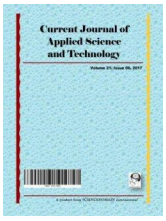

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Supplementation of Selected Tanniniferous Phyto-sources at Graded Level Decreases Methane Production *in vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LB and RB designed the study. Author LB performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors LB and PKM managed the analyses of the study. Authors APK and AD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was carried out to determine the effect of graded level supplementation of selected tanniniferous phyto-sources, viz. *Syzygium cumini*, *Machilus bombycina* and *Acharas zapota* on *in vitro* methane, fermentation characteristics and rumen protozoa population in order to determine the optimum dose of supplementation for the inclusion in the ruminant diet to achieve methane reduction.
Study Design: This study was design based on previous screening studies carried out in our laboratory. A graded level study was carried out here to determine the optimum dose of inclusion of these phyto-sources in the ruminant diet.
Methodology: *In vitro* gas production test was carried out by adding these sources at different levels viz. 0%, 5%, 10%, 15% and 20% to the basal diet consisting finger millet straw (*Elusine*

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coracana) as roughage source and concentrate in 70:30. The gas samples obtained after 24 h of incubation were analysed using gas chromatograph. The effect of phyto-sources on rumen fermentation characteristics and protozoa population was determined using standard methodologies.

Results: The addition of these phyto-sources significantly (*P*=.05) reduced the total gas production at 10% in case of *Machilus bombycina* and above 10% in *Syzygium cumini,* while *Acharas zapota* had reduce gas production at the highest inclusion level. However, all the phyto-sources reduced methane production as the percentage of inclusion increased. Effect on fibre digestion was also observed. A significant effect (*P*=.05) on rumen ammonia-N and protozoa were observed. An overall reduction in TVFA was also recorded in this study.

Conclusion: This study confirms that the selected phyto-sources were potent methane inhibitor. Considering 200 mg of basal diet, *S. cumini* and *A. zapota* can be included within 10% of the basal diet and *M. bombycina*, at a maximum of 5% to reduce methane without affecting rumen fermentation unfavourably. However, long-term *in vivo* trials should be conducted to determine the efficacy of these phyto-sources on enteric methane emission.

Keywords: Methane; tannins; rumen fermentation; protozoa; optimisation.

1. INTRODUCTION

Methane (CH_4) is an unavoidable by-product of enteric fermentation involving a consortium of rumen microbes. During the fermentation reduced cofactors (NADH, NADPH, and FADH) are re-oxidised (NAD^+, $NADP^+$, FAD^+) through dehydrogenation reactions. The released hydrogen (H_2) in the rumen is converted to CH_4 by the rumen methanogens. This process also acts as a primary sink for H_2, and thereby helps in maintaining a low partial pressure. Rumen methanogens are accountable for a loss of 4-10% of gross energy [1], in addition, CH_4 emission also contributes immensely to the carbon footprint. Considering the energy content of CH_4, which is 55.22 MJ/kg [2], loss of CH_4 significantly affects rumen productivity. Hence decreasing CH_4 from ruminants; is not only desirable with an animal productivity perspective but also from the point of global CH_4 reduction.

After the ban of growth-promoting antibiotics in the animal feeds within European Union; there began an increased exploration of plant bioactive components to manipulate rumen fermentation which has the potential to reduce enteric CH_4 [3]. Among the various plant bioactive compounds, tannins are widely explored as rumen manipulators as phyto-sources contain tannin naturally and it is available in abundance. Tannins are polyphenolic compounds and are produced in the plants, mainly for self-defence mechanism. Even though tannins are known for their anti-nutritional effect, but on supplementing them at a low level usually have a beneficial effect on nitrogen (N) utilisation of ruminants as well as on animal health and CH_4 emission [4]. However, the effect of tannin varies according to their concentration, structural variation, molecular weight and type of tannins. Thus, the preliminary step for using tannin sources is to screen whether it has the potential to reduce CH_4, and once the CH_4 mitigation potential is established then further studies required for determining the optimum dose of inclusion in the diet at which CH_4 is reduced without hampering the rumen fermentation.

In the present study, three tanniniferous phyto-sources were selected [5], from the previous studies by the authors. Therefore sources were evaluated *in vitro* at different grades to determine the optimum dose of their inclusion in the ruminant diet for CH_4 reduction without any adverse effect on rumen fermentation.

2. MATERIALS AND METHODS

2.1 Test Sources and Basal Diet

Based on previous screening studies three phyto-sources were selected. Among these *Syzygium cumini* contains hydrolyzable tannins (HT) [5]; while *Machilus bombycina* possess condensed tannins (CT) [5] and *Acharas zapota* contains both CT and HT [5]. These phyto-sources were collected during the month of April 2014 from Jorhat district (94° 12′ 11"E longitude to 26°45'27"N latitude), Assam. A total of four trees were used for each phyto-source available in that area to collect the sample. The study material includes both mature and immature leaves of each phyto-source so that tannin content from a particular source should represent tannin from both old and new leaves.

