rumen ciliate protozoal count was observed just before feeding with increase in total and spirotrich protozoal number at 6 h (peak value) followed by a gradual decrease at 8 to 10 h post-feeding in the calves of both the

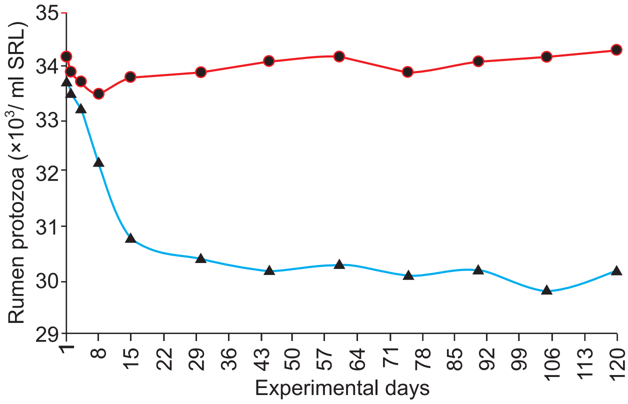

Fig. 1. Effect of dietary supplementation of *Mallotus philippensis* leaf meal on rumen total protozoal population in the growing calves during total experimental period (G1: (•) = un-supplemented; G2 (Δ) = supplemented with *Mallotus philippensis* leaf meal).

experimental groups. Feed offer was associated with an abrupt increase in the total as well as differential protozoal population within 6 h of post feeding in the present experiment, which could be due to migration of ciliate protozoa from the reticulo-rumen wall from where they sequestered to the rumen medium in response to chemical stimuli originating from the diet (Santra and Karim 2002, Santra *et al.* 2016). The migration of ciliates from the reticulo-rumen wall into rumen liquor was due to chemotactic movement of protozoa towards the feed entering the rumen. After the feed was utilized, the protozoa gradually migrated back to the reticulo-ruminal wall resulting in the drop in their numbers in the rumen liquor at 8 to 10 h post-feeding in the experimental calves of G1 and G2 group as observed in the present experiment (Fig. 1). The total rumen protozoal number as well as holotrich and spirotrich protozoal number decreased ($P<0.01$) in the calves of G2 groups. Dietary supplementation of *Mallotus philippensis* leaf reduced the rumen protozoal population in the present experiment which may be due to presence of

Table 3. Effect of dietary supplementation of Kamela (*Mallotus philippensis*) leaf meal as herbal feed additive on rumen protozoal population (× 10^3/ml) in growing cross-bred calves*

Attribute	Level of kamela leaf (%)		SEM	Level of significance
	0 (G1)	2 (G2)		
Large holotrich	1.2	0.8	0.03	P<0.01
Small holotrich	3.5	2.9	0.07	P<0.01
Total holotrich	4.7	3.7	0.11	P<0.01
Large spirotrich	11.5	9.7	0.16	P<0.01
Small spirotrich	24.6	21.8	0.39	P<0.01
Total spirotrich	36.1	31.5	0.83	P<0.01
Total rumen protozoa	40.8	35.2	0.97	P<0.01

SEM, Standard error of mean. *Each value is an average of 16 separate observations and each observation is an average of the protozoa count in 30 microscopic fields (magnification 100×).

plant secondary metabolites. It was also reported earlier by many workers that feeding of selected tropical/Indian tree leaves reduced rumen protozoal population in ruminant animals due to presence of plant secondary metabolites like tannin, saponin, essential oils and flavonoids (Meel *et al.* 2015, Santra *et al.* 2016, Malik *et al.* 2017, Cherdthong *et al.* 2019).

Rumen metabolites and enzyme profile: The *p*H of rumen liquor was lower (P<0.05) while TVFA and propionic acid concentration was higher (P<0.05) in *Mallotus philippensis* leaf meal fed calves (Table 4). Dietary supplementation of *Mallotus philippensis* leafs had no effect on ruminal total nitrogen and TCA-soluble-nitrogen concentration. However, ammonia nitrogen concentration was lower (P<0.01) in the rumen of the calves of G2 than G1 group. The lowest rumen *p*H was observed in G2 group at 3 h post feeding, i.e. the rumen *p*H decreased from 0 to 3 h, followed by increase at 6 to 12 h post feeding. In the present experiment, over all rumen *p*H was lower and the drop in rumen *p*H was much higher after ingestion of feed in the cows supplemented with *Mallotus philippensis* leaf meal as feed additive (G2), indicating that ciliate protozoa seem to have a *p*H stabilizing effect in the rumen due to engulfment of excess starch from the rumen (Hristov *et al.* 2001). It is hypothesized that the shift in VFA composition is usually associated with fibre degradation and a reduction in fibrolysis accounts less acetate production (Malik *et al.* 2017). Condensed tannin (CT) alter the proportions of VFA, particularly the ratio of acetate : propionate (Jayanegara *et al*. 2012). It is known that effect of condensed tannin on rumen fermentation is depend on its source and form, and even minor change in structure may affect the magnitude of action (Bhatta *et al*. 2009). Higher TVFA and propionate production while lower acetate to propionate ratio in *Mallotus philippensis* leaf meal fed calves (G2) in the present experiment might be due to presence of plant secondary metabolites particularly condensed tannin in the tested tree leaves. It was reported that *Mallotus philippensis* leaf contain various phytochemicals like tannin, saponin, flavonoids, etc. (Velangann *et al*. 2011).

