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Rumen fermentation pattern, enzyme profile and ciliate protozoal population in betel (*Piper betle*) leaves fed lactating crossbred cows

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ABSTRACT

The aim of this study was to evaluate the effect of supplementation of *Piper betle* (local name 'Pan') leaf (Bangla variety) on rumen ciliate protozoal population, enzyme profile and fermentation characteristics in lactating crossbred cows. Twelve lactating crossbred (Jersey × Tharparker) cows with an average milk yield of 5.4 ± 0.78 kg/d were divided into equal groups (G1 and G2) and fed individually under stall feeding for 70 days on a mixed ration containing green fodder (maize) and concentrate mixture. The G1 group was supplemented with 100 g *Piper betle* leaves powder/animal/day along with concentrate mixture. No significant differences were noticed between the 2 groups for daily dry matter intake and nutrient digestibility. 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 *Piper betle* leaves. Rumen enzyme activities were similar in both the experimental group. It is concluded that supplementation of *Piper betle* leaves have a potential for reducing ciliate protozoal population and rumen ammonia nitrogen concentration without affecting rumen polysaccharide degrading enzyme activities and nutrient digestibility in dairy cow.

Key words: Ciliate protozoa, Dairy cows, Nutrient digestibility, *Piper betle* leaf, Rumen enzyme

The symbiosis of protozoa and methanogenic archae in the rumen is well established and therefore, suppression of protozoa is suggested to be a promising approach to reduce methane production which leads to better utilization of dietary energy (Goel *et al*. 2008). Moreover, rumen protozoa possess strong proteolytic activity and are contributed to intra-ruminal recycling of microbial nitrogen which should be avoided or minimized for efficient utilization of dietary protein (Jouany 1996). Therefore, elimination of protozoa from the rumen (defaunation) results in an increased growth rate and feed conversion efficiency in young ruminants due to better dietary energy and protein utilization (Ivan *et al*. 2004). Defaunation is presently not feasible practically due to unavailability of suitable defaunating agent. However, a considerable reduction in rumen protozoal population may also be beneficial for improving the productive performance of the animals as it increased milk yield in dairy cows and growth rate of young ruminants (Ivan *et al*. 2004).

Plants containing secondary metabolites like saponins, tannins, essential oils etc. are reported to be effective against ruminal methanogenesis and ciliate protozoa (Ramachandran *et al*. 2014, Bhatta *et al*. 2015, Pathak *et al*. 2015). Wall *et al*. (2014) reported that feeding of a blend of cinamaldehyde and eugenol along with the basal diet, increased daily dry matter intake and milk production in lactating dairy cows. *Piper betle* leaf contained 0.08 to 0.2 g essential oil/100 g fresh leaf (Guha 2006). Rumen ciliate protozoal population reduced significantly in steer by feeding essential oil (Ando *et al*. 2003). Essential oil mixture extracted from *Anethum graveolens* decreased ruminal methane production (Patra and Saxena 2010) indicating better dietary energy utilization. Therefore, this experiment was conducted with the objectives to study the efficiency of dietary supplementation of *Piper betle* leaves to suppress the rumen ciliate protozoal population and to measure the effect on nutrient digestibility, rumen fermentation pattern and enzyme profile in dairy cows.

MATERIALS AND METHODS

Preparation of Piper betle *leaf powder:* Fresh leaves of *Piper betle* were collected locally and the taxonomic identities of this leaves were determined in the Department of Botany, Kalyani University, West Bengal, India. *Piper betle* leaves were washed under running tap water after collection, air-dried and homogenized to fine powder and stored in airtight container for further use.

Experimental animals and feeding schedule: Lactating crossbred (Jersey × Tharparker) cows (16) having an average body weight of 295.2 ± 3.16 kg with an average daily milk yield of 5.4 ± 0.78 kg during mid lactation were selected for conducting the experiment. Cows were divided

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into equal groups (G1 and G2) of 8 cows each in such a way that the average milk yield of each group becomes similar. All the lactating crossbred cows were fed individually for 70 days on a mixed ration containing green fodder (maize) and concentrate mixture. Animals were dewormed once at the beginning of the experiment. Weighed quantity of concentrate mixture was given to the experimental animals in 2 equal installments at 7.00 AM and 3.00 PM daily before half an hour of milking. Green maize fodder was offered *ad lib.* daily to the all animals at 10.00 AM and 5.00 PM and the leftover feed in 24 h was weighed, recorded and discarded daily during the entire experimental feeding period. Cows of G1 group were supplemented with sun dried and ground 100 g *Piper betle* leaves (Bangla variety)/animal/day along with concentrate mixture in the morning. Cows of G2 group were not supplemented with *Piper betel* leaves and remained as control.

