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ORIGINAL ARTICLE

Microbial profiles, in vitro gas production and dry matter digestibility based on various ratios of roughage to concentrate

Sanjay Kumar · Sumit Singh Dagar · Sunil Kumar Sirohi · Ramesh Chandra Upadhyay · Anil Kumar Puniya

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Abstract The study assessed the effects of different roughage to concentrate ratios on enteric methane production, rumen fermentation and microbial counts. These ratios were 80:20, 50:50, and 20:80 for diets 1, 2, and 3, respectively. No significant differences were observed in total gas production among diets; however, methane emissions increased ($P<0.05$) with increased roughage in diet. The pH was greater ($P<0.05$) in diet 1 compared to diets 2 and 3 (6.38 vs 6.17 and 6.07). In vitro dry matter digestibility increased with decreased roughage ratios (47.67, 61.67, 67.33 % for diets 1, 2 and 3, respectively). Similarly, total volatile fatty acids (mM/100 mL) also increased with decreased roughage ratios [diet 1 (5.38); diet 2 (6.30); diet 3 (7.37)]. Methanogen counts, total bacterial counts and protozoal counts were lower ($P<0.05$) in diet 3 compared to diet 1 and 2. However, total fungal counts were higher in diet 1 compared to diet 2 and 3. The results indicate that methane emission, enteric fermentation patterns, and change in methanogens population appear only with higher level of roughage. These findings are important for reducing methane without any impact on rumen performance.

S. Kumar · S. S. Dagar · A. K. Puniya (✉)
Dairy Microbiology Division, National Dairy Research Institute, Karnal 132001 Haryana, India
e-mail: akpuniya@gmail.com

A. K. Puniya
e-mail: akp@ndri.res.in

S. K. Sirohi
Dairy Cattle Nutrition Division, National Dairy Research Institute, Karnal 132001 Haryana, India

R. C. Upadhyay
Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal 132001 Haryana, India

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Introduction

Enteric emissions of greenhouse gases are of major concern, especially due to their role in climate change (Kumar et al. 2009). Methane is one of the most potent greenhouse gases contributing to farm level emissions, when expressed in CO_2-eq to account for warming potential (Beauchemin et al. 2010; Veysset et al. 2010). A negative implication of methane production by ruminants is a loss of 2–12 % of gross feed energy. These values can be affected by factors such as the type of feed, feed intake and/or compounds affecting methanogenesis (Johnson and Johnson 1995). Therefore, mitigation of methane emissions from ruminants can lower greenhouse gases and increase the efficiency of livestock production (Kumar et al. 2009). Different strategies, such as dietary manipulations (Beauchemin et al. 2008), use of chemical feed additives (AO 2008), halogenated methane analogues (Anderson et al. 2008), probiotics (Newbold and Rode 2006), bacteriocins (Sar et al. 2004), plant extract (Patra et al. 2011), etc., can be used to solve this problem; however, no ideal solution has been achieved yet.

Feeding high-concentrate or restricted-roughage rations was found to increase ruminant productivity and decrease methanogenesis per unit of the feed ingested (Martin et al. 2010). This might be due mostly to the shifting of rumen fermentation towards propionogenesis, whereas fibrous diets result in the preferential production of acetate, butyrate and methane compared to a concentrate diet. However, limited information is available on the levels of roughage and concentrate ratios suited to enhanced animal productivity. Therefore, the present study was designed to assess the effects of diets on methanogenesis, digestibility patterns and different rumen microbial groups for methane mitigation.

Materials and methods

Diets and batch fermentations

Fresh rumen liquor was collected from fistulated Murrah buffalo, maintained at National Dairy Research Institute, Karnal, on a standard wheat straw based diet (concentrate/roughage ratio; 40: 60), just before morning feeding in a pre-warmed (39 °C), CO_2-flushed, insulated flask. The liquor was brought immediately to laboratory and used as the source of inoculum within 30 min of sampling.

