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Methane production and microbial protein synthesis in adult sheep fed total mixed ration as mash and as complete feed block

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ABSTRACT

Sixteen rams in two groups were fed *ad libitum* total mixed ration as mash (TMRm) and as block (TMRb) for 90 days, and methane (CH_4) emission was estimated by standard SF_6 tracer technique. Feed samples were also incubated *in vitro* for calculating CH_4 emission. *In vitro* incubation revealed lower ($P < 0.05$) gas production and CH_4 from TMRb. Rams fed on TMRb consumed higher ($P < 0.05$) dry matter (DM), digestible crude protein (CP) and metabolizable energy (ME). The digestibility of nutrients was similar between the groups except for CP, which was higher in TMRb. The CH_4 emission per unit digestible DM and organic matter intake was lower ($P < 0.05$) from TMRb, resulting in lower energy loss as percentage of dietary energy and higher ME intake as compared to TMRm. Microbial protein synthesis (MPS) as assessed from urinary purine derivatives was higher in TMRb. The rams on TMRb also exhibited improved N utilization compared to TMRm. Post-feeding (4 h) ruminal attributes in TMRm showed higher total N and lower acetic and butyric acid with an increased total ciliate protozoa and entodinia population. It may be concluded that TMR offered in block form emits less CH_4 and saves ME, besides improving CP digestibility, MPS and N utilization.

Introduction

Methane (CH_4) production in ruminants is a significant loss of energy accounting 13.4% of the digestible energy (DE) in low digestibility diet and 10.3% in high digestibility diets. It is one of the main greenhouse gases (GHG), with a global warming potential (GWP) 23 times that of carbon dioxide (CO_2) on a 100-year time scale [1]. A reduction in CH_4 production as 'feed quality' improves has also been observed [2,3]. Grinding or pelleting has been shown to decrease CH_4 per unit of feed intake by 20–40% [4] and accounts for the increase in feed efficiency [5]. Simultaneous ruminal availability of carbohydrate and N sources maximizes microbial growth [6,7]. But sheep fed with moderate- to high-concentrate diets suggest that ruminal synchrony has little effect on ruminal or whole animal metabolism [7]. Although pelleting of first-cut alfalfa reduced CH_4 production, dry matter (DM) digestibility and urinary energy loss, the decline in energy loss as CH_4 was not sufficient to justify the extra mechanical energy spent in this process [8]. The feeding of complete feed stabilized rumen fermentation, minimized fermentation losses and ensured better rumen ammonia utilization [9].

Despite the importance of feeding system for livestock production and environmental impact, very few studies have compared the different forms of total mixed ration (TMR) on CH_4 production in ruminants. It is necessary to confirm the advantages of TMR in terms of a stable ruminal pH and nutrient digestibility, and to understand how its ruminal fermentation characteristics affected ruminal CH_4 production.

The objective of this study is to understand the effects of feeding total mixed ration as mash (TMRm) or in the form of a compact block (TMRb) on CH_4 emission and microbial protein synthesis (MPS) in adult rams. The results were also compared with *in vitro* ruminal incubation in a steady-state fermentation system.

Materials and methods

The study was carried out from May through July 2016 at the Central Sheep and Wool Research Institute, Avikanagar (Rajasthan, India). The mean minimum and maximum ambient temperature ranged from 25.68 to 26.68 °C and 32.45 to 42.46 °C, respectively, with relative humidity from 40.42 to 86.68%. The animal care, handling and sampling

procedures were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Experimental diet

TMR was prepared by mixing concentrate, roughage and molasses at 35, 60 and 5 kg, respectively, per 100 kg of feed. The composition of concentrate was maize 45, barley 45, groundnut cake 4, mustard cake 3, mineral mixture 2 and salt 1 kg/100 kg. The roughage moiety was chaffed (1–3 cm) *Cenchurus ciliaris* hay. In TMRm, both concentrate and roughage were mixed together with molasses in the feed mixture while in TMRb, the mixture was converted into feed block by compressing at 5000 psi (351.5 kg/cm^2) using a horizontal feed block-making machine developed by the National Agricultural Research Project (NARP), Department of Agricultural Research and Education (DARE), New Delhi. Samples of TMRm and TMRb were collected and dried in a forced-air oven at 55–60 °C for 48 h for assessing DM content.

In vitro gas production and fermentation constants

In vitro gas production was determined by the method of Menke and Steingass [10]. Three blank samples were run containing 30 mL of the medium. The syringes were incubated in a hot water bath cum shaker at 39 °C. Gas production was recorded at 2, 4, 6, 8, 10 12, 24, 36, 48, 72 and 96 h of incubation. Net gas production by each sample during the above-mentioned period was calculated by subtracting the gas produced by the blank. The data so generated was processed with Sigmastat software (v. 5.0) for calculating the $t^{1/2}$(h), potential gas production and rate constant.

The gas production kinetic parameters were calculated from the time-dependent (0–96 h) *in vitro* cumulative gas production data by applying a single-pool logistic model as depicted below, using the Sigmastat software (v. 5.0). After subtraction of

gas production from blank bottles, data were fitted to a one-phase exponential decay equation (Eq1) (GraphPad Prism 8.0; GraphPad Software LLC, La Jolla, USA):

$$Y = (Y_0 - \text{Plateau}) * \exp(-K * X) + \text{Plateau}$$

$$\text{Half-way time } (t_{1/2}) = \ln(2)/K: \text{time to reach half-way gas production (Eq2)}$$
$$\text{Inflection time } (\mu) = 1/K: \text{time to reach maximum fermentation rate or microbial growth (Eq3)}$$

where Y_0 or lag time (L) is the Y value when X (time, t) is zero; Plateau (potential gas production, b) is the Y value at infinite time; and K is the rate constant, expressed as a reciprocal of the x-axis time units.

In vitro gas production and methane estimation

After calculation of fermentation constants, two sets of samples each in triplicate were run simultaneously. In set 1, 200 mg (and in set 2, 400 mg) of oven-dried samples were incubated in 100-mL glass syringes fitted with plungers. In set 1, 30 mL of medium consisting of 10 mL rumen fluid and 20 mL buffer solution was used [10], whereas in set 2, 40 mL (10 mL of rumen fluid and 20 mL of buffer solution) of the medium was added as per the modified method [11]. Samples were incubated in a hot water bath cum shaker at 39 °C for up to 24 h, then total gas production was recorded and a gas sample from the first set was analyzed for CH_4 concentration. The fermentation was terminated by keeping the syringes in ice water. Methane was analyzed using a DANI make Gas Chromatograph (Model 1000, Series 011124002, Italy) using a flame ionization detector (FID) with a packed column (Chromatopak, 2 m length, 1/8 inch diameter packed with 10% SP-1000 on 80/100 mess Chromosorb WHP). The CH_4 production was calculated using the following equation (Eq4):

$$CH_4(\text{mL/g digested DM}) = \big[\{t^{1/2}\text{substrate gas production (mL/g)} \times t^{1/2}\text{substrate } CH_4\%$$
$$- t^{1/2}\text{blank (mL)} \times t^{1/2}\text{blank } CH_4\%\}/100\big] \times \text{Substrate DM digestibility}$$

The incubated sample in set 2 was quantitatively transferred to a 600-mL spoutless beaker with added 100mL of neutral detergent solution. The content was refluxed at boiling temperature for 1 h and then pressure filtered through pre-

weighed sintered G1 glass crucibles using a pressure pump. The insoluble residue was washed off the crucibles with detergent and hot water and then finally with acetone. Crucibles were dried in a hot-air oven at 100 °C for 24 h, then weighed. The crucible with the sample was incinerated in a muffle furnace at 600 °C for 4 h and weighed again after cooling. The DM and organic matter (OM) digestibility was calculated after subtracting the empty crucible's weight from the crucible weight after drying and incineration. Necessary corrections for blank samples were also made. Partitioning factor (PF), as an index of efficiency of MPS, was calculated from the ratio of OM degraded (mg) to gas volume (mL) at 24 h incubation [12].

Animal feeding and metabolism trial

Sixteen adult male rams of Malpura breed were divided into two equal groups, T1 and T2. They were kept in a shed provided with individual feeding arrangements and offered *ad libitum* total mixed ration in a mash form in T_1 and as a complete feed block in T_2. A daily record of feed intake was maintained during the entire experimental period of 91 days. Water was available to the rams *ad libitum* during the experiment. A metabolic trial on six representative rams of each group was conducted for 10 days at the end of the experiment, which included 4 days of adaptation and 6 days of collection. Daily feed offered, residue left, feces voided and urine excreted were recorded on a 24-h basis. The feed and feces samples were dried in a forced-air oven at 55–60 °C to constant weight for DM estimation. Samples were ground to pass a 1-mm screen and preserved for chemical analysis. Daily fresh samples of feces were preserved with 1:4 sulfuric acid for N assay. Similarly, aliquots of urine were preserved for estimation of N. Urine excreted was collected in a container with 100 mL 10% sulfuric acid, and after recording urine was diluted with water to 1.0 L. A urine sample (20 mL) was stored at −20.0 °C for purine derivatives (PD) analysis.

At the end of the metabolic trial, rumen liquor samples (50 mL) were drawn from all the rams 4 h post-feeding using a stomach tube connected to a suction pump. Each sample was placed in a 100 mL glass jar and pH was recorded using a digital pH meter within 4–5 min of sampling. The rumen fluid was brought to the laboratory and strained through four layers of muslin cloth.

Analysis

The feed and faeces samples were ground with a Willey mill having a 1-mm sieve and were analyzed in triplicates for ash and crude protein (CP) as per the standard methods described in (Association of Official Analytical Chemists) AOAC [13]. The neutral detergent fiber (NDF) was assayed with a thermo-stable amylase and expressed exclusive of residual ash and acid detergent fiber (ADF) and lignin (by solubilization of cellulose with sulfuric acid), which were analyzed sequentially [14]. Hemicellulose and cellulose were calculated by the difference between NDF and ADF and ADF and lignin, respectively. The stored urine samples were analyzed for PD after dilution [15].

For protozoa count, 1 mL of the fluid was pipetted into a scintillation vial using a wide orifice pipette, to which 1 mL of formalinized physiological saline and two drops of brilliant green dye were added and mixed in. Total and differential ciliate protozoa counts were made [16]. The strained rumen liquor (SRL) was preserved by adding a few drops of saturated mercuric chloride solution and kept in labelled polypropylene bottles at − 20 °C. The samples were analyzed for total N (micro-Kjeldahl), ammonia N [17], and short-chain fatty acids (SCFA) [18]. The GC (DANI make Gas Chromatograph, Model 1000, Series 011124002, Italy) analysis of SCFA was done by injecting 1 μL of sample to flow through a packed column (10% SP-1200) conditioned with programmable temperature and flow rate (viz. injection port temperature 220 °C, column temperature stepped up from 115 °C to 150 °C in 15 min, and FID temperature 250 °C with carrier gas (N) flow rate 30 mL/min; hydrogen 30 mL/min; air 300 mL/min).