Approximately 500 g of leaves were harvested from each source and they were shed dried for a period of 10 days for reducing the moisture content. Leaf samples containing both mature and immature leaves of each source were pooled individually and brought to the laboratory. Thereafter, the samples were ground individually to pass through a 1-mm sieve. Thus three airtight plastic containers containing three different test sources were prepared and preserved until further analysis. A basal diet was prepared by mixing ground *Eleusine coracana (*Ragi) straw as a roughage source and concentrate (40% maize, 35% soya, 22% bran, 2% mineral mixture and 1% salt) at 70: 30 ratio.

2.2 Chemical Composition Analysis

The phyto-sources were analyzed for crude protein (CP) as per AOAC [6], where CP refers to (Nitrogen content X 6.25). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) fractions were analyzed by Van Soest et al. [7]. Total phenol (TP) and total tannins (TT) were estimated by the Folin-Ciocalteu method using polyvinylpolypyrrolidine to separate non-tannin phenols from tannin phenols [8]. Absorbance for TP and TT were recorded at 725nm. The CT was analysed by Butanol- HCl-iron method and the absorbance were recorded at 550 nm [8]. Both TP and TT were expressed as gallic acid equivalents and CT as leucocyanidin equivalents. Finally, tannin content was reported as g/Kg DM content for each source. All the analysis was carried out in triplicates.

2.3 Gas Production Study

The gas production study of the graded level supplementation of these phyto-sources was carried out following Menke et al. [9] using *in vitro* gas production technique (IVGPT). The phyto-sources were incubated at 0, 5.0, 10.0, 15.0 and 20.0% (fresh basis) on over and above the basal diet [4] (Table 1). To calculate the dry matter content of each phyto-source, a sample of individual phyto-source were kept for dry matter analysis on the same day. To carry out IVGPT, rumen liquor was collected before feeding at 9.00 h from two cannulated crossbred male cattle fed on maintenance diet in accordance with ICAR (2013). Their diets were comprised of finger millet (*Eleusine coracana*) straw and concentrate mixture in 70:30 ratio. The rumen fluid collected from both the cattle was pooled and filtered through a muslin cloth into a pre-warmed beaker followed by mixing with McDougall buffer. The media prepared was maintained at 39°C under an anaerobic condition to provide similar rumen environment. About 30 ml of buffered rumen liquor was dispensed into 100 ml calibrated syringe containing 200 mg of fresh sample [9]. The syringes were then placed in a water bath maintained at 39°C. The initial plunger position was recorded for each syringe and the syringes were monitored to observe the gas production. Incubation was terminated after 24 h. Syringes without leaf sample and basal diet were served as blank per incubation. To check day-to-day variation in the quality of inoculum standard syringe containing concentrate and hay from Hohenheim University was used. The gas produced due to substrate fermentation after 24 h was calculated by subtracting the gas produced at 24 h from the blank syringe. The gas produced in 24 h was collected in an evacuated labelled glass vial and used for CH_4 estimation.

2.4 Methane Estimation

For estimation of CH_4, 1ml of gas was withdrawn using an airtight Hamilton syringe from the glass vial filled with collected gas and injected into the gas chromatograph (Chemito GC-1000, India) equipped with a thermal conductivity detector and Porapak Q column. Temperatures of injector, column, and detector were set at 60°, 100° and 110°C, respectively. The standard gas used for CH_4 estimation (Chemix specialities gases, Bangalore) was composed of 23.01% CH_4 and 33.5% CO_2. The peak of CH_4 gas was identified based on the retention time of standard CH_4 gas and the area obtained was used to calculate CH_4 percentage in the gas sample. This was followed by the calculation of CH_4 in ml.

Calculation of methane (%) and methane (ml)

$$CH4\% = (Concentration\ of\ standard\ gas)X(Area\ of\ test\ sample) / (Area\ of\ standard\ gas)$$

$$\mathrm{CH4}\ (ml) = \mathrm{CH4}(\%)\ X\ Netgas\ (ml) in\ 24h$$

2.5 *In-vitro* dry Matter Digestibility

In vitro dry matter digestibility (IVDMD) was estimated as per Goering and Van Soest [10]. In brief, test samples (500 mg on fresh basis) were used and gas production test was carried out *in vitro* similar as mentioned above. Here double strength buffer was used [11]. After 24 h of

Table 1. Diet preparation for the *in vitro* optimisation study (% of phyto-sources included in fresh basis to the basal diet)

Graded levels of phyto-source used in the study (%)	Basal diet (mg)	Phyto-source (mg)	Total sample incubated (mg)
0	200	0	200
5	190	10	200
10	180	20	200
15	170	30	200
20	160	40	200

incubation, dry matter digestibility was measured by transferring the contents into labelled fibre bags. Then the syringe was re-washed to remove the remaining residual feed and the contents were poured into fibre bags. The IVDMD was analysed by boiling the contents in 100 (ml/sample) of NDS solution for 1 h and once the boiling was over, the samples were filtered and kept for drying in the hot air oven at 90°C. The residue left is weighed after 24 h to determine IVDMD percent.