Different diets were prepared by mixing roughage [wheat straw and Berseem (*Trifolium alexandrinum*) in 70:30 ratio] and concentrate (maize, 33; groundnut cake, 21; mustard cake, 12; wheat bran, 20; deoiled rice bran, 11; mineral mixture, 2 and salt, 1 %) in different ratios of 80:20; 50:50 and 20:80 for diets 1, 2 and 3, respectively.

The in vitro Hohenheim gas test apparatus was used as described by Menke and Steingass (1988). Three sets of syringes were prepared in triplicate using three different diets (200 mg) as substrate in the test syringes. The buffered medium (30 mL) containing rumen microflora was dispensed into the syringes and incubated at 39 °C for 24 h.

Fermentation characteristics

After incubation for 24 h, total gas was recorded from the calibrated scale on syringes and the methane was analyzed as described by Kumar et al. (2012).

In vitro dry matter digestibility (IVDMD) was determined using the method of Tilley and Terry (1963), and total volatile fatty acids (TVFA) were calculated as described by Barnett and Reid (1957). Before measuring the pH, the syringe content was centrifuged at 6,000*g* for 5 min.

After 24 h of incubation, 1.0 mL content from diets was immediately poured in anaerobic diluent (Joblin 2005) and serially diluted for microbiological analysis. For methanogen counts, tenfold dilutions (10^{-4} to 10^{-11}) were inoculated into serum bottles containing 'BY' medium (Joblin 2005; Kumar et al. 2012). Each bottle was flushed with a mixture of 80 % H_2 and 20 % CO_2 under 200 kPa pressure. The bottles were incubated at 39±0.5 °C and manually mixed once each day. After 20 days, the level of methane in the headspace gases was determined as described by Kumar et al. (2012). Tubes with methane concentrations >100 ppm (μg/mL) were counted as positive for the determination of methanogens by the 'most probable number' method. Its values were calculated from the methane positive tubes as described by Clarke and Owens (1983). Fresh rumen fluid was used as a positive control.

Fungal and bacterial counts were taken as thallus forming units (TFU) and colony forming units (CFU) per milliliter, respectively, using the roll-tube method (Joblin 1981; Miller and Wolin 1974). For TFU count, 1.0 mL of 10^{-1} to 10^{-6} dilutions were inoculated in serum bottles containing Joblin's agar medium (Dagar et al. 2011) supplemented with antibiotics (penicillin and streptomycin), rolled and incubated at 39±0.5 °C for 4 days. For total bacterial count, 1.0 mL of 10^{-1} to 10^{-10} dilutions were inoculated with the help of sterile CO_2 flushed syringes in the serum bottles containing total bacterial agar medium (McSweeney et al. 2005) supplemented with antibiotics (nystatin and cycloheximide), rolled and incubated at 39±0.5 °C for 24 h. In roll tubes, the viable count for fungi and bacteria were taken as the mean of the three roll tube counts at the appropriate dilution.

For protozoal counts, a uniform aliquot of syringe contents after 24 h of incubation was mixed with an equal volume of preservative solution [bromocresol green/formalin (30/40 w/v HCHO in water)/saline, 0.06/0.14/0.8, w/v/v] and kept at 4 °C until analyzed. Protozoa were enumerated using the method of Goel et al. (2008).

Statistical analysis

All the experiments used a completely randomized design. The data were analyzed statistically using one way analysis of variance to compare the means as per the procedure of statistical analysis system (SAS/ SPSS 1999 version 10.0 for windows). Significant differences ($P<0.05$) among treatment mean values were determined by the Duncan's multiple range test according to the principles of Steel and Torrie (1980).

Results and discussion

Effect of diets on rumen fermentation parameters

No significant differences were observed in total gas production among different diets (Table 1). Consistent with our results, Getachew et al. (2005) found that gas production and estimated metabolizable energy during 24 h was not affected among seven different forages and nine different concentrates. Eun et al. (2004) also reported that total gas production was not affected by forage:concentrate ratios. However, methane emission (mmol/g substrate) was lower in diet 3 compared to diet 1 and 2. These findings are in agreement with those of Yanez-Ruiz et al. (2008), who reported that increasing the level of concentrate in diet decrease methane emissions. Similarly, Johnson and Johnson (1995) and Whitelaw et al. (1984) reported that methane production decreased when diet changed from a forage-based diet to a high concentrate-based diet. Furthermore, Eun et al. (2004) found that methane production was highest with high (70 %) forage diet compared to medium (50 %) or low (30 %) forage diets.

Table 1 Effect of different roughage to concentrate ratios on rumen fermentation parameters. Values are the mean ± standard error of three replicates; means in the same column with the same lower case letter differ significantly ($P<0.05$). *IVDMD* In vitro dry matter digestibility, *TVFA* total volatile fatty acids

Different roughage to concentrate ratio(s)[a]	Rumen fermentation parameters				
	Total gas (mL)	Methane 1 (mmol/g substrate)	pH	IVDMD (%)	TVFA (meq/100 mL)
Diet 1 (80:20)	52.2±1.70 a	6.17±0.51 a	6.38±0.01 a	47.67±0.59 a	5.38±0.09 a
Diet 2 (50:50)	51.9±2.05 a	5.66±0.75 a	6.17±0.02 b	61.67±0.36 b	6.30±0.10 b
Diet 3 (20:80)	63.0±0.47 a	4.38±0.44 b	6.07±0.02 b	67.33±0.95 c	7.37±0.11 c

[a] Diet 1: Roughage: concentrate (80:20); Diet 2: Roughage: concentrate (50:50); Diet 3: Roughage: concentrate (20:80)

It is well-known that a sub-acute rumen acidosis is associated with the level of concentrate in the diet (Desnoyers et al. 2008). Therefore, pH measurement can be used as a tool to evaluate the fermentation process in the rumen. The pH was greater ($P<0.05$) in diet 1 (high roughage) compared to diet 2 and 3. Desnoyers et al. (2008) observed that with the high concentrate diet, the fermentation process increases and decreases the buffering capacity in the rumen. Therefore, the pH values decreased, as seen in the high concentrate diet.

Lana et al. (1998) also reported the role of pH in regulating methane and ammonia production. Therefore, the findings of methane production and pH are consistent with other research studies confirming that methane production decreases with increasing levels of concentrate in the diet, as well as increasing pH values with increasing levels of roughage in the diet.

The IVDMD system is correlated most highly with in vivo digestibility (Marten and Barnes 1979); many factors can influence IVDMD, including the source and activity of inoculums. The IVDMD increased ($P<0.05$) with increasing levels of concentrate (Table 1). This is likely due to the difference in roughage to concentrate ratios. Similar results for IVDMD were reported by Santra and Karim (2009).

In addition, TVFA increased as the level of roughage decreased, being highest ($P<0.001$) in diet 3 followed by diets 2 and 1. This finding is related closely to lower level of structural carbohydrates in concentrates versus roughage leading to an increased ratio between propionate and acetate. Therefore, a reduction in methane production was observed when the level of concentrate increased (Johnson and Johnson 1995). As a result, livestock performance increases with increasing levels of concentrate (O'Mara 2004). However, care must be taken when a high concentrate diet is fed due to the possibility of acidosis.

Effect of diet on rumen microbial groups

There were no significant differences in methanogen counts between diet 1 and diet 2 (Fig. 1); however, a significant decrease ($P<0.05$) was recorded with diet 3. Walichnowski and Lawrence (1982) reported that a low roughage diet increases propionate, which leads to a decrease in pH, thus reducing methanogenic activity or counts. In addition, the symbiotic association of hydrophobic methanogens with hydrogen producers is usually realized by attachment or by floc formation (Lange et al. 2005). Among these ciliates, protozoa are the only organisms for which such interaction can be demonstrated microscopