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Modulating Natural Methane Release from Rumen Fermentation through the Use of *Ficus glomerata* Leaf Tannins in Murrah Buffalo (*Bubalus bubalis*)

Ram Kumar Singh, Avijit Dey * and Mala Singh

Division of Animal Nutrition and Feed Technology, ICAR-Central Institute for Research on Buffaloes, Hisar 125 001, India

* Correspondence: avijit.dey@icar.gov.in or avijitcirb@gmail.com; Tel.: +91-9728-334810; Fax: +91-1662-275004

Abstract: Enteric fermentation is one of the largest contributors of methane release to the environment from the livestock sector. Plant bioactive compounds can modulate rumen fermentation for reduced methanogenesis and fatty acid biohydrogenation. The present study investigates the effects of tannin extract from *Ficus glomerata* (FG) leaves on the rumen fermentation, methanogenesis, feed digestibility and fatty acid biohydrogenation of a total mixed ration with the aim of developing a feed supplement for enhanced livestock production and product quality with lower methane emission. The tannin extract (70% aqueous acetone extract) of FG leaves in the total mixed ration (oat hay/concentrate mixture; 1:1) was studied at four graded dose regimens (0.0 (control), 0.25 mL (FG-0.25), 0.50 mL (FG-0.50) and 1.0 mL (FG-1.0) per 60 mL of buffered rumen fluid) in three replicates for each treatment in a radio-frequency-based automatic gas production system (ANKOM-RF) at 39 °C for 24 h following the standard in vitro gas production protocol. The total gas production (mL or mL/g incubated dry matter (DM)) was gradually reduced ($p < 0.01$) at dose levels of FG-0.50 and FG-1.0; however, it remained intermediary and comparable ($p > 0.05$) for FG-0.25 with the control and FG-0.50. Compared to the control, the methane concentration (%) in the head space gas, as well as the total methane production (mL or mL/g DM incubated, or mL/g DM digested), were found to be gradually reduced ($p < 0.01$) with increasing doses (0.25–1.0 mL) of FG extract. The reduced ($p < 0.05$) feed degradability at higher levels (0.50–1.0 mL) of FG extract supplementation and the comparative ($p > 0.05$) effects with the control at a lower level of supplementation (FG-0.25) are suggestive of the dose-responsive detrimental effects of tannins on fibrolytic microbes in the rumen. However, the ammonia concentration decreased ($p < 0.05$) in all of the incubations compared to the control. Among the volatile fatty acids, acetate remained comparable ($p > 0.05$) with enhanced ($p < 0.05$) propionate at a lower dose (FG-0.25); however, a dose-dependent reduction was evident at higher dose levels (FG-0.50 and FG-1.0). The production of stearic acid (C18:0), which is a product of the rumen biohydrogenation process, was reduced ($p < 0.05$), irrespective of the concentration of the FG extract. Compared to the control, the concentration of *t*-vaccenic acid (C18:1), which is a precursor of conjugated linoleic acid (CLA) in animal products, was increased in all the FG-extract-supplemented groups. It may be concluded that *Ficus glomerata* leaf tannins can modulate rumen fermentation for reduced methanogenesis and fatty acid biohydrogenation in a total mixed ration. As a higher level of inclusion negatively affects feed digestibility, a lower dose (0.25 mL FG extract per 60 mL fermentation fluid or 4.17 mL FG extract per L of fermentation fluid) is suggested to achieve desirable effects on methane abatement (30%) and an improvement in fatty acid profiles in animal products.

Keywords: enteric methane emission; rumen biohydrogenation; *Ficus glomerata*; leaf tannins; buffalo

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1. Introduction

The rumen is a natural fermenter in livestock and is responsible for degrading inedible feedstuffs by means of a diverse population of microorganisms to produce volatile fatty

acids, which are a source of energy for the purposes of animal maintenance and production. The byproducts of this fermentation process are carbon dioxide and hydrogen, which form methane using the archaea that are present in the rumen and are emitted in the environment, mainly via eructation [1]. Globally, ruminants are one of the major sources of anthropogenic methane and nitrous oxide emissions, which have attracted the attention of environmentalists. The health of the environment is deteriorating day by day, and the ill effects of climate pollution are already being witnessed. Various pandemics, disease occurrences and the shrinking lifespan of human beings are directly or indirectly associated with climatic conditions; even international agencies [2] are addressing the correlation of various health hazards with climate safety. Moreover, the World Economic Forum report recently addressed the catastrophic Environmental Performance Index (EPI) of many developing countries compared with the global scenario [3]. The feed fermentation inside ruminants leads to significant dietary losses (6–10% gross energy loss and around 8–14% digestible energy loss), which results in higher input rearing costs [4].

Phytochemicals have strong antimicrobial properties and the potential to modify rumen fermentation to reduce enteric methane emission from ruminants [5]. The antimicrobial effect of essential oils suggests their relevance as anti-methanogenic feed additives, but it is challenging to maintain feed digestibility due to their general inhibitory effects on rumen microbes. Tannins are polyphenolic compounds and have the ability to modulate various rumen functions, viz., feed digestion, methane production, protein degradation, fatty acid biohydrogenation, etc., depending on the dose of supplementation. Condensed tannins near neutral pH form complexes with dietary proteins, carbohydrates, alkaloids, nucleic acids and minerals in the rumen, resulting in suppressed feed digestibility and the release of H_2 [6]. Tannins, especially condensed tannins, have marked impacts on rumen microbial proliferation [7]. However, the intensity of the effect varies with the source, type and concentration of tannins in the fermentation media. They may detrimentally affect rumen fermentation and reduce the production performance of animals [8]; however, their strategic use could improve animal health, milk production and the fatty acid profile in the milk of ruminants with a reduction in enteric methane and ammonia emissions [9].

Ficus glomerata, a species of plant in the family *Moraceae*, is native to Australia and tropical Asia. The leaves of the plant are rich in tannins, especially condensed tannins [10]. Therefore, the present study is conducted to examine the graded dose response of *Ficus glomerata* leaf extract on rumen fermentation, methane production and fatty acid biohydrogenation under an in vitro fermentation system with buffalo (*Bubalus bubalis*) rumen inoculum.

2. Results and Discussion

The modulation of rumen fermentation by plant bioactive compounds with the aim of reducing enteric methane production and enhancing the efficiency of animal production is an important research area for animal scientists as well as environmentalists. Tannins are naturally occurring plant secondary compounds that exhibit diversified action in animal systems depending upon their source, type, concentration, and delivery system to animals, basal diet, frequency of feeding, etc. [11]. The leaves of *Ficus glomerata* (FG) were found to be rich in polyphenolic compounds, with a significant concentration of condensed tannins (Table 1), which have the potential to modulate rumen fermentation [10]. The chemical composition of the oat hay/concentrate mixture (Table 1) used as a substrate for in vitro studies exhibited values within the range for Indian feeds and fodders [12]. The total gas production (mL or mL/g dry matter (DM) incubated) was gradually reduced ($p < 0.01$) at the dose levels of FG-0.50 and FG-1.0 (Figure 1); however, it remained intermediary and comparable ($p > 0.05$) for FG-0.25 with the control and FG-0.50, indicating the effect of tannins on rumen microbial activity. Compared to the control, the methane concentration (%) in the head space gas, as well as total methane production (mL or mL/g DM incubated, or mL/g DM digested), were found to undergo a gradual reduction ($p < 0.01$) with increasing doses (0.25–1.0 mL) of FG extract. Tannins have inhibitory effects on rumen

protozoa and methanogenic archaea depending on the source and dose levels [13,14]. *Ficus glomerata* leaf extracts are rich in condensed tannins (Table 1), which could have role in reducing the archaeal population, thereby lowering methane production (Figure 2). However, no effects on methane production by tannins have been reported by many researchers [15,16], probably due to the addition of lower dose levels in their experiments.

Table 1. Chemical composition (% dry matter basis) of mixed feed (oat hay/concentrate mixture) used as substrate during in vitro studies.

Attributes	Oat Hay	Concentrate Mixture [1]	*Ficus glomerata* Leaf Meal
Chemical composition			
Organic matter	93.30 ± 1.12	91.07 ± 1.01	88.50 ± 1.23
Crude protein	7.80 ± 0.56	19.50 ± 0.67	14.85 ± 0.35
Ether extract	3.07 ± 0.60	4.12 ± 0.45	4.54 ± 0.24
Total ash	6.70 ± 0.72	8.93 ± 0.63	11.50 ± 0.82
Neutral detergent fiber	58.40 ± 1.35	44.60 ± 1.23	38.70 ± 1.67
Acid detergent fiber	43.70 ± 1.73	21.06 ± 0.96	31.52 ± 1.11
Phenolic fractions			
Total phenolics [2]	-	-	16.92 ± 0.52
Total tannin phenolics [2]			15.71 ± 0.41
Non-tannin phenolics [2]			1.22 ± 0.06
Condensed tannins [3]			10.64 ± 0.33

[1] Composed of the following ingredients (% parts): wheat grains, 20; maize grains, 15; mustard cake, 20; groundnut cake, 10; wheat bran, 32; mineral mixture, 2.0; and common salt, 1.0. [2] As tannic acid equivalent. [3] As leucocyanidin equivalent.

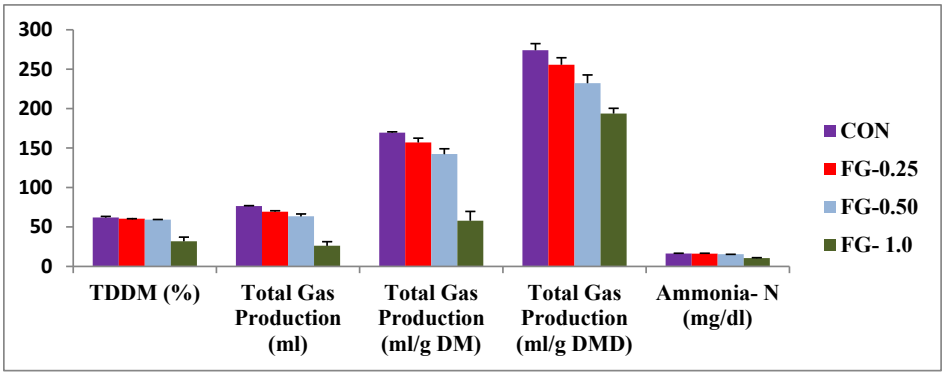

Figure 1. Effects of FG leaf extract supplementation in mixed feed substrate on truly degradable dry matter, total gas production and ammonia N.

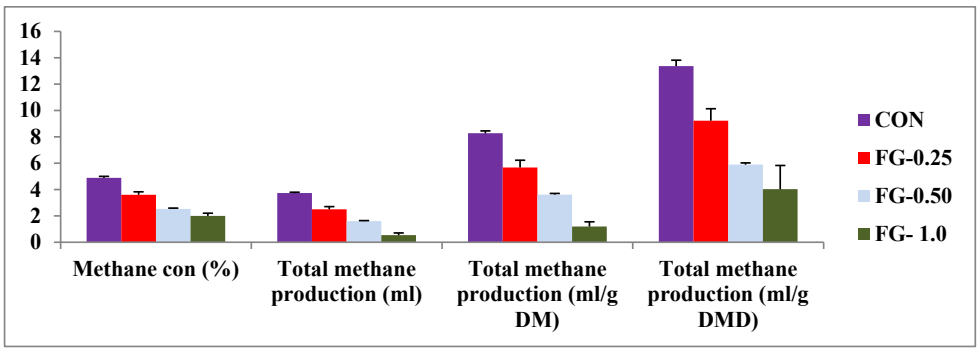

Figure 2. Effects of the FG leaf extract supplementation in mixed feed substrate on ruminal methanogenesis.

The reduced ($p < 0.05$) feed digestibility at higher levels (0.50–1.0 mL) of FG extract supplementation and comparative ($p > 0.05$) effects with the control (Table 2) at lower levels (FG-0.25) are suggestive of dose-responsive detrimental effects of tannins on fibrolytic microbes in the rumen. Reductions in anaerobic fungi, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* at 1 mg/mL of quebracho, mimosa and chestnut tannins were described [17]. Tannins at higher concentrations in diets remain free after binding with proteins and suppress fiber digestion by complexing with lignocellulose [18]. The reduction in gas production and methanogenesis at higher levels of FG extract (Figure 1) could also be attributed to reduced feed digestibility. The ammonia concentration decreased ($p < 0.05$) gradually in all the incubations in comparison to the control (Table 1). Tannins have an affinity for bacterial and plant proteins and reduce ruminal degradation [10,19], and thereby, ruminal ammonia production. However, ruminal protein protection and ammonia production depend on the type of tannins (hydrolysable or condensed tannins) [20]. The supplementation with FG leaf extract, which is rich in condensed tannins, might be responsible for the protection of substrate proteins, hence the reduced ammonia-N in the fermentation fluid (Figure 1). Supplementation with FG extract affected volatile fatty acid production in a dose-dependent manner. The molar proportion of acetate remained similar ($p > 0.05$) to that of the control in FG-0.25; however, it was reduced at higher doses (FG-0.50 and FG-1.0). Propionate production was enhanced ($p < 0.01$) in FG-0.25 but remained comparable ($p > 0.05$) in FG-0.50 with the adverse effect in FG-1.0 treatment. Butyrate production was comparable ($p > 0.05$) in all the treatments, except FG-1.0, where a reduction ($p < 0.01$) was observed. As the propionate production was enhanced ($p < 0.01$) in FG-0.25 and FG-0.50, the ratio of acetate to propionate was reduced; however, it remained comparable ($p > 0.05$) to the control in FG-1.0. The effects of tannins on rumen fermentation depend on the source, dose level as well as the substrate [5]. FG leaf extract is rich in condensed tannins (Table 1), which might have negative effects on fibrolytic rumen microbes at higher doses (FG-0.50 and FG-1.0), resulting a reduction in feed degradability and volatile fatty acid production [17]. While examining the effects of increasing doses of condensed tannin extract from *Cistus ladanifer* on in vitro ruminal fermentation and biohydrogenation, Guerreiro et al. [21] demonstrated a reduction in individual fatty acid production at all the studied doses. In the present study, the positive effect on propionate production at a lower dose level (FG-0.25) could be due to a shift in rumen fermentation via the modulation of rumen microbes and the reduction in methane production [9,13].

The modulation of rumen fermentation for reduced biohydrogenation of fatty acids is imperative for improving the quality of animal products [22]. Plant bioactive compounds, especially tannins, could modulate the biohydrogenation process to enhance mono- and poly-unsaturated fatty acids in milk and meat [14]; however, the effect varies with the type of tannins and their concentration [23]. *Ficus glomerata* leaves are rich in condensed tannins, and their extracts at graded levels of supplementation were found to modify ($p < 0.05$) various individual saturated fatty acids; however, the production of stearic acid (C18:0), a product of the rumen biohydrogenation process, was reduced ($p < 0.05$), irrespective of the concentration of FG extract (Table 3). Compared to the control, the concentration of *t*-vaccenic acid (C18:1), a precursor for conjugated linoleic acid (CLA) in animal products, was increased in all the FG extract-supplemented groups (Table 4). In an in vitro experiment by Carreño et al. [24] with four different tannin extracts (grape, quebracho, oak and chestnut), the inhibition of biohydrogenation with increased vaccenic acid concentration was described; however, a difference in their effects was delineated depending on the source and concentration. The supplementation of condensed tannins from *Acacia mearnsii* in a grass clover hay-based diet was demonstrated to enhance the intermediary products of biohydrogenation (cis-9, trans-11, cis-15 C18:3, trans-11, cis-15 C18:2 and trans-11 C18:1), suggesting the effectiveness of *Acacia mearnsii* CT for modulating biohydrogenation by removing potential microbes involved in BH. The tannin extracts of FG leaves, in the present study, might have modified the ruminal microbes, resulting in an increase (Figure 3) in *t*-vaccenic acid in the fermentation fluid.

Table 2. Effects of graded doses of tanniferous *Ficus glomerata* (FG) leaf extract on in vitro gas production, truly degradable dry matter, methanogenesis, volatile fatty acid production and fermentation characteristics of mixed feed substrate.

Attributes	Control	FG-0.25	FG-0.50	FG-1.0	SEM	*p* Value
Total gas production (mL)	$76.55^{c} \pm 0.47$	$69.32^{bc} \pm 1.32$	$63.48^{b} \pm 3.06$	$26.16^{a} \pm 5.27$	6.85	<0.001
Total gas production (mL/g DM)	$169.52^{c} \pm 1.14$	$157.07^{bc} \pm 5.57$	$142.36^{b} \pm 6.85$	$57.95^{a} \pm 11.83$	15.35	<0.001
Methane concentration (%)	$4.88^{c} \pm 0.12$	$3.60^{b} \pm 0.23$	$2.53^{a} \pm 0.06$	$2.0^{a} \pm 0.21$	0.41	<0.001
Total methane production (mL)	$3.73^{d} \pm 0.07$	$2.50^{c} \pm 0.20$	$1.60^{b} \pm 0.04$	$0.54^{a} \pm 0.16$	0.43	<0.001
Total methane production (mL/g DM)	$8.27^{d} \pm 0.18$	$5.67^{c} \pm 0.56$	$3.61^{b} \pm 0.09$	$1.19^{a} \pm 0.36$	0.96	<0.001
Total methane production (mL/g DMD)	$13.36^{c} \pm 0.46$	$9.22^{b} \pm 0.91$	$5.89^{ab} \pm 0.13$	$4.03^{a} \pm 1.80$	1.37	0.003
TDDM (%)	$61.96^{c} \pm 1.46$	$60.45^{c} \pm 0.03$	$56.28^{b} \pm 0.21$	$31.82^{a} \pm 5.32$	4.49	0.001
Ammonia N (mg/dL)	$16.33^{c} \pm 0.47$	$16.10^{b} \pm 0.70$	$15.40^{b} \pm 0.0$	$10.50^{a} \pm 0.70$	0.84	0.002
Acetate (mM/dL)	$3.46^{c} \pm 0.08$	$3.40^{c} \pm 0.07$	$2.96^{b} \pm 0.02$	$2.19^{a} \pm 0.07$	0.10	0.002
Propionate (mM/dL)	$0.88^{b} \pm 0.02$	$1.02^{c} \pm 0.04$	$0.84^{b} \pm 0.06$	$0.57^{a} \pm 0.07$	0.04	0.002
Butyrate (mM/dL)	$0.25^{b} \pm 0.01$	$0.23^{b} \pm 0.02$	$0.24^{b} \pm 0.01$	$0.15^{a} \pm 0.01$	0.02	0.001
A:P ratio	$3.93^{c} \pm 0.02$	$3.33^{a} \pm 0.01$	$3.52^{b} \pm 0.05$	$3.84^{c} \pm 0.04$	1.02	0.001

Control, FG-0.25, FG-0.50 and FG-1.0 are treatment groups with 0.0, 0.25, 0.50 and 1.0 mL of FG leaf extract/60 mL of buffered rumen fluid (BRF), respectively. Mean values bearing a, b, c and d superscripts in a row vary significantly ($p < 0.01$).

Table 3. In vitro fatty acid biohydrogenation (mg/incubation) profile upon incubation of mixed feed substrate with buffered rumen fluid supplemented with graded levels of tanniferous *Ficus glomerata* (FG) leaf extract.

Fatty Acids	Control	FG-0.25	FG-0.50	FG-1.0	SEM	*p* Value
Saturated fatty acids (SFA)						
C4:0	$36.75^{bc} \pm 0.22$	$37.11^{c} \pm 0.05$	$36.29^{b} \pm 0.19$	$0.0^{a} \pm 0.0$	4.79	<0.001
C6:0	$3.92^{c} \pm 0.09$	$3.34^{b} \pm 0.06$	$3.42^{b} \pm 0.04$	$0.0^{a} \pm 0.0$	0.47	<0.001
C8:0	$0.0^{a} \pm 0.0$	$3.55^{b} \pm 1.78$	$9.30^{c} \pm 0.46$	$2.72^{ab} \pm 0.18$	1.09	0.001
C10:0	$3.82^{b} \pm 0.09$	$4.04^{c} \pm 0.08$	$4.20^{c} \pm 0.07$	$0.0^{a} \pm 0.0$	0.53	<0.001
C11:0	$4.48^{c} \pm 0.09$	$4.71^{c} \pm 0.12$	$4.00^{b} \pm 0.07$	$0.0^{a} \pm 0.0$	0.58	<0.001
C12:0	$5.74^{a} \pm 0.09$	$5.64^{a} \pm 0.07$	$5.22^{a} \pm 0.01$	$10.95^{b} \pm 0.49$	0.72	<0.001
C13:0	$2.13^{a} \pm 0.09$	$2.31^{a} \pm 0.23$	$2.78^{a} \pm 0.09$	$13.17^{b} \pm 0.88$	1.42	<0.001
C14:0	$6.74^{a} \pm 0.04$	$5.15^{a} \pm 0.11$	$5.27^{a} \pm 0.05$	$43.76^{b} \pm 4.8$	5.07	<0.001
C15:0	$2.71^{a} \pm 0.10$	$2.13^{a} \pm 1.4$	$0.37^{a} \pm 0.37$	$10.48^{b} \pm 0.80$	1.27	<0.001
C16:0	$16.47^{d} \pm 0.39$	$13.52^{c} \pm 0.09$	$10.29^{b} \pm 0.13$	$0.0^{a} \pm 0.0$	1.87	<0.001
C18:0	$15.27^{b} \pm 0.14$	$5.40^{b} \pm 0.15$	$0.6.32^{c} \pm 0.22$	$2.86^{a} \pm 0.08$	0.39	<0.001
Total SFA	88.05 ± 0.29	87.93 ± 0.52	87.49 ± 0.19	84.32 ± 3.64	0.89	0.53
C14:1	$11.94^{d} \pm 0.29$	$10.89^{c} \pm 0.19$	$9.05^{b} \pm 0.12$	$7.69^{a} \pm 0.44$	0.50	<0.001
Trans-vaccenic acid (C18:1)	$0.0^{a} \pm 0.0$	$2.89^{ab} \pm 0.39$	$3.46^{ab} \pm 0.29$	$7.99^{b} \pm 3.49$	1.16	0.063
Total unsaturated fatty acids	11.94 ± 0.29	13.07 ± 0.52	12.51 ± 0.19	15.68 ± 3.67	0.89	0.53

Control, FG-0.25, FG-0.50 and FG-1.0 are treatment groups with 0.0, 0.25, 0.50 and 1.0 mL of FG leaf extract/60 mL of buffered rumen fluid (BRF), respectively. Mean values bearing a, b, c and d superscripts in a row vary significantly ($p < 0.001$).

Table 4. Modulation (%) of rumen bio-hydrogenation of fatty acids, total gas production, methane production and truly degradable dry matter upon incubating graded doses of tanniferous FG leaf extract with mixed feed substrate.

Dose Incubated (mL/60 mL BRF)	SFA Inhibited	*t*-Vaccenic Acid (C18:1) Enhanced	Gas Production Inhibited	Methane Production Inhibited	TDDM Inhibited
0.25	0.14	100	9.44	32.98	2.01
0.50	0.64	100	17.07	57.10	8.73
1.0	4.24	100	65.83	85.52	48.64

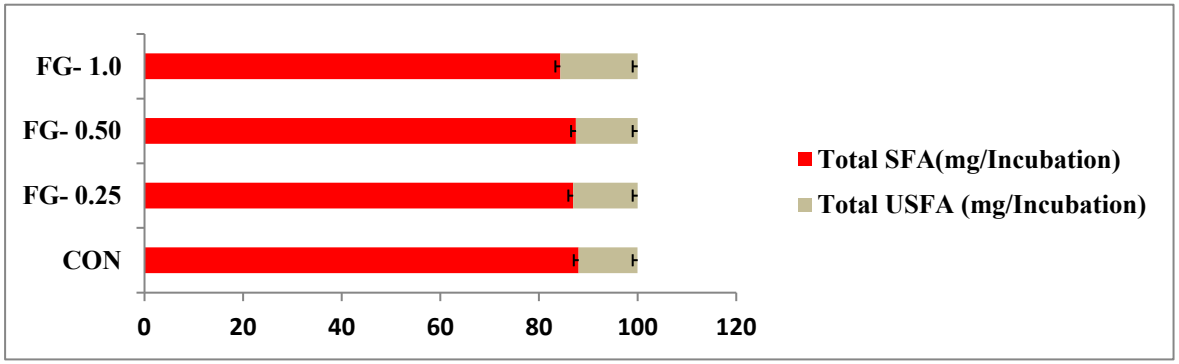

Figure 3. Effects of FG leaf extract supplementation with mixed feed on rumen biohydrogenation characteristics.

3. Materials and Methods

The experiment was conducted in the Division of Animal Nutrition and Feed Technology, ICAR—Central Institute for Research on Buffaloes (CIRB), Hisar, Haryana, India (29.1203_N, 75.8069_E). The guidelines of the Institutional Animal Ethics Committee of the CIRB were compiled (IAEC-CIRB/19-20/A/010 dated 5 August 2019) for the care of the rumen-fistulated animals and for rumen fluid collection.

3.1. Experimental Design and Substrate

A total mixed feed (500 mg) consisting of oat hay/concentrate mixture (1:1) was used as a substrate for this in vitro fermentation study. Tannin extracts (70% aqueous acetone extract) of *Ficus glomerata* (FG) leaves were prepared [25] and examined at four graded concentrations (0 mL, 0.25 mL, 0.50 mL and 1.0 mL per 60 mL of buffered rumen fluid) on the basis of preliminary laboratory screening. Three bottles per treatment per run with two incubation runs were prepared to obtain representative results.