Calculation:

$$\text{IVDMD (\%)} = \frac{\text{Wt. of DM incubated} - \text{Wt. of NDF residue}}{\text{Wt. of DM incubated}} \text{X } 100$$

2.6 Fermentation Characteristics

After termination of incubation, rumen fluid was transferred to a pre-cooled 50 ml centrifuge tube and pH was recorded immediately using a pH meter (Eutech Instruments). The rumen fluid was also analysed for ammonia-N using method prescribed by Conway [12]. To estimate total volatile fatty acid (TVFA), 2 ml of buffered rumen fluid and 2 ml of TVFA buffer (potassium oxalate + oxalic acid) were taken in a Markham apparatus and the distillate collected in an ice cold conical flask was titrated against 0.01N sodium hydroxide [13].

2.7 Enzyme Assay of Fermented Rumen Liquor

After 24 h of the incubation, the content of each syringe was taken in 100 ml beaker and enzymes were extracted as per Agarwal et al. [14]. Buffered rumen liquor was treated with carbon tetrachloride and 0.4% lysozyme solution at the rate of 5 ml each per 30 ml of syringe content and incubated at 39°C for 3 h followed by sonication (Vibra cell model VCX-130). The sonicated samples were centrifuged at 24,000 g for 20 min at 4°C (Kuboto 6500, centrifuge) and the supernatant was used for enzyme estimation. The protein content of the enzymes was estimated as per Lowry et al. [15] using BSA as standard.

2.8 Rumen Protozoa Enumeration

After 24 h of completion of *in vitro* gas production test, the rumen fluid was preserved for protozoa enumeration by mixing it with formaldehyde at 1:2 ratio in a 5 ml microcentrifuge tube. The protozoa were identified morphologically as per Hungate [16] and calculated as per Kamra et al. [17].

2.9 Statistical Analysis

Data analysis for this study was carried out using SPSS [18] version 20.0 software package. Data were analysed by one way analysis of variance (ANOVA) with a model that includes treatment effect and experimental error. Individual levels were considered as experimental units and the same procedure was carried out for all the three samples. Differences between means were compared by Tukey's method and considered significant at *P* value <.05. The results were presented as means with standard error and *P* value. Superscripts have been placed wherever means were significant at 5%.

3. RESULTS AND DISCUSSION

3.1 Tannin Content of Basal Diet and Chemical Composition

The tannin content and chemical composition of the basal diets comprising three phyto-sources at graded level are presented in table 2. The organic matter content of the selected phyto-sources varies between 847-864 (g/Kg DM). Among the phyto-sources, *M. bombycina* has the highest crude protein content and *A. zapota* has the lowest. The fibre fraction study shows *M. bombycina* of having the highest NDF and ADF.

The highest fat content was recorded in *A. zapota*. The total phenolic study shows *S. cumini* with the highest TP, TT and HT, while *M. bombycina* had the highest CT content. *A. zapota* has a mixed composition of both the tannin. The non-tannin phenolics ranged between 8.3-21.4 (g/kg DM).

3.2 Total Gas and Methane

Supplementation of phyto-sources at increasing dose in the basal diet significantly (*P*=.05) influenced total gas production (Table 3). At 5% inclusion of *M. bombycina* in the basal diet, a significant (*P*=.05) reduction in total gas was recorded, while at 15% in case of *S. cumini.* On the other hand, *A. zapota* has affected gas production when included at 20% in the basal diet. To understand this variable effect, effective tannin concentration at each dose for individual phyto-source were compared (Table 2). At 5% supplementation of *M. bombycina* in the basal diet, the effective tannin contribution was 0.50% of CT and 0.04% HT, while *S. cumini* at 15% contributes 0.71% CT and 4.98% HT to the basal diet to bring about a significant (*P*=.05) change in total gas.

This observation clearly suggests the role of CT in influencing gas production. This could be due to the presence of the large number of free phenolic hydroxyl groups in CT which forms a strong complex with carbohydrate [19], thereby reducing their degradability and hence reduces the total gas production. Contrary to them, *A. zapota* was effective at its highest inclusion level (20%) in the diet where it contributes 1.76% CT and 2.08% HT to the basal diet. To achieve a significant effect on gas production, *A. zapota* needs more CT contribution to the diet, which shows the presence of a different kind of CT in *A. zapota*. As CT is oligomeric or polymeric

Table 2. Tannin content in the basal diet and chemical composition of the phyto-sources

	S. cumini	***M. bombycina***	***A. zapota***	**SEM**	***P*-Value**
Organic matter[1](g/Kg DM)	863.6[a]	847.2[c]	851.8[b]	0.244	<0.001
Crude protein[1](g/Kg DM)	78.0[b]	123.3[a]	69.9[c]	8.39	<0.001
NDF[1](g/Kg DM)	405.3[c]	520.8[a]	469.2[b]	16.8	<0.001
ADF [1](g/Kg DM)	378.5[b]	469.2[a]	337.1[c]	19.5	<0.001
Ether Extract[1](g/Kg DM)	19.0[c]	52.1[b]	54.3[a]	0.62	<0.001
Phenolic content					
Total phenol[2] (g/Kg DM)	211.4[a]	62.7[c]	107.6[b]	15.1	<0.001
Non tannin phenol[2](g/Kg DM)	21.4[a]	8.3[c]	10.9[b]	1.38	<0.001
Total Tannin[2] (g/Kg DM)[*]	190.0[a]	54.4[c]	96.7[b]	13.7	<0.001
Condensed tannin[2](g/KgDM)[**]	23.5[c]	50.4[a]	44.4[b]	2.76	<0.001
Hydrolysable tannin[2] (g/KgDM)	166.4[a]	4.05[c]	52.1[b]	16.4	<0.001
Tannin content in the basal diet	***S. cumini***				
Level of phyto-sources % in the basal diet	0	5	10	15	20
Total tannin %	0.0	1.90	3.80	5.70	7.60
CT %	0.0	0.24	0.47	0.71	0.94
HT%	0.0	1.66	3.32	4.98	6.64
Tannin content in the basal diet	***M. bombycina***				
Level of phyto-sources % in the basal diet	0	5	10	15	20
Total tannin %	0.0	0.54	1.08	1.62	2.16
CT %	0.0	0.50	1.00	1.50	2.00
HT%	0.0	0.04	0.08	0.12	0.16
Tannin content in the basal diet	***A. zapota***				
Level of phyto-sources % in the basal diet	0	5	10	15	20
Total tannin %	0.0	0.96	1.92	2.88	3.84
CT %	0.0	0.44	0.88	1.32	1.76
HT%	0.0	0.52	1.04	1.56	2.08

NDF= Neutral detergent * Tannic acid equivalent; Leucocyanidin equivalent 1 represents three replicate of each; 2 represent six replicate of each; Mean values bearing different superscripts in a column differ significantly (p<0.05); SEM= standard error of means*

proanthocyanidins consisting of cathecin units which are formed by successive condensation of the single building blocks with a degree of polymerisation between two and greater than fifty blocks, different kind of CT can be expected to exist in nature [20]. Thus the number and pattern of polymerisation might affect the biological activity of one CT from other. A view on the CH_4 ml production at various levels shows that tannin effect from these phyto-sources is more prominent on CH_4 as compared to total gas. *S. cumini* decreased 2.35 ml of CH_4 on an average compared to the basal diet as its supplementation increased, while *M. bombycina* shows an average decrease of 2.22 ml and 1.3 ml of CH_4 in case of *A. zapota*. At a percentage level of CH_4 reduction, these phyto-sources could reduce 23.8-43.7% of CH_4 as compared to the basal diet at their highest inclusion level (Table 3). The prominent effect on CH_4 as compared to total gas certainly confirms that gas production is related to the fibre degradation, while the reduction in CH_4 could be due to the more direct effect on the rumen microbes responsible for CH_4 production. Upon considering the proportion of tannin and its contribution toward CH_4 reduction, *S. cumini* at 5% inclusion level contributes 0.24% CT and 1.66% HT, while *M. bombycina* contributes 0.50 % CT and only 0.04% HT. On the other hand, *A. zapota* contributes comparable concentration of CT (0.44%) as that of *M. bombycina,* but little more (0.52%) of HT to the basal diet. A careful observation of tannin fraction contribution by the three phyto-source shows that for reducing CH_4, both HT as well as CT are required. This result can be supported by the previous study carried out by Jayanegara et al. [21] which suggest HT reduces CH_4 emission by directly targeting the activity of methanogens or hydrogen producing bacteria as compared to CT, where the CH_4 abatement is associated with the reduction in fibre digestion. While comparing the CH_4 reduction in percentage among the three phyto-sources, *M. bombycina* and *S. cumini* reduce more CH_4 as compared to *A.zapota*. This difference in effect surely reflects the different type of CT composition in *A. zapota*. Different types of CTs are formed with different ortho-phenolic groups in them. Haslam [22], said that the biological activity of tannin depends mainly on the molar concentration of ortho-phenolic groups in the tannin. Apart from HT and CT content, the presence of high concentration of non-tannin phenolics in *S. cumini* and *A. zapota*, might have additive effect with HT in reducing CH_4, as non-tannin phenols determine the tannin bioactivity of a phyto-source and account for CH_4 reduction without compromising the protein and nutrient utilization [23,24]. However, from this study, it can be inferred that a phyto-source with both types of tannin content in almost equal proportion might not necessarily have a strong CH_4 reduction capability compared to the phyto-source which have either of the tannins in a larger extent. This result is different from a previous study carried out by Bhatta et al. [25] who reported that both HT and CT together reduces more CH_4 as compared to individual HT and CT. This difference of response could be due to the difference in nature of HT and CT composition of our studied phyto-source.