In vivo methane emission assessment

In vivo CH_4 emission from experimental rams was determined by the SF_6 tracer technique [19] with appropriate modifications to suit the experimental animal. A suitable canister was designed to fit in the animal that was placed above the shoulder. It is connected to a halter with an inlet filter attached with capillary and PTFE (Polytetrafluoroethylene) tubing via a reducing union. The halter unit was positioned above the nose without hindering the animal's prehension and normal respiration activities (as shown in Figure 1). A permeation tube with a predetermined release rate was used to orally administer SF_6 into the rumen. The ram was adapted to this canister and permeation tube for 5 days. Exhaled air from
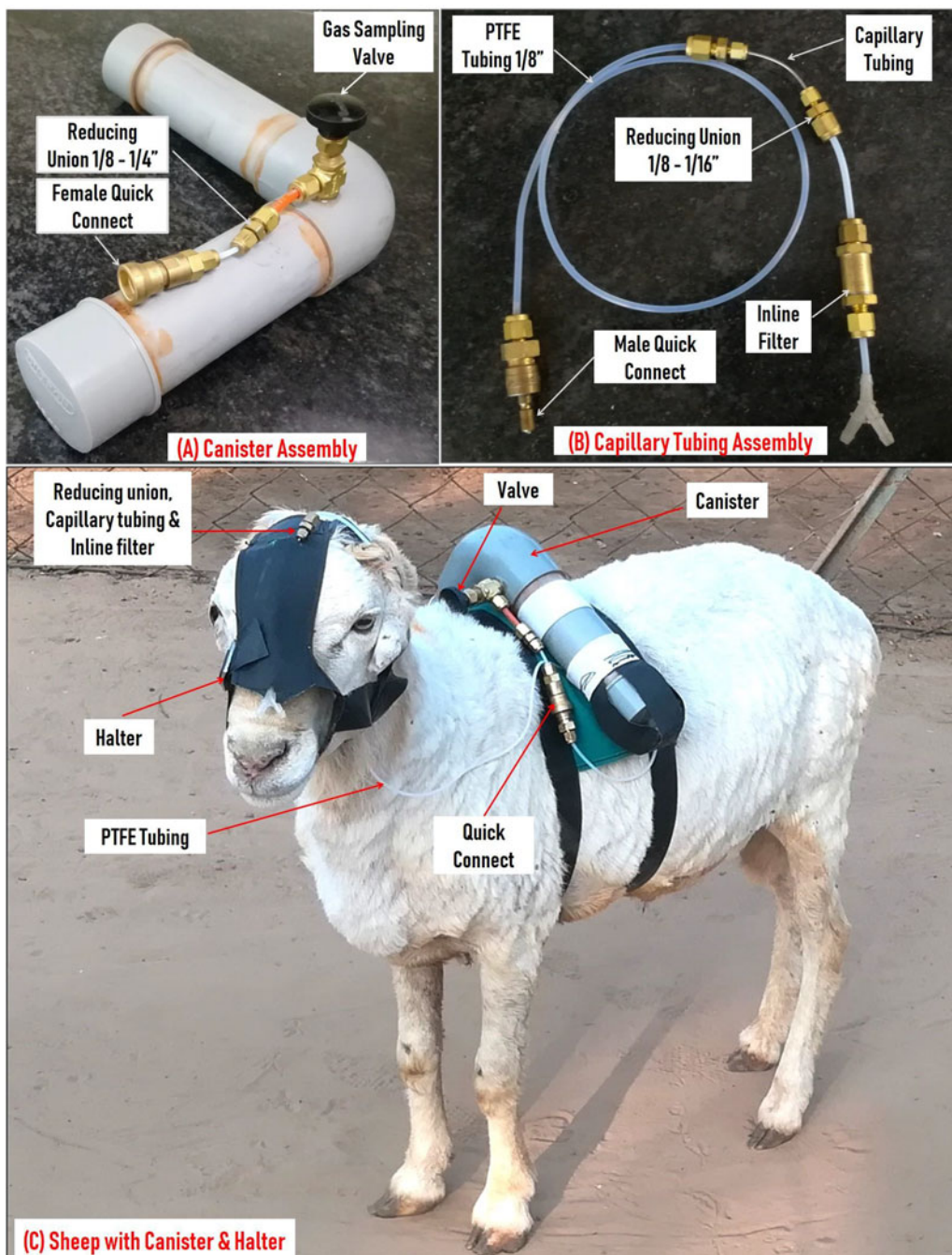
Figure 1. Sheep fitted with canister and halter for collecting exhaled gases to determine methane using the SF_6 technique.

around the animal's nose and mouth is drawn continuously for 24 h (one cycle) into the evacuated canister. Initial (600–650 mm Hg) and final (200–300 mm Hg) negative pressure of the canister were recorded, and then the canisters were filled with N gas to bring in positive pressure (500–600 mm Hg). Gas samples (n = 5) from the canister were analyzed for SF_6 and CH_4 concentration by gas chromatograph (DANI Master GC, Series 100922002, Italy) in a packed column (molecular sieve 5A, length 180 cm, outer diameter 3.17 mm) fitted to a packed injector and electron capture detector (ECD). The temperature at the injection port was 220 °C, that of the column was 85 °C and that of the detector was 280 °C, with N used both as carrier (flow rate 30 mL/min)

and auxilary (20 mL/min). Area of unknown sample was compared with that of standard sample and enteric CH_4 production was estimated by multiplying the CH_4/SF_6 ratio by the known permeation tube release rate, corrected for actual duration of sample collection and background CH_4 concentration [20]. The loss of energy through CH_4 was calculated by multiplying the CH_4 emission values (g) with its energy value [21].

Statistical analysis

The data on various parameters were analyzed using SPSS Base16.0 (SPSS Software products, Marketing Department, SPSS Inc. Chicago, IL 60606-6307, USA).

The significance of the difference between the treatments was determined using Tukey's studentized range test, and significance was declared at $P < 0.05$. Data are presented as means and pooled standard errors of the mean.

Results and discussion

Chemical composition

The chemical composition of TMRm and TMRb was similar (Table 1). It is only the process involved in preparation of TMR that differs, and hence with 11% CP, the diet is found to be sufficient for the maintenance requirements of the rams [22].

In vitro ruminal feed evaluation

In vitro ruminal fermentation kinetic assay revealed lower potential gas production ($P < 0.001$),

Table 1. Chemical composition (g/kg dry matter) of total mixed ration fed to sheep.

Chemical composition	TMRm	TMRb
Dry matter	896.5	905.8
Organic matter	910.9	909.1
Total ash	89.1	90.9
Crude protein	110.9	109.2
Ether extract	26.8	26.2
Total carbohydrates	773.2	773.7
Neutral detergent fiber	595.2	596.8
Acid detergent fiber	330.6	374.9
Hemicellulose	224.6	221.9
Cellulose	263.1	299.8
Lignin	75.7	75.1

Total mixed ration (TMR) provided as mash (TMRm) or compact block (TMRb).
TMR constituents (kg/100 kg): Concentrate* 35, Cenchurus ciliaris hay 60 and molasses 5 Concentrate constituents (%): maize 45, barley 45, oil extracted groundnut cake 4, oil extracted mustard cake 3, mineral mixture 2 and salt 1.

increased halfway time ($P = 0.032$) and inflection time ($P = 0.039$) in TMRb as compared to TMRm (Table 2; Figure 2). Initial gas production is basically accounted from the fermentation of soluble substrates, and thereafter gradual accumulation of residual insoluble substrates limits the rate of fermentation in this steady-state fermentation system [23]. A high substrate fermentation potential might be related to the profile of volatile fatty acids (VFAs) produced, although the in vitro observation may not directly correspond to in vivo ruminal fermentation data on these VFA metabolites. Gas is produced mainly when substrate is fermented to acetate and butyrate, while fermentation to propionate yields relatively lower gas due to buffering of the acid [3]. Lower gas production with propionate is related to the absence of CO_2 production, while higher acetate and butyrate production yielded more CO_2, consequently increasing the volume of gases. The lag time showed different onset times of fermentation for TMRm and TMRb, which was indicative of time span for the substrate-specific microbial colonization [24], and it contributed to higher $t_{1/2}$ and μ in TMRb compared to TMRm. The rate constant of gas production is thus proportional to concurrent microbial mass and the level of substrates available for degradation. Reasonably, the halfway time to the maximum gas volume is associated with speed of microbial attachment, duly contributed from degradation of soluble nutrients (e.g. carbohydrates, proteins), which ultimately decides substrate degradability [18]. This was quite evident from the gas curve (Figure 2) that showed a sigmoidal behavior with a

Table 2. In vitro ruminal gas production, digestibility and methane emission from total mixed ration.

Parameters	TMRm	TMRb	SEM	P value
Potential gas production (b; mL/200 mg DM)	48.5b	42.2a	0.83	<0.001
Rate constant (K)	0.061	0.058	0.0039	0.911
Lag time (L)	−0.283	−0.869	0.827	0.789
Half time ($t_{1/2}$; h)	11.28a	12.00b	0.172	0.032
Inflection time (μ; h)	16.28a	17.31b	0.231	0.039
Residual sum of square (R2)	0.992	0.960	—	—
Digestibility (g/kg)				
Dry matter (DM)	604	573	10.6	0.149
Organic matter (OM)	611	591	10.7	0.176
Partitioning factor (PF)	3.36	3.59	0.088	0.105
In vitro methane production				
Gas production (mL/200mg DM)	36.4b	32.9a	0.65	0.008
Methane concentration (%)	26.7	25.9	0.92	0.800
Methane production (mL/200 mg DM)	9.73b	8.51a	0.296	0.029
Methane emission (g/kg DM)	35.3b	30.8a	1.08	0.009
Methane emission (g/kg digestible DM)	58.4b	53.8a	1.15	0.049
Methane emission (g/kg OM)	39.2b	34.3a	1.19	0.009
Methane emission (g/kg digestible OM)	64.2b	58.0a	1.21	0.036

Total mixed ration (TMR) provided as mash (TMRm) or compact block (TMRb).
The partitioning factor is the ratio of digestible OM to fermentation gases at 24 h of incubation.
SEM: Standard error of the mean.
Treatment means with different superscript letters differ significantly ($P < 0.05$).
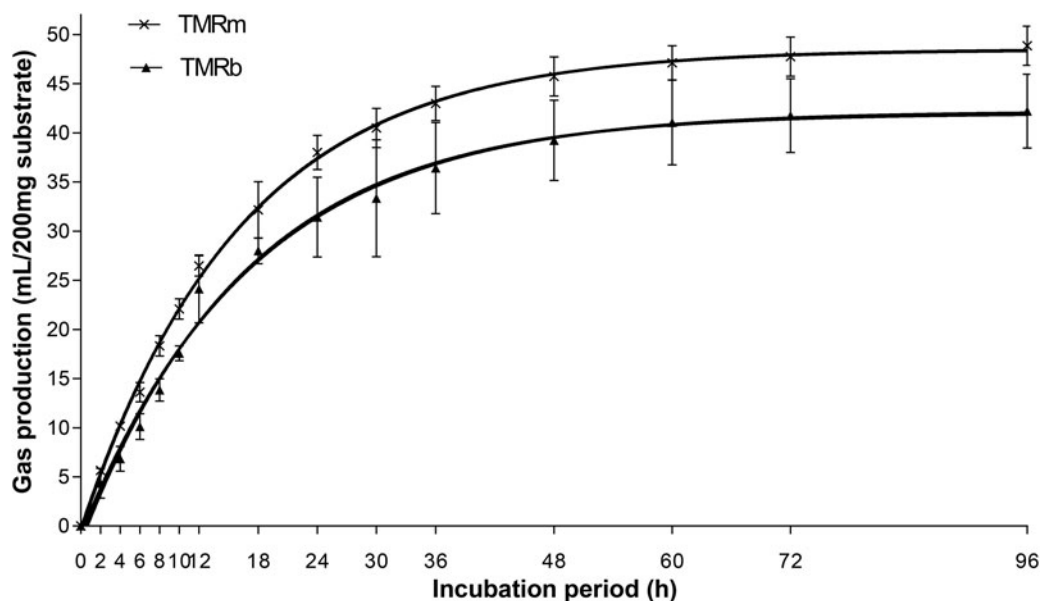
Figure 2. *In vitro* gas production pattern of total mixed ration (TMR) as mash (TMRm) and compact block (TMRb).

fixed point of inflection at 16.28 and 17.31 h of incubation for TMRm and TMRb, respectively. The relationship between μ and substrate availability is best described by this type of curve where ruminal microbial growth peaked as a function of concurrent substrate concentration [25]. The above kinetic parameters, as assessed by applying a one-phase exponential model, showed goodness of fit with a R^2 value > 0.96.

The partitioning of nutrients for microbial growth accrued from carbon (C) and N sources due to substrate degradation, metabolite production and gas production has a definite relationship [26], and a higher PF often indicates a higher proportion of degraded substrate being partitioned toward microbial cells [12]. Therefore, low gas production with high PF is generally indicative of high efficiency of microbial production (EMP) [27]. It often emphasized for simultaneous availability of carbohydrate and N sources to maximize rumen microbial growth [28]. TMRm and TMRb exhibited similar DM and OM digestibility, but varied gas production. This led to an alteration in the fermentation pattern with a non-significantly (P = 0.105) higher PF and lower absolute CH_4 production from TMRb. Higher degradability of DM/OM is preferential as the nutrients are more readily available and can therefore be more effectively utilized by the animal [29]. Feed ingredients that have a high *in vitro* degradability but low gas production per unit of truly degraded substrate (OM) should be selected for ruminants [18,23]. Total gas and CH_4 production after 24 h of *in vitro* incubation was lower (P < 0.05) in TMRb, which showed a reduction in CH_4 emission to the tune of 12.5% when

expressed on a substrate DM basis, and 9.7% on a digestible OM basis. The lower gas and CH_4 production in TMRb could thus be attributed to better utilization of fermentable energy substrates and metabolites for microbial growth. In this experiment, TMR in mash and block form differs only with respect to processing, where TMRb was subjected compression by applying 5000 psi, which decreased its bulk or volume but increased the density of forage particles. Also, some amount of heat is produced due to mechanical compaction, and all these processes might have altered the physico-chemical properties of the TMR. Therefore, there were differences in initiation of fermentation process (L), $t_{1/2}$, μ and b between TMRm and TMRb. Alteration in this *in vitro* ruminal fermentation pattern subsequently affected substrate degradability, resulting in decreased CH_4 production from TMRb compared to TMRm. In a similar vein, Karimizadeh *et al*. [30] also discussed the possible effect of processing on nutrient intake, digestibility and rumen fermentation of lambs of diets with different physical form.

Intake and digestibility of nutrients

The complete feed is a quantitative mixture of all dietary ingredients, blended thoroughly to prevent separation and selection by the animals. Between the two forms of diet that were offered to rams, one as mash and the other as compact feed block, the possibility for selection does exist with the mash form. The animal will try to search for the more palatable concentrate mixture, partially leaving aside the roughage moiety, even though they

were mixed with a binder molasses. This resulted in a difference (P < 0.05) in nutrient intake (e.g. DM, digestible CP and metabolizable energy, ME) between the two groups, being higher in TMRb than TMRm (Table 3). Densification of feed ingredients resulted in higher voluntary feed intake and contributed to increased CP and energy (ME) intake. In agreement with these findings, it is also reported that the block form of the diet increased bulk density by 3.60 times compared with its mash form, which resulted in a higher voluntary intake in buffaloes and in crossbred calves [31,32]. An increase in feed intake by the lambs due to compaction of diet during complete feed block (CFB) making was also observed earlier [30,33]. The more the ruminal ballast is bulky, in volume or in weight, the more the intake decreases with or without digestibility modification [34] as the intake appears limited by the maximal volume that the digestive tract can accommodate [35,36]. Moreover, DM intake is strongly correlated to both nutrient digestibility and animal requirements [37]; hence, a lower intake in TMRm was nevertheless deficient to meet the ME requirements for maintenance [22] and the rams in this group left more of the roughage moiety after meeting their requirements from the available concentrates.

The digestibility of nutrients except that of CP did not differ significantly between the groups (Table 3). There were some numerical differences showing non-significantly (P > 0.05) higher digestibility of fiber components (NDF and ADF), perhaps being assisted by higher CP digestibility (P = 0.036). Similarly, higher digestibility of OM and CP was observed in animals fed the CFB versus other diets [30]. In TMRm, the possibility of accessing

concentrate and the resultant preferential uptake by the rams compared to the roughage moiety delivered more degradable nutrients to the rumen for early fermentation. In contrast, TMRb offered a balanced supply of both concentrates and roughage at the prescribed mixture of 40:60 as a maintenance ration to rams. In consequence with this possible change in uptake of feed and nutrients, a higher (P < 0.05) concentration of rumen total N, ammonia N and total VFA was observed in the SRL as assessed at 4 h post feeding (Table 4). Thus, the post-feeding uptake and availability of nutrients for rumen micro-organisms over the rest of the day was being limited in TMRm, thereby possibly affecting the digestibility of fibrous feeds. In TMRb, there was limited scope for selection, and thus a uniform supply with better synchronization of energy and protein would have resulted in better utilization of protein and fiber fractions. A synchronized supply of energy and N in the rumen enhances rumen fermentation efficiency, thereby improving nutrient utilization [37]. In the CFB system of feeding, the ruminant animals have continuous free choice availability of uniform feed mixture, resulting in a more uniform load on the rumen and less fluctuation in the release of

Table 3. Body weight, nutrient intake and digestibility of sheep fed on *total mixed ration.*

Parameters	TMRm	TMRb	SEM	P value
Alteration in live weight (LW)				
Initial LW (kg)	49.3	49.5	1.25	0.437
Final LW (kg)	49.4	51.2	1.98	0.662
Nutrient intake				
Dry matter (g/day)	926[a]	1255[b]	25.23	0.037
Digestible crude protein	37.9[a]	42.8[b]	2.53	0.001
ME intake (Mcal/day)	1.87[a]	2.21[b]	0.080	<0.001
ME intake (Kcal/W$^{0.75}$)	100[a]	131[b]	7.2	0.006
Digestibility (g/kg)				
Dry matter	567	552	11.8	0.783
Organic matter	588	585	10.9	0.851
Ether extract	648	620	13.3	0.202
Crude protein	431[a]	466[b]	8.3	0.036
Total carbohydrates	604	589	12.0	0.916
Neutral detergent fiber	480	507	10.3	0.264
Acid detergent fiber	421	450	12.4	0.178

Total mixed ration (TMR) provided as mash (TMRm) or compact block (TMRb).
ME: Metabolizable energy. SEM: Standard error of the mean.
Treatment means with different superscript letters differ significantly (P < 0.05).

Table 4. Rumen fermentation metabolites and ciliate protozoa population of sheep fed on *total mixed ration.*

Parameter	TMRm	TMRb	SEM	P value
Rumen metabolites				
pH	6.53	6.44	0.036	0.220
Total N (mg/dL)	76.35[b]	68.70[a]	2.067	0.028
Ammonia N (mg/dL)	7.62[b]	5.46[a]	0.493	0.028
VFA concentration (mM/L)				
Acetic acid (c2)	34.63[b]	28.55[a]	1.871	0.046
Propionic acid (c3)	10.76[b]	6.71[a]	1.043	0.036
Isobutyric acid (c4i)	0.312[b]	0.166[a]	0.029	0.008
Butyric acid (c4)	5.889[b]	4.254[a]	0.358	0.016
Isovaleric acid (c5i)	0.260[b]	0.160[a]	0.015	0.006
Valeric acid (c5)	0.210[b]	0.108[a]	0.022	0.013
Branched-chain fatty acids	0.783[b]	0.434[a]	0.067	0.010
Total VFA	51.57[b]	39.94[a]	2.784	0.009
VFA proportion				
Acetic acid (%)	66.51[a]	71.55[b]	1.294	0.045
Propionic acid (%)	20.34[a]	16.88[b]	1.032	0.036
Butyric acid (%)	11.62	10.48	0.461	0.300
c2:c3 ratio	3.62[a]	4.24[b]	0.176	0.041
c3:(c2 + c4) ratio	0.275[b]	0.206[a]	0.015	0.011
Non-glucogenic:glucogenic ratio	4.58	5.22	0.271	0.108
Ciliate protozoa population (×10^4 cell/mL SRL)				
Holotrichs	24.21	21.09	3.029	0.635
Entodini[a]	221.0[b]	163.2[a]	14.14	0.036
Total protozo[a]	245.0[b]	181.2[a]	10.21	0.032

Total mixed ration (TMR) provided as mash (TMRm) or compact block (TMRb).
Branch-chain fatty acids (BcFA) = valerate + isovalerate + isobutyrate (c5 + c5i + c4i).
NGR (nonglucogenic:glucogenic ratio) = [(acetate + 2 × butyrate + BcFA) ÷ (propionate + BcFA)] or [(c2 + 2c4 + c5 + c5i + c4i) ÷ (c3 + c5 + c5i + c4i)].
SEM: Standard error of the mean. SRL: Strained rumen liquor. VFA: Volatile fatty acids.
Treatment means with different superscript letters differ significantly (P < 0.05).

ammonia, which supports more efficient utilization of C and N for microbial proliferation.

Rumen fermentation metabolites

The post-feeding ruminal attributes showed pH at 6.53 in TMRm and 6.44 in TMRb, which may be considered optimal in this type of feeding regimen. Feeding TMR eliminates the need to feed large meals of concentrate, and thus may be beneficial in stabilizing the rumen pH, to prevent it from falling rapidly toward acidic soon after feeding. As per the earlier discussion, there was a preferential intake of concentrate in TMRm, but the pH did not become mor acidic, probably due to simultaneous intake of proteinaceous feed that contributed to higher total and ammonia N to stabilize the pH. The concentration of ruminal short and branched-chain fatty acids (BcFA) was higher ($P <$ 0.05) in TMRm than in TMRb. The overall rumen fermentation characteristics in TMRb indicated a shift away from propionate toward acetate with a proportional distribution (%) of acetate (c2), propionate (c3) and butyrate (c4) at 71.55, 16.88 and 10.48 compared to 66.51, 20.34 and 11.62 in TMRm. Consequently, the c2:c3 ratio was wider ($P = 0.041$) with a narrower ($P = 0.011$) c3:(c2 + c4) ratio. The observation on increased gas production from TMRm during *in vitro* ruminal incubation corresponded well with the increase in acetate and butyrate production *in vivo*. Fermentation gases are produced predominantly when the substrates are fermented to acetate and butyrate, while lower amounts of gases are associated with propionate production [3]. *In vivo* ruminal metabolism has a different effect with regards to concentration of VFA; less acid and ammonia accumulation leads to better utilization of carbon skeleton for MPS, which leads in turn to efficient fermentable energy utilization [24,25]. Thus, feeding of TMRb effected better stabilization of rumen fermentation by minimizing fermentation loss, and ensuring better ammonia utilization. Further, production of acetate and butyrate from pyruvate is accompanied by the production of H_2, whereas propionate production utilizes H_2, the major substrate for methanogenesis [38].