Digestion trial: A digestion trial was conducted after 55 days of experimental feeding in all experimental cows. The digestion trial lasted for 10 days i.e., 3 days adaptation followed by seven days of sample collection. During this period, daily feed offered, feed refused and faeces voided by individual cows were collected and recorded. Twenty-four hour faecal output of each animal 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 and 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 for consecutive 3 days from all experimental cows after 67 days of experimental feeding at 0, 3, 6, 9 and 12 h post feeding by stomach tube. The collected rumen fluid was strained through 2 layers of muslin cloth having 100 µm pores to remove feed particles. After collection of the rumen liquor, pH was recorded immediately and a sub samples were preserved at -25 ºC 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 per Barnett and Reid (1957) and VFA fractionations were estimated in a gas chromatograph as described by Cottyn and Boueque (1968). Total and differential count of rumen protozoa were done (Kamra *et al*. 1991). Ammonia nitrogen concentration in the rumen liquor was estimated as per Weatherburn (1967). For estimation of enzyme activity, rumen liquor was processed (Patra *et al*. 2006) and enzyme activity was estimated (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 offered concentrate mixture and maize fodder contained 19.2 and 10.7 % CP and 13.1 and 31.1 % cellulose on DM basis, respectively (Table 1). The chemical composition of maize fodder used in the experiment was comparable to the values reported by Kathirvelan and Tyagi (2009). *Piper betle* leaves (Bangle variety) contained 12.4 % DM on fresh basis along with 10.2 % CP, 24.1 % cellulose and 2.7 % acid detergent lignin on DM basis and 3.4 kcal gross energy/g DM.

Nutrient intake, nutrient digestibility and plane of nutrition: Daily dry mater intake and nutrient digestibility

Table 1. Chemical composition (% DM) of experimental ration

Attributes	Concentrate mixture	Maize fodder	*Piper betle* leaves
Organic matter (OM)	88.5	90.9	96.2
Crude protein (CP)	19.2	10.7	10.2
Neutral detergent fibre (NDF)	39.9	74.5	60.7
Acid detergent fibre (ADF)	18.7	37.6	27.3
Cellulose	13.1	31.1	24.1
Acid detergent lignin (ADL)	4.6	6.1	2.7
GE (kcal/kg DM)	4.3	4.1	3.4

Table 2. Effect of dietary supplementation of *Piper betle* leaves on nutrient digestibility in lactating cows

Attributes	G1	G2	SEM	Level of significance
Dry matter intake				
Green maize fodder (kg/d)	4.7	4.6	0.06	NS
Concentrate mixture (kg/d)	4.9	4.8	0.07	NS
Total (kg/d)	9.6	9.4	0.14	NS
kg/100 kg body weight/d	3.3	3.2	0.05	NS
g/kg $W^{0.75}$/d	13.6	13.4	0.16	NS
Nutrient intake and its digestibility				
Organic matter				
Intake (kg/d)	8.5	8.4	0.13	NS
Digestibility (%)	63.1	63.7	0.39	NS
Crude protein				
Intake (kg/d)	1.36	1.34	0.03	NS
Digestibility (%)	67.6	66.9	0.51	NS
Cellulose				
Intake (kg/d)	2.2	2.1	0.03	NS
Digestibility (%)	47.4	49.3	0.96	NS
Gross energy				
Intake (Mcal/d)	40.3	39.5	1.19	NS
Digestibility (%)	59.2	58.4	0.68	NS
Nutritive value of experimental diet				
Digestible crude protein (g/kg DM)	95.3	92.6	0.81	NS
Digestible energy (Mcal/kg DM)	2.5	2.4	0.07	NS

was not influenced by the dietary supplementation of *Piper betle* leaves to the experimental cows (Table 2). Apparent digestibility of the nutrient and nutritive value of the experimental diet e.g., DCP and DE content of the diet were not influenced by the dietary supplementation of *Piper betle* leaves. *Piper betle* leaves (Bangla variety) contains 0.08 to 0.2 g essential oil / 100 g fresh leaf and this essential oils is constituted by a mixture of about twenty-one different compounds of which eugenol is the chief ingredient constituting about 29.5g /100 g of the oil (Guha 2006). Yang *et al*. (2007) reported that feeding of 2 g of juniper berry essential oils (containing 35 % á-paine) in cows and 750 mg or 2 g of an essential oil mixture to dairy cattle (Benchaar *et al*. 2007) did not influence feed intake. Therefore, the present observation e.g., dietary supplementation of *Piper betel* leaves which contain essential oils, had no effect on daily dry matter intake in lactating cows was similar to the earlier findings. The lack of influence of essential oils on total tract digestibility of DM and OM in the present study is in agreement with previous observation with dairy cows (Benchaar *et al*. 2006).

Rumen ciliate protozoa and enzyme profile: The ciliate protozoal population present in the rumen of the experimental cows in both the experimental groups were B type due to presence of *Epidinium* sp, and the absence of *Polyplastron multivesiculatum* (Coleman 1980). Numerically spirotrich protozoa comprised more than 90 % of total protozoal population in the cows of both the experimental groups (Table 3) in the present experiment is also similar to the earlier findings. Santra and Karim (2002) also reported that spirotrich protozoa comprised more than 80 % of total rumen protozoal population under Indian condition. The lowest number of ciliate protozoal count was observed just before feeding with increase in total, Isotrichidae and Entodiniomorphid protozoal number at 3 h (peak value) followed by a gradual decrease at 6 to 12 h post-feeding in the cows of both the experimental groups (Figs 1–3). Feed offered was associated with an abrupt increase in the total as well as differential protozoal population within 3 h of post feeding in the present experiment, which could be due to migration of ciliate protozoa from the reticulo-rumen wall

Table 3. Effect of dietary supplementation of *Piper betle* leaves on rumen protozoal population ($\times 10^3$/ml) in lactating cows*

Attributes	G1	G2	SEM	Level of significance
Large isotrichidae	0.1	0.6	0.02	P<0.01
Small isotrichidae	0.4	1.9	0.05	P<0.01
Total isotrichidae	0.5	2.5	0.09	P<0.01
Large entodiniomorphid	2.4	4.5	0.13	P<0.01
Small entodiniomorphid	10.2	22.4	0.31	P<0.01
Total entodiniomorphid	12. 6	26.9	0.95	P<0.01
Total rumen protozoa	13.1	29.4	0.85	P<0.01

* Each value is an average of sixteen separate observations and each observation is an average of the protozoa count in 30 microscopic fields (magnification 100 ×).

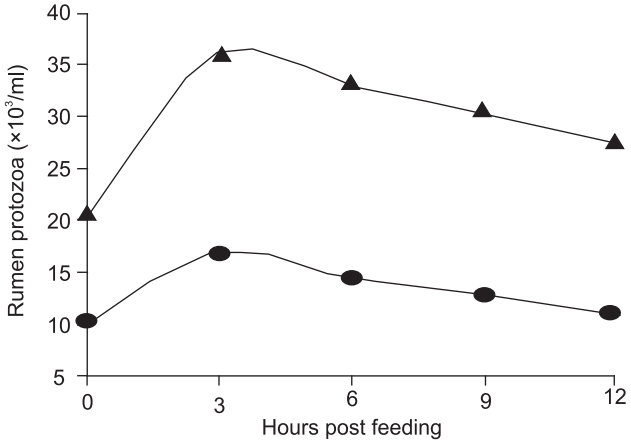

Fig. 1. Effect of dietary supplementation of *Piper betle* leaves on rumen total protozoal population in the lactating cows at different hours of post feeding (G1: ●, supplemented with *Piper betle* leaves; G2: ▲, unsupplemented).