Table 4. Effect of dietary supplementation of Kamela (*Mallotus philippensis*) leaf meal as herbal feed additive on rumen fermentation and enzyme profile in growing calves

Attribute	Level of kamela leaf (%)		SEM	Level of significance
	0 (G1)	2 (G2)		
Rumen metabolites				
*p*H	6.69	6.39	0.021	P<0.05
TVFA (mM/100 ml)	7.10	8.20	0.13	P<0.05
Acetate (mol/100 mol)	68.70	67.70	0.25	P<0.05
Propionate (mol/100 mol)	22.10	22.90	0.17	P<0.05
Butyrate (mol/100 mol)	9.20	9.40	0.09	NS
Acetate: propionate ratio	3.10	2.90	0.01	P<0.05
Total–N (mg/100 ml)	67.30	65.70	1.92	NS
TCA-soluble-N (mg/100 ml)	32.70	31.50	0.67	NS
NH_3–N (mg/100 ml)	8.40	7.30	0.16	P<0.01
Rumen enzyme activity (IU/100 ml)				
Carboxy methyl cellulase	95.60	114.30	3.27	P<0.01
Xylanase	126.50	147.80	4.09	P<0.05
β-glucosidase	13.90	17.30	0.29	P<0.05
Amylase	208.70	215.20	10.38	NS

NS, Non-significant; SEM, Standard error of mean.

The concentration of total nitrogen and NH_3–N in the rumen liquor peaked at 3 h post-feeding followed by a gradual decrease, achieving their lowest value at 0 h post-feeding. Decrease in ammonia nitrogen concentration in the rumen fluid of calves supplemented with *Mallotus philippensis* leaf meal as feed additive, probably due to the reduction of rumen protozoal population. The presence of protozoa in the rumen ecosystem is associated with increased recycling of microbial nitrogen in the rumen and therefore, decreased protozoal population in the rumen are usually associated with lowered ammonia nitrogen concentrations, primarily as a result of a decrease in proteolysis of bacterial protein by ruminal protozoa (Hristov *et al*. 2005). Moreover, several studies have suggested that plant secondary compounds and their active components may conserve amino acids from ruminal degradation by inhibiting microbial deamination (Newbold *et al*. 2004, Cherdthong *et al*. 2019, Wann *et al.* 2019).

Ruminal enzyme activity e.g. activity of carboxy methyl cellulase, xylanase, β-glucosidase were significantly higher in *Mallotus philippensis* leaf meal fed calves (G2). However, amylase enzyme activity was similar in both the experimental groups. Generally, presence of ciliate protozoa, inhibit the cellulolytic activity of rumen fungi (Widyastuti *et al*. 1995) as well as rumen bacteria (Lee *et al*. 1999). Decreased protozoal population in the rumen are usually associated with higher rumen bacterial and fungal population as evident from the defaunated animals (Chaudhary *et al*. 1995). Therefore, activity of fibre degrading enzymes, e.g. carboxymethyl cellulase, xylanase and β-glucosidase were improved by the dietary supplementation of *Mallotus philippensis* leaf meal in the present study although the rumen protozoal population become decreased.

It is concluded from the present study that dietary supplementation of *Mallotus philippensis* leaf in growing crossbred calves significantly reduce the rumen ciliate protozoal population and ammonia nitrogen concentration while increased the ruminal fibre degrading enzyme activities which leads to improved organic matter and fibre digestibility resulting in higher TVFA and propionate production. *Mallotus philippensis* leafs may be used to manipulate rumen fermentation particularly for higher TVFA and propionate production and reducing the rumen ciliate protozoal population for efficient utilization of dietary energy and protein.

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