Variation in DM intake leads to variation in CH_4 production on a per-day basis when cattle were fed *ad libitum* [39], which was also observed in this experiment (Table 5). The ciliate protozoa population showed an increased count of entodinia leading to higher total counts in TMRm, which can be

Table 5. Methane emission and its contribution to energy loss in sheep fed on *total mixed ration.*

Parameter	TMRm	TMRb	SEM	P value
Methane emission				
g/day	27.9	26.9	1.09	0.697
g/kg DM intake	30.3[b]	21.4[a]	1.80	0.003
g/kg digestible DM intake	53.3[b]	38.8[a]	2.75	0.010
g/kg digestible OM intake	56.2[b]	43.5[a]	2.82	0.014
Loss of energy through methane				
Energy loss/day (Kcal)	372	359	14.6	0.683
Energy loss (% of GE)	9.46[b]	7.68[a]	0.121	0.029
Energy loss (% of DE)	16.1[b]	13.1[a]	0.18	0.021
Energy loss (% of ME)	19.8[b]	16.2[a]	0.25	0.033

Total mixed ration (TMR) provided as mash (TMRm) or compact block (TMRb).
DE: Digestible energy. DM: Dry matter. GE: Gross energy. ME: Metabolizable energy. OM: Organic matter. SEM: Standard error of the mean.
Treatment means with different superscript letters differ significantly ($P < 0.05$).

correlated to initial selective consumption of concentrates from the TMR. There was simultaneous access to both concentrate and roughage from TMRm, and as a matter of preference sheep consumed proportionately higher concentrate during the first few hours, which increased the population of ciliate protozoa and eventually led to an increase in total N and ammonia N levels during 4 h post-feeding. Santra *et al.* [40] also attributed such alteration in ruminal fermentation attributes to feeding diets of higher digestibility. Higher ammonia N in TMRm was also associated with higher protozoa population that effected higher proteolytic and deamination activity [41]. Increased ciliate protozoa population are also linked to higher butyric acid production in its VFA metabolism. Taken together, higher concentrations of butyrate and BcFA can be correlated to increased proteolysis associated with an upsurge in protozoa population.

Methane emission and energy loss

The CH_4 emission was estimated by employing the SF_6 technique with suitable modification to fit it to grazing sheep (Figure 1). The daily emission of CH_4 was similar in the two groups (Table 4), which is in line with the earlier reports [42,43]. However, significant ($P < 0.05$) differences were observed when CH_4 emission was expressed in terms of intake of DM, digestible DM and digestible OM, with significantly lower ($P < 0.05$) values in TMRb. The percentage reduction in CH_4 was to the tune of 23–29% in TMRb, which had a significant bearing on energy loss and final uptake of ME by the rams. Similar to the present results, a reduction of 20 to 40% per unit of DM was reported from another study at high levels of intake [44]. There was

35.5% higher DM intake from TMRb compared to TMRm. Studies have revealed that variation in DM intake accounts for 52 to 64% of the variation in CH_4 production on a per-day basis when cattle are fed *ad libitum* [38,43]. Thus, more often a strong relationship between DM intake and ruminal CH_4 production has been reported [45–47]. The evidence that increasing feed intake decreased CH_4 yield can be explained by a decrease in mean rumen retention time and the extent of rumen fermentation compared to low intake levels [48]. Although retention time and ruminal passage rate of the feed were not measured, decreased CH_4 production in TMRb could be related to higher DM intake. Feed processing (e.g. making TMR into CFB) thus contributes to improvement in DM digestibility with a reduction in CH_4 output per unit of gain by increasing the energy available for productive purposes and diluting the CH_4 associated with maintenance [49,50].

Similarly, daily loss of energy through CH_4 was non-significantly different between the groups, but it was significantly lower in TMRb when expressed as a percentage of GE, DE and ME intake. There was conservation of fermentable CH_4 energy due to reduced loss by emission of 16.2% in TMRb compared to 19.8% in TMRm. Further, a higher DM intake in TMRb (1255 g/d) than TMRm (926 g/d), with a relatively similar digestibility but improved efficiency of energy utilization as evidenced by positive rumen fermentation attributes, and a reduced loss of energy through fermentable gases including CH_4, contributed to higher ME intake and accumulation of live weight (0.1 vs 1.7 kg) in TMRb compared to TMRm. Karimizadeh *et al.* [30] also observed improved performance of lambs on CFB compared to two other physical forms of the diets, viz. mash and pellet due accompanied by improved efficiency of nutrient utilization with enhanced digestibility and rumen fermentation. It is reported that higher energy intake level in cattle resulted in a lower percentage of DE intake being converted to CH_4 [51]. Higher CH_4 emission in TMRm can be correlated with higher protozoa population, because they have been shown to be responsible for 9–25% of methanogenesis in the rumen [52]. The CH_4 production in the rumen increased exponentially with the increase in protozoa population. Concomitantly, a decrease in CH_4 production both *in vitro* and *in vivo* was observed in the absence of protozoa [53]. Methanogens associated with protozoa reached a maximum (10 to 100 times pre-feeding levels) after feeding,

because they migrate and stick to feed particles as well as onto the surface of protozoa [54]. The present observation of increased entodinia and total ciliate population 4 h post-feeding in TMRm confirms this phenomenon due to selective consumption of more concentrates by the rams compared to the synchronized and uniform supply of both concentrate and roughage at a 40:60 ratio in TMRb.