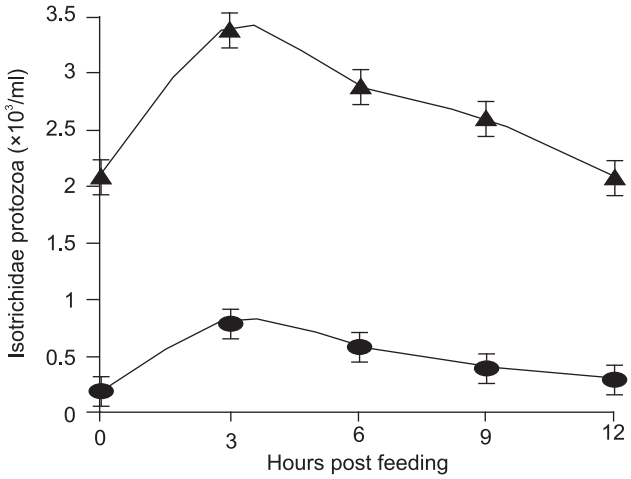

Fig. 2. Effect of dietary supplementation of *Piper betle* leaves on Isotrichidae protozoal population in the rumen of lactating cows (G1: ●, supplemented with *Piper betle* leaves; G2: ▲, unsupplemented).

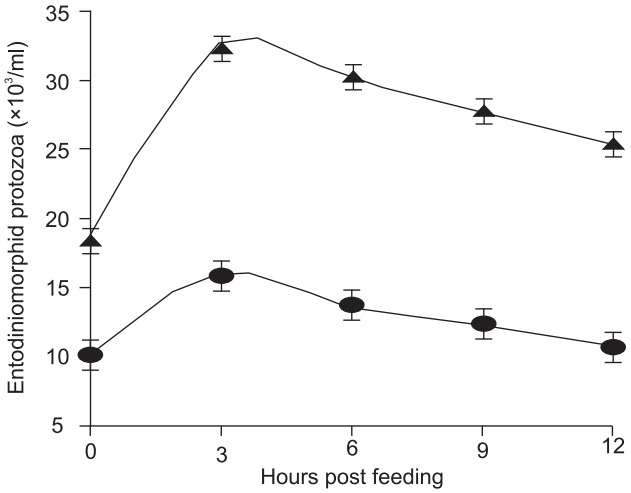

Fig. 3. Effect of dietary supplementation of *Piper betle* leaves on Entodiniomorphid protozoal population in the rumen of lactating cows (G1: ●, supplemented with *Piper betle* leaves; G2: ▲, unsupplemented).

from where they sequestered to the rumen medium in response to chemical stimuli originating from the diet (Santra and Karim 2002). 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 observed drop in their numbers in the rumen liquor at 6 to 12 h post-feeding in the experimental cows of G1 and G2 group as observed in the present experiment (Figs 1–3). The total rumen protozoal number as well as holotrich and spirotrich protozoal number decreased (P<0.01) in the cows of G1 groups. Dietary supplementation of *Piper betle* leaf reduced the rumen protozoal population in the present experiment may be due to the presence of essential oils in it. Little information is available on the effect of essential oils and their compounds on ciliate protozoal populations in the rumen. Ando *et al*. (2003) showed that feeding 200 g/d of dried peppermint to cannulated steers significantly decreased protozoa numbers by approximately 50 %. Rasmussem *et al*. (2005) also reported that essential oil present in rosemary (*Rosmarinus officinalis*) had a notable inhibitory effect on rumen protozoa.

Rumen metabolites and enzyme profile: The pH of rumen liquor was higher (P<0.05) in G2 group, whereas TVFA concentration and propionic acid production was higher (P<0.05) in G1 group (Table 4). Dietary supplementation of *Piper betle* leaves 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 cows of G1 than G2 group. The lowest rumen pH was observed in G1 group at 3 h post feeding: the rumen pH decreased from 0 to 3 h, followed by increase at 6 to 12 h post feeding (Fig. 4). In the present experiment over all rumen pH was lower and the drop in rumen pH was much higher after ingestion of feed in the cows supplemented with *Piper betle* leaves (G1), indicating that ciliate protozoa seem to have a pH stabilizing effect in the rumen due to

Table 4. Effect of dietary supplementation of *Piper betle* leaves on rumen fermentation and enzyme profile in lactating cows

Attributes	G1	G2	SEM	Level of significance
Rumen metabolites				
pH	6.5	6.8	0.11	P < 0.05
TVFA (mM/100 ml)	9.5	8.2	0.39	P < 0.05
Acetate (mol/100 mol)	67.2	68.9	0.32	P < 0.05
Propionate (mol/100 mol)	23.9	22.3	0.21	P < 0.05
Butyrate (mol/100 mol)	8.9	8.8	0.11	NS
Acetate: propionate ratio	2.8	3.1	0.01	P < 0.05
Total – N (mg/dl)	77.2	73.1	2.15	NS
TCA-soluble-N (mg/dl)	34.1	32.8	1.47	NS
NH_3–N (mg/dl)	9.1	10.9	0.32	P<0.01
Rumen enzyme activity (IU/dl)				
Carboxy methyl cellulase	15.5	17.4	1.21	NS
Xylanase	14.6	15.2	1.64	NS
b-glucosidase	0.52	0.48	0.09	NS

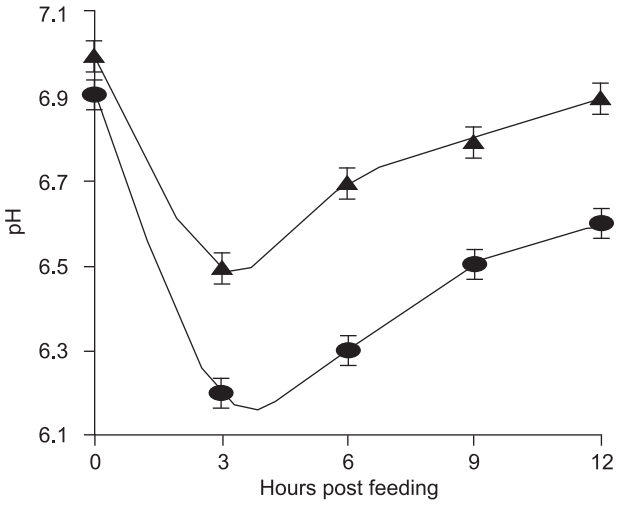

Fig. 4. Effect of dietary supplementation of *Piper betle* leaves on rumen pH in lactating cows (G1: ●, supplemented with *Piper betle* leaves; G2: ▲, unsupplemented).

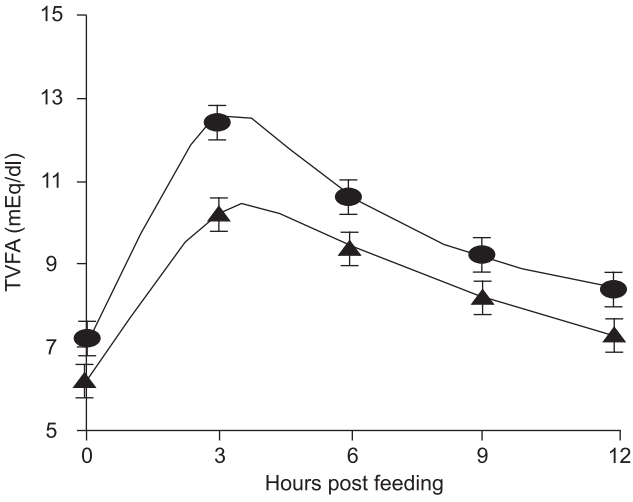

Fig. 5. Effect of dietary supplementation of *Piper betle* leaves on TVFA concentration in the rumen of lacting cows (G1: ●, supplemented with *Piper betle* leaves; G2: ▲, unsupplemented).

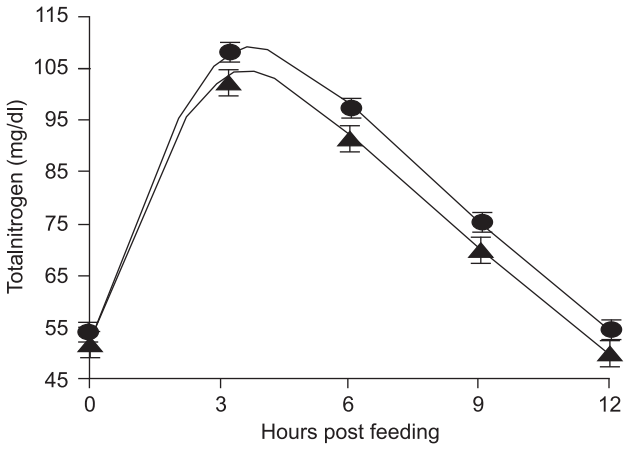

Fig. 6. Effect of dietary supplementation of *Piper betle* leaves on total nitrogen concentration in the rumen of lactating cows (G1: ●, supplemented with *Piper betle* leaves; G2: ▲, unsupplemented).