Oats fodder was cultivated at ICAR—the Central Institute for Research on Buffaloes, Hisar, Haryana, India, harvested at the pre-bloom stage, oven dried at 65 °C for 48 h and ground to pass through 1.0 mm sieve for subsequent use. A concentrate mixture was prepared by mixing the following ingredients (% parts): wheat grains, 20; maize grains, 15; mustard cake, 20; groundnut cake, 10; wheat bran, 32; mineral mixture, 2.0; and common salt, 1.0.

3.2. Collection of Rumen Inoculum

Rumen liquor was collected from two rumen-fistulated Murrah buffalo steers (*Bubalus bubalis*) maintained on roughage-based diet at the animal farm of the institute. The mixed semisolid contents of the rumen digesta were collected manually from different locations at different depths in the early morning before offering feed and water to the animals and hand-squeezed to obtain the liquid portion of the rumen fluid. An equal volume of rumen fluid from both the fistulated animals was pooled to completely fill a 1.0 L pre-warmed

oxygen-free thermos flask and brought to the laboratory. The rumen fluid was filtered through four layers of muslin cloth under continuous flow of CO_2 to use as a source of inoculums for the in vitro investigations.

3.3. In Vitro Incubation

Investigations were carried out using a control (CON) of total mixed feed as substrate and various treatment groups with different graded dose regimen (0 mL, 0.25 mL, 0.50 mL and 1.0 mL per 60 mL of buffered rumen fluid) of FG leaf extract. The in vitro incubations were carried out in 250 mL capacity ANKOM bottles fitted with radio-frequency (RF)-based pressure- and temperature-sensitive modules (ANKOM-RF Gas Production System). The day before the incubation, 500 mg mixed feed substrate was weighed into the bottles. On the day of in vitro incubation, the various concentrations of FG leaf extract were added to the respective bottles in duplicate. Incubation medium (60 mL) consisting of rumen liquor and in vitro buffer medium in a 1: 2 ratio [26] were added to the bottles under continuous gassing with CO_2 before being capped and returned to the incubator rack set at 39 °C and 85 oscillations/minutes for 24 h. Three bottles containing only incubation media without substrate were used as blanks for the experiment and three replicates for each treatment were maintained.

3.4. Estimation of Fatty Acid Biohydrogenation

After 24 h of fermentation, sample preparation for studying the in vitro fatty acid biohydrogenation of the substrate was carried out in accordance with the protocol of Mandal et al. [27], and finally, the sample was subjected to GC analysis. Incubated buffered rumen fluid (BRF) was initially prepared for esterification compounds, i.e., FAME (fatty acid methyl esters). For this, 20 µL of fermented BRF was added to Pyrex culture tubes (dimension, 16 × 125) and mixed with 0.35 mL of 10 N KOH and 2.65 mL of methanol. The contents were subjected to 1.5 h of incubation at 55 °C with 5 s of vigorous shaking at every 20 min interval. After incubation, the sample was subjected to cooling under running tap water, and 0.29 mL of 24 N H_2SO_4 was added with inverse mixing. The contents were again incubated and cooled in a similar manner. Finally, 1.5 mL n-hexane was mixed in the sample and vortexed for 5 min, and afterwards, subjected to centrifugation at 2500 rpm for 5 min. The acquired glassy transparent supernatant n-hexane layer containing FAME was subjected to GC analysis (Agilent GC system-8890, Agilent Technologies, Palo Alto, CA, USA), maintained at a 260 PSI inlet pressure, a 175 °C column temperature and a 260 °C detector temperature. The individual fatty acid concentration was calculated as follows:

$$\text{Individual fatty acid concentration (g/100 g of fatty acid methyl ester)} = \frac{Area\ of\ individual\ fatty\ acid}{Total\ area\ of\ fatty\ acid} \quad (1)$$

3.5. Estimation of Gas and Methane Production

After exactly 24 h of incubation, the recording was stopped and the total gas production (mol) in the bottles was recorded automatically through cumulative pressure and temperature using an ANKOM RF Gas Production System, and the total gas was determined as follows:

$$\text{Total gas production (mol)} = \frac{Pressure\ in\ ANKOM\ RF\ Gas\ Production\ bottle\ (KPa) * Head\ space\ volume\ of\ ANKOM\ RF\ Gas\ Production\ bottle\ (mL)}{Temperature\ of\ ANKOM\ RF\ Gas\ Production\ bottle\ (Kelvin)\ * Avogadro's\ number\ (8.3144)} \quad (2)$$