3.3 Dry Matter Digestibility

In vitro dry matter digestibility (IVDMD) was affected with increased phyto-source inclusion in the basal diet (Table 3). The immediate effect on IVDMD was recorded with CT rich *M. bombycina,* where significant (P=.05) effect on IVDMD was recorded at 5% of its inclusion in the diet. It reduces 0.76% of digestibility at the significant levels compared to the basal diet. On the other hand, *A. zapota* had significantly reduced IVDMD at 15%, while *S. cumini* was effective at 10%. It is observed from the study that *M. bombycina* at 5% inclusion level in the basal diet contributes 0.50% CT and 0.04% HT. While *A. zapota* at 15% inclusion level contributes 1.32% CT and 1.56% HT, whereas *S. cumini* contributes 0.47 % CT and 3.32% HT at its significant level. The CT fraction present in these phyto-sources binds to the fibre and hence prevents the interaction of fibrolytic microbes thereby affecting digestibility. Apart from that some CT covalently linked via ether bridges C-4 to carbohydrates in analogy to the C-C bridges in CT which also impacts fibre digestion [26]. However, Mueller-Harvey et al. [26] study also mention that binding between polyphenols and polysaccharides depends on the molecular size of the polyphenol and its conformational flexibility which in turn determines the impact of polyphenols (tannins) on IVDMD. On the other hand, the role of HT cannot be ignored as the reduction in IVDMD in case of *S. cumini* relies mainly on the HT fraction. A study carried out by Jayanegara et al. [27] using Sumach tannin (HT) shows its toxicity towards *Ruminococcus flavefaciens,* which is a rumen cellulolytic bacteria, thus giving an idea that HT in our study might be toxic to certain fibre degrading bacteria. Even the large content of non-tannin phenolics might have contributed to reducing digestibility. Since non-tannin phenolics

Table 3. Comparison of effect of inclusion of phyto-source in the basal diet on total gas, methane percent in total gas, methane ml, reduction in methane in percentage and IVDMD

Source	***S. cumini* (HT source)**						
Level of phyto-sources % in the basal diet	0	5	10	15	20	SEM	*P*-Value
24 h gas production (ml 200-1 mg DM)	35.1^{a}	34.9^{a}	34.4^{ab}	33.7^{b}	32.7^{c}	0.183	0.001
CH_4% of total gas	20.6^{a}	16.3^{b}	15.2^{c}	13.7^{d}	12.4^{e}	0.530	0.001
CH_4 ml 200^{-1} mg DM	7.2^{a}	5.7^{b}	5.2^{c}	4.6^{d}	4.0^{e}	0.204	0.001
CH_4 reduction (%)	0^{a}	21.0^{b}	27.2^{c}	36.1^{d}	43.7^{e}	2.79	0.001
IVDMD%	65.4^{a}	65.2^{ab}	64.8^{b}	64.1^{c}	64.0^{c}	0.120	0.001
Source	***M. bombycina* (CT source)**						
Level of phyto-sources % in the basal diet	0	5	10	15	20	SEM	*P*-Value
24 h gas production (ml 200-1 mg DM)	35.8^{a}	35.1^{b}	34.7^{b}	33.3^{c}	33.0^{c}	0.212	0.001
CH_4% of total gas	20.3^{a}	16.0^{b}	15.1^{c}	14.9^{c}	13.4^{d}	0.438	0.001
CH_4 ml 200^{-1} mg DM	7.3^{a}	5.6^{b}	5.3^{c}	5.0^{d}	4.4^{e}	0.181	0.001
CH_4 reduction (%)	0^{a}	23.2^{b}	28.2^{c}	31.8^{d}	39.5^{e}	2.49	0.001
IVDMD%	65.5^{a}	65.0^{b}	64.4^{bc}	64.2^{c}	64.1^{c}	0.139	0.001
Source	***A. zapota* (CT+HT Source)**						
Level of phyto-sources% in the basal diet	0	5	10	15	20	SEM	*P*-Value
24 h gas production (ml 200-1 mg DM)	35.2^{a}	35.2^{a}	34.8^{a}	34.5^{ab}	33.2^{b}	0.162	0.001
CH_4% of total gas	20.5^{a}	18.1^{b}	17.2^{bc}	16.7^{c}	16.5^{c}	0.305	0.001
CH_4 ml 200^{-1} mg DM	7.2^{a}	6.3^{b}	6.0^{b}	5.8^{bc}	5.5^{c}	0.119	0.001
CH_4 reduction (%)	0^{a}	12.5^{b}	16.0^{b}	20.1^{c}	23.8^{d}	1.56	0.001
IVDMD %	65.6^{a}	65.4^{a}	65.1^{a}	64.6^{b}	64.3^{b}	0.096	0.001

**CT=Condensed tannin, HT= Hydrolysable tannin, DM= Dry matter, IVDMD= In vitro dry matter digestibility, SEM= Standard error of means, Mean values bearing different superscripts in a row differ significantly (p<0.05)*

like the derivatives of cinnamic acid, ferulic and p-coumaric acids influence IVDMD by esterifying to the carbohydrates in plant cell walls [26].

3.4 Rumen Fermentation Characteristics

There was no significant effect of the phyto-source supplementation at the graded level on rumen pH. The pH reported in this study is in the acceptable range for an adequate process of fermentation. Even though there is a graded effect of the phyto-sources on IVDMD, Van Soest [28] mentioned that the decrease in cellulose digestibility may not always be the result of a reduction in ruminal pH. A similar effect of tannin on rumen pH was also observed by Oliveira et al. [29], upon studying the effect of low and high tannin content sorghum silage on CH_4 emission and associated fermentation parameters. TVFA in the rumen is produced mainly by the fermentation of organic matter in the rumen and is influenced by substrate composition, fibre depolymerisation, microbial population and substrate availability. The ability of tannin to complex with macromolecules, influences the fibre depolymerisation and also substrate availability, thus reflects through affecting TVFA production. This effect was evident in the case of *M. bombycina* where TVFA reduction and its effect on IVDMD were comparable at 5% of its inclusion in the basal diet. Thus suggesting both the effect is a result of fibre binding and reduction in substrate availability. However, all CT do not behave similarly due to the difference in their structure. For example, at 10-15% inclusion level of *S. cumini* and 15% of *A. zapota* a significant (*P*=.05) change in TVFA was recorded. In this study it is also observed that both HT and HT+CT source had a greater impact on CH_4 production as compared to the TVFA suggesting the reduction of CH_4 is primarily due to antimethanogenic activity rather than tannin's negative effect on digestibility. For instance, *S. cumini*, a HT source could affect TVFA at 10-15% inclusion level in the basal diet, while at a very lower level inclusion it could reduce CH_4 reinforcing the effect it had on rumen methanogens rather than on fibre degradation. Apart from fibre, tannin form complex with protein, thus preventing its degradation at rumen pH. A large number of the free phenolic group present in tannin forms strong hydrogen bonds at multiple sites with proteins [30] affecting ammonia-N. In this study, ammonia-N showed a

considerable reduction (*P*=.05) in all treatments (*P*=.05) compared to the control. Significant (*P*=.05) change in ammonia-N was noted at 5% inclusion level in case of *S. cumini* and *M. bombycina,* whereas 10% in case *of A. zapota* in the basal diet (Table 4). The contribution of CT fraction from both *M. bombycina* and *A. zapota,* might be responsible for binding protein strongly, thus affecting ammonia-N at those levels in the diet. Among the two sources, the effective CT contribution at those level by *M.bombycina* is 0.5% while 0.88% by *A.zapota*. The difference in the type and quantity of CT might be responsible for showing the significance effect at different inclusion levels by the phyto-sources. Our study is supported by the meta-analysis report of Jayanegara and Palupi [30] on CT effect on nitrogen digestion, which states that CT has a negative effect on protein digestibility due to its ability to form a complex with protein and therefore affects rumen ammonia-N production. Their report also mentions that proteolytic microbes are more sensitive to tannin than fibrolytic bacteria. The structural variation between CT and HT leads to the more protein protection ability of CT than HT, thereby leading to less emission of ammonia from animals consuming CT supplemented diet [31]. However, in CT+HT rich phyto-source, the proportion of individual components also determines the kind of effect it has on ammonia as rumen bacteria can dissociate protein-HT complex easily as compared to protein-CT complex [25]. Thus the observed differences in ammonia-N with CT+HT rich sample (*A. zapota*) could also be due to the ratio of HT to CT in the phyto-source, apart from the difference in CT type. In the case of *S. cumini* which contributes 1.66% HT and only 0.24% CT, at 5% inclusion level suggest that the reduction in ammonia-N observed in *S. cumini* is due to the HT fraction rather than CT. Abarghuei et al. [32] in an *in vivo* study using HT from oak leaves (*Quercus libaniOliv*.) on sheep found significant (*P*=.05) reduction in the proteolytic bacteria suggesting the toxicity of HT on proteolytic bacteria. Thus the ammonia-N reduction observed using *S. cumini* might be due to its toxicity towards proteolytic bacteria rather than forming HT-protein complex.

3.5 Enzyme Activity

The effect of graded level of phyto-sources on enzyme activities is presented in table 5. In the present study, tannin from the three sources at different levels has impacted enzyme activity significantly (*P*=.05). The increase or decrease in enzyme activity could be due to the change of conformation of the enzyme which results in inhibition or activation of enzyme depending on the exposing of the catalytic site of the enzyme to the substrate [33]. Overall, a reduction of 1.4-5.7% carboxy methyl cellulose (CMC) activity was recorded at the respective significant level of the phyto-source inclusion in the diet was observed. In case of HT source, reduction of CMC at 5% supplementation could be due to formation of complex by HT with cellulase enzyme, which can precipitate, resulting loss in its cellulolytic activity [34]. The overall effect on CMC is also associated with rumen protozoa population as they contribute to this enzyme largely [35]. The decrease in rumen protozoa number due to the supplementation of the CT and the CT+HT source additionally reduced the CMC activity. The ability of CT fraction from both *M. bombycina* and *A. zapota* might form a complex with polysaccharide leading to less substrate availability for the enzyme to act on, thus reducing enzyme activity [35]. While in case of amylase, a reduction up to 8.2% was recorded for *A. zapota* at 5% of its inclusion in the basal diet. At that level *A. zapota* contributes 0.44% of CT to the diet. On the other hand, the CT source could depress amylase activity significantly (*P*=.05) at 15% of inclusion in the basal diet. Thus with increasing CT concentration, a decrease in amylase activity was observed. A similar observation was recorded by Gonçalves et al. [36] where increase CT concentration decreased α-amylase activity. They also mentioned that activity depends on the structure of CT. This was also observed in our study where *S. cumini* could affect amylase activity at 10% supplementation in the basal diet, but it is interesting to note that at 10% *S. cumini* contributes only 0.47% CT, which is less than the primary CT dense source used in this study to bring about the change. The hydroxyl groups present in the tannin structure might influences amylase activity which was also proved by Kato et al. [37] where they carried out a study using both CT and HT on human amylase activity. In this study, protease activity was significantly (*P*=.05) reduced in all the three phyto-sources. The way tannin act on the proteolytic bacteria and protozoa could be the reason for its effect on the protease enzyme. The ability of CT to form complex with the bacterial cell as well as with bacterial enzymes alter the growth of bacteria thereby influencing protease activity [38]. Abarghuei et al. [39] have found a similar response upon replacing *Alfa alfa* with grape pomace (a source of tannin) where the response

Table 4. Effect of phyto-sources on rumen fermentation parameters

Level of	*S. cumini*			*M. bombycina*			*A. zapota*		
(% of phyto-source in basal diet)	**pH**	**TVFA**	**Ammonia-N**	**pH**	**TVFA**	**Ammonia-N**	**pH**	**TVFA**	**Ammonia-N**
0	6.74	14.1^{a}	23.3^{a}	6.83	14.0^{a}	23.1^{a}	6.83	13.9^{a}	25.9^{a}
5	6.74	14.0^{ab}	21.7^{b}	6.83	13.9^{b}	20.5^{b}	6.83	13.7^{ab}	24.9^{a}
10	6.73	13.9^{bc}	21.5^{b}	6.83	13.8^{c}	19.4^{c}	6.83	13.7^{ab}	23.3^{b}
15	6.73	13.8^{c}	20.5^{b}	6.82	13.6^{d}	17.9^{d}	6.83	13.6^{b}	21.7^{c}
20	6.73	13.5^{d}	20.5^{b}	6.82	13.4^{e}	16.1^{e}	6.82	13.5^{b}	20.5^{c}
SEM	0.001	0.038	0.237	0.001	0.052	0.452	0.001	0.042	0.388
***P*-Value**	0.158	0.001	0.001	0.097	0.001	0.001	0.158	0.005	0.001

**Mean values bearing different superscripts in a column differ significantly (p<0.05); SEM= standard error of means, TVFA = total volatile fatty acids, is expressed in mmol/dl and ammonia-N in mg/dl.*

Table 5. Effect of graded level supplementation of phyto-sources on rumen enzyme activities

Phyto-source	***S. cumini***		
Level of inclusion in the basal diet (%)	CMC	Amylase	Protease
0	19.7^{a}	13.6^{a}	33.9^{a}
5	19.3^{b}	13.2^{ab}	32.9^{ab}
10	19.2^{b}	13.1^{b}	32.1^{b}
15	19.1^{b}	13.0^{b}	32.0^{b}
20	18.9^{b}	12.9^{b}	31.6^{b}
SEM	0.069	0.072	0.227
p-Value	0.001	0.011	0.001
Phyto-source	***M. bombycina***		
Level of inclusion in the basal diet (%)	CMC	Amylase	Protease
0	13.8^{a}	13.1^{a}	36.7^{a}
5	13.7^{ab}	12.8^{ab}	35.4^{a}
10	13.6^{bc}	12.6^{ab}	35.2^{a}
15	13.6^{c}	12.3^{b}	33.2^{a}
20	13.6^{c}	12.3^{b}	32.2^{b}
SEM	0.023	0.079	0.322
p-Value	0.001	0.001	0.001

Phyto-source	***A. zapota***		
Level of inclusion in the basal diet (%)	CMC	Amylase	Protease
0	17.5^{a}	20.8^{a}	35.6^{a}
5	17.2^{a}	19.1^{b}	33.5^{b}
10	17.1^{a}	17.5^{bc}	33.5^{b}
15	16.5^{b}	16.3^{cd}	32.9^{b}
20	15.8^{c}	15.6^{d}	32.2^{c}
SEM	0.132	0.418	0.307
P-Value	0.001	0.001	0.001

**1 IU= ug of glucose/ml/min (CMC and Amylase),1IU=ug of hydrolysed protein/min/ml, CMC= Carboxy methyl cellulase, SEM= standard error of means*

of cellulolytic and proteolytic bacteria reverted significantly (*P*=.05) with the addition of PEG to the diet suggesting the influence of tannin on rumen microbes. The impact of the HT fraction of the phyto-sources on the enzyme activity could also vary due to the presence of tanninolytic microbes which degrade them, thus influencing the bound tannin at a particular time.

3.6 Rumen Protozoa

The effect of graded level of phyto-sources on rumen protozoa population is presented in table 6. In this study, a significant (*P*=.05) effect of the phyto-sources on different categories of protozoa was observed. It is found that rumen ciliates especially entodina were majorly affected by all the three phyto-sources and this reduction were prominent at higher inclusion level. It was noted that as tannin percentage was increasing, the rumen protozoa population started to decrease. In the case of *S. cumini* there was partial reduction in entodina population at 10% of its inclusion which contributes (0.47% CT and 3.32% HT) to the diet, but as the inclusion level reaches 20% of basal diet where the effective tannin concentration increased to 0.94% CT and 6.64% HT, it could bring significant (*P*=.05) effect on rumen entodina population. In the case of *M. bombycina*, with 10% inclusion in the diet, it could bring 13% reduction in total protozoa population. At this level, the effective tannin contribution to the diet is 1% CT and 0.08% HT. A reduction of 87% in holotricha population was recorded at 20% inclusion (2% CT and 0.16% HT) of *M. bombycina*. On the other hand, *A. zapota* at 15% inclusion level could significantly (*P*=.05) affect entodina population. It could reduce 11.5% of the entodina population and 71% holotricha population compared to the basal diet at this level. The high susceptibility of holotricha observed with *A. zapota* and *M. bombycina* samples could be due to the high crude fat content of both the phyto-source, as fat limits the metabolising capability of protozoa and thereby reduces its number. A similar effect was observed by Yanez et al. [40] upon using olive leaf having high crude fat content affecting the

Table 6. Effect of graded level of phyto-source on rumen protozoa (X 10^5 ml)

Phyto-source	***S. cumini***		
Level of phyto-source in the basal diet	**Entodina**	**Holotricha**	**Total Protozoa**
0	0.236^{a}	0.006	0.243^{a}
5	0.223^{ab}	0.006	0.229^{ab}
10	0.208^{bc}	0.006	0.215^{bc}
15	0.207^{bc}	0.006	0.213^{bc}
20	0.195^{c}	0.006	0.201^{c}
SEM	0.004	0.000	0.004
P-Value	0.001	1.00	0.001
Phyto-source	***M. bombycina***		
Level of phyto-source in the basal diet	Entodina	Holotricha	Total Protozoa
0	0.241^{a}	0.008^{a}	0.249^{a}
5	0.218^{ab}	0.006^{ab}	0.225^{ab}
10	0.212^{b}	0.004^{ab}	0.216^{b}
15	0.203^{b}	0.003^{ab}	0.206^{b}
20	0.200^{b}	0.001^{b}	0.200^{c}
SEM	0.004	0.001	0.004
P-Value	0.001	0.011	0.001
Phyto-source	***A. zapota***		
Level of phyto-source in the basal diet	Entodina	Holotricha	Total Protozoa
0	0.236^{a}	0.007^{a}	0.243^{a}
5	0.231^{ab}	0.004^{ab}	0.236^{ab}
10	0.219^{ab}	0.003^{ab}	0.221^{ab}
15	0.214^{ab}	0.003^{ab}	0.215^{b}
20	0.212^{b}	0.002^{b}	0.215^{b}
SEM	0.003	0.001	0.003
P-Value	0.021	0.038	0.012

Mean values bearing different superscripts in a column differ significantly (p<0.05); SEM= standard error of means

holotrich population in the rumen liquor. The effect of tannin on total protozoa is supported by the effect of tannin on ammonia N as well since protozoa contribute a lot towards ammonia-N due to its high deaminase activity [41]. The previous report from Bhatta et al. [25] shows protozoa are more sensitive towards CT and also towards HT+CT containing samples as compared to HT containing samples, but in this study, HT supplemented basal diet has also affected total protozoa population. However, there were reports which stated that tannins had no effect on rumen protozoa as in case of Benchaar et al. [42] where supplementation of CT from Quebracho trees (150g CT/cow/day) had no effect on the numbers and generic distribution of rumen protozoa. Such discrepancy could be due to the type of tannin, origin, and level of application [39].

4. CONCLUSION

It can be concluded from this study that selected phyto-sources are a potent CH_4 inhibitor. Considering all the major rumen fermentation parameters, it can be inferred from this study that *S. cumini* (HT source) and *A. zapota* (HT+CT source) can be included at a maximum of 10% in the basal diet (200 mg), while the CT source (*M. bombycina*) can be included at a maximum of 5%, so that CH_4 reduction could be achieved without hampering any physiological parameters. However, long-term *in vivo* trials should be conducted to determine the efficacy of these phyto-sources on enteric CH_4 emission as well as its effect on rumen fermentation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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