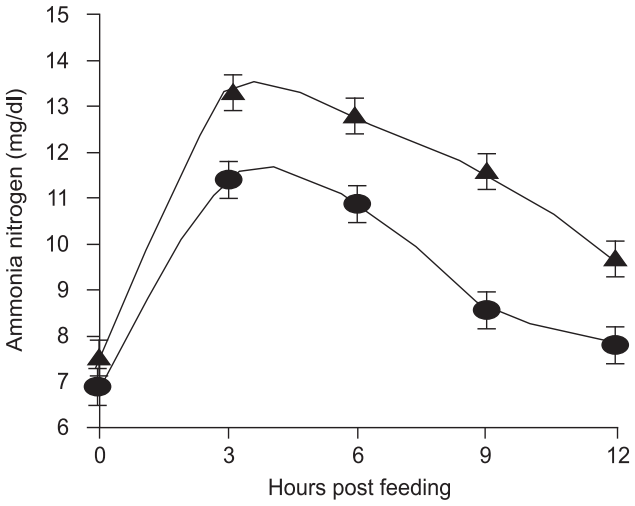

Fig. 7. Effect of dietary supplementation *Piper betle* leaves on ammonia nitrogen concentration in the rumen of cows (G1: ●, supplemented with *Piper betle* leaves; G2: ▲, unsupplemented).

engulfment of excess starch from the rumen (Hristov *et al*. 2001). It is a great challenge to identify the appropriate dose rate for various essential oils or essential oil active components that favorably alter aspects of rumen metabolism without reducing total VFA concentration and, supplementation with essential oils or essential oils compound has increased total VFA concentration in a limited number of studies (Benchaar *et al*. 2008). In one such *in vitro* study, addition of 1.5 mg/l of essential oil increased total VFA concentration in continuous cultures, although there was no concomitant increase in organic matter digestibility (Castillejos *et al*. 2005) which was similar to the findings of the present study. Further, higher ruminal propionate and lower acetate production along with lower acetate to propionate ratio due to dietary supplementation of *Piper betle* leaves in the present experiment is in agreement with some earlier observations. Busquet *et al*. (2005) reported that the molar proportion of acetate was reduced (P<0.01) by 11 % when 312 mg garlic oil/liter was added to *in vitro* batch culture rumen fermentation at a constant pH. Similarly, Spanghero *et al*. (2008) observed that a blend of essential oils shifted the end products of fermentation, in particular a reduction in the acetate proportion and the acetate to propionate ratio. The lack of an effect of *Piper betle* leaves on molar proportion of butyrate in the rumen is consistent with the results obtained from dairy cows supplemented with a mixture of essential oils, including thymol, eugenol, vanillin, galacol and limonene (Benchaar *et al*. 2007). TVFA production at similar time intervals exhibited reversed trend (Fig. 5) to that of rumen pH.

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 (Fig. 6 and 7). Decrease in ammonia nitrogen concentration in the rumen fluid of cows supplemented with *Piper betle* leaves, is 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 essential oils and their active components may conserve amino acids from ruminal degradation by inhibiting microbial deamination (Newbold *et al*. 2004). However, Molero *et al*. (2004) speculated that the effects of essential oils on nitrogen metabolism may result from the inhibition of proteolytic activity or a decrease in the attachment and colonization of feed by proteolytic microbes.

Ruminal enzyme activity e.g., activity of carboxy methyl cellulase, xylanase, b-glucosidase and amylase were 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 b-glucosidase was not affected by the dietary supplementation of *Piper betle* leaves in the present study although the rumen protozoal population decreased.

It is concluded from the present study that dietary supplementation of *Piper betle* leaf in dairy cows significantly reduced the rumen ciliate protozoal population and anmmonia nitrogen concentration with out affecting the activity of polysaccharide degrading enzymes and nutrient digestibility. *Piper betle* leaves may be used to manipulate rumen fermentatiom particularly for reducing the rumen ciliate protozoal population for efficient utilization of dietary protein.

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