The correlation between rumen fermentation metabolites and methanogenesis also confirms the reduced CH_4 emission in TMRb. The production of acetate and butyrate from pyruvate is accompanied by the production of H_2, whereas propionate production utilizes H_2, which is the major substrate for methanogenesis [24,55]. A lower acetate and butyrate production in TMRb can thus be correlated to lower CH_4 emission. On the contrary, an increase in CH_4 with TMRm might be due to an increase in acetate resulting from the difference in uptake and ruminal degradation of structural and non-structural carbohydrates between the two feeding forms. It is suggested that the DM partitioning between MPS and fermentation influences H_2 production and hence methanogenesis [56]. The iso-fatty acids had a positive correlation with the efficiency of microbial growth [57], but they were not distinctly related to MPS in TMRm which exhibited higher concentration.

In vivo CH_4 emission followed a similar trend to that observed during *in vitro* ruminal feed evaluation. The observed differences are due to different methodology applied for CH_4 estimation. Moreover, the *in vitro* incubation method simulates the ruminal fermentation of feed and may be considered a useful tool for screening of feed resources including TMR. Under *in vivo* experimentation the animals were allowed long-term adaptation to the tested feedstuffs, and the emission is quantified based on maintaining a constant release rate from permeation tubes, effect of release rate upon emission rate of CH_4, background level determination and consistency in applicability of the SF_6 methodology. A similar discrepancy in assessing CH_4 emission by SF_6 tracer technique vis-à-vis the *in vitro* gas method was reported in another study [58].

Microbial protein synthesis and N utilization

Urinary excretion of PD revealed significantly higher (P < 0.05) allantoin and total PD in TMRb than TMRm (Table 6). The PD excretion seemed to be dependent on digestible OM and N intake [59],

Table 6. Microbial protein synthesis and N utilization in sheep fed on *t*otal mixed ration during the metabolism trial.

Parameters	TMRm	TMR[b]	SEM	P value
Purine derivatives excretion (meq/L)				
Allantoin	4.13[a]	6.18[b]	0.613	0.002
Xanthine + hypoxanthine	0.48	0.68	0.041	0.385
Uric acid	0.96	1.53	0.134	0.051
PD excretion	5.58[a]	8.40[b]	0.744	0.003
PD absorption	5.85[a]	9.59[b]	1.023	0.003
Microbial N synthesis				
DOMI (g/day)	629	652	13.4	0.123
Microbial N (g/kg DOMI)	6.96[a]	11.06[b]	1.303	0.002
MCP (g/100 g DOMI)	4.35[a]	6.91[b]	0.814	0.002
N balance (g/day)				
N intake	19.6[a]	23.6[b]	1.03	0.048
Faecal N excretion	11.3	12.9	0.72	0.306
Urinary N excretion	3.98	4.67	0.20	0.080
N balance	4.34[a]	6.00[b]	0.45	0.060
N balance (% intake)	22.21[a]	25.25[b]	1.129	0.198
N balance (% absorbed)	45.88[a]	55.73[b]	2.246	0.019

Total mixed ration (TMR) provided as mash (TMRm) or compact block (TMRb).
DOMI: Digestible organic matter intake. MCP: microbial crude protein. PD: purine derivatives. SEM: Standard error of the mean.
Treatment means with different superscript letters differ significantly (P < 0.05).

and it was evident in TMRb. Microbial N synthesis per kg digestible OM intake (DOMI) and microbial CP per 100 g DOMI were higher (P < 0.05) in TMRb. It can also be substantiated with lower gas production from feed samples when incubated *in vitro*. Thus, energy saved through less CH_4 emission was utilized for microbial growth because microbial cell synthesis is dependent on total (Adenosine Triphosphate) ATP availability as well as the efficiency of ATP use for biomass production [60]. Thus, an increase in DM intake contributed to higher N and ME availability for microbial growth in TMRb, resulting in higher MPS. Improved MPS with increased OM and CP digestibility on feed block-containing diets has also been reported [61].

In response to higher feed intake in TMRb, the N balance data (Table 6) revealed significantly higher (P < 0.05) N intake, balance and its utilization expressed as percentage of N intake and absorbed. In TMRm, concentrate was preferentially consumed and degraded in the rumen, leading to higher ammonia N in the first few hours and very much less over the rest of the day, thereby affecting both the N and fiber utilization, whereas in TMRb uptake of both N and carbohydrates was uniform and synchronous. This might have altered the ruminal fermentation attributes, leading to greater efficiency of microbial degradation and usage of dietary N. A probable coincidence of optimized availability of C and N skeleton with the rate of fermentation reaching maximum would have been reflected through higher microbial protein synthesis and better N balance in TMRb. This implies that the supply of available N in the rumen is relatively well synchronized with the slow release of energy for MPS in TMRb. But there is little experimental evidence to support the synchrony of energy and N release in the rumen, although it has been demonstrated that altering the degree of synchrony in the rates of ruminal release of energy and N has a marked effect on MPS [7,57]. Theoretically, synchronization of energy and N supply in the rumen should allow more efficient use of nutrients by rumen microbes and increase microbial protein and fermentation end products, thus increasing the available nutrients in the small intestine.

Conclusion

The physical form of TMR offered as a mash (TMRm) or a compact feed block (TMRb) has a significant bearing on DM and nutrient intake, which ultimately regulates ruminal fermentation process toward reduced methanogenesis with concomitant energy saving, leading to higher ME intake and improved microbial protein synthesis. Further, conservation of fermentable CH_4 energy (3.6% of ME) through reduced CH_4 emission would have provided a different H_2 sink for efficient usage of N and energy toward production. Provision of TMR in block form had a positive effect on feed protein digestibility and improved efficiency of energy utilization, as evidenced from positive rumen fermentation attributes and reduced loss of energy through fermentable gases including CH_4, which contributed to higher ME intake, which can be used for production and reducing the feed and environmental cost of C emission.

Acknowledgements

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