The methane concentration (%) in the head space gas was estimated after 24 h of incubation using a gas chromatograph (Nucon-5700, New Delhi, India) installed with a flame ionization detector (FID) and a column (Porapak 'Q'). The gas sample (200 µL), with the help of a 1000 µL graduated gas-tight micro-syringe (Hamilton, Switzerland), was taken from the bottles and injected into the injector port of the GC. A mixed gas (CH_4:CO_2: 50:50) (Centurion Scientific, New Delhi, India) was used as a standard for comparison. The temperatures of the injector, detector and oven of the GC were maintained at 140 °C, 200 °C and 70 °C, respectively, and the column pressure for the carrier gas, hydrogen, was

set at 10 psi. The proportion of methane (%) in the total gas concentration was calculated as follows:

$$\text{Methane in head space } (\%) = \frac{[Area\ covered\ by\ the\ sample] * 50}{\text{Area covered by standard of gas}} \quad (3)$$

The total methane production was calculated by multiplying the total gas production by the methane concentration.

3.6. Volatile Fatty Acid (VFA) Estimation

After the termination of incubation (24 h), 1 mL of the supernatant of each bottle's content was added to a micro-centrifuge tube containing 0.20 mL of 25% metaphosphoric acid. The mixture was allowed to stand for 2 h at room temperature and centrifuged at 5000× *g* for 10 min to obtain a clear supernatant and stored at −20 °C for subsequent VFA determination. On the day of analysis, a sample of 1 µL was taken using a Hamilton syringe and injected manually into the gas chromatograph (NUCON-5700, Nucon Engineers, New Delhi, India), which was equipped with a glass column packed with chromosorb 101 and flame ionization detector (FID). The column pressures for hydrogen and zero-moisture air were 20 psi and 10 psi, respectively. The temperature of the column oven was increased from 170 °C to 230 °C at 3 °C/min and held for 7 min. The injector and detector temperature of the FID were set at 240 °C and 250 °C, respectively. Three blanks were included for the correction of VFA produced from the inoculum in each run, and two runs were executed for each sample.

3.7. In Vitro Dry Matter Degradability and Ammonia Production

The content of the each ANKOM bottle was transferred into a spoutless beaker via repeated washings with neutral detergent solution. After refluxing the contents for 1 h, the residue was recovered in pre-weighed filter crucibles (G1). After drying the crucibles to a constant weight, ashing was performed at 550 °C. The truly degradable dry matter (TDDM) was calculated as follows:

$$\text{TDDM } (\%) = \frac{(Dry\ weight\ of\ substrate\ incubated - Dry\ weight\ of\ residue\ leftover) * 100}{Dry\ weight\ of\ substrate\ incubated} \quad (4)$$

The ammonia nitrogen (NH_3-N) concentration was estimated in accordance with the Conway microdiffusion disk method [28].

3.8. Chemical and Statistical Analysis

Mixed feed substrates (oat hay/concentrate mixture) were chemically analyzed in accordance with the methods of the Association of Official Analytical Chemistry [29]. However, the fiber constituents were analyzed using the method of Van Soest et al. [30]. The extraction of dried *Ficus glomerata* leaves was performed using aqueous acetone (30:70), and the tannin fractions were analyzed [25]. The data obtained were subjected to analysis of variance (ANOVA) using SPSS 16.0 software [31], and the treatment means were ranked using Duncan's multiple range tests according to Snedecor and Cochran [32]. The mean differences were described via Tukey's HSD test, where the significance levels were $p \leq 0.05$, which corresponds to 95% confidence, and $p \leq 0.01$, which corresponds to 99% confidence.

4. Conclusions

Ficus glomerata leaf tannins have the ability to modulate rumen fermentation for reduced methanogenesis and fatty acid biohydrogenation in a total mixed ration. At all the experimental dose levels in this study, reductions in methane and ammonia production with enhanced *t*-vaccenic acid, a precursor of conjugated linoleic acid (CLA), were evidenced. As at higher levels (FG-0.50 and FG-1.0) of *Ficus glomerata* leaf extracts inclusion in in vitro fermentation medium, negatively affected feed digestibility and volatile fatty acid production, a lower dose (0.25 mL/60 mL or 4.17 mL/L) is suggested to achieve the

desirable effects of a reduction in methane production and fatty acid biohydrogenation without affecting feed digestibility. However, an in vivo study would validate these results and determine its effectiveness for use as a feed supplement for the abatement of enteric methane emission and enhancing unsaturated fatty acids in animal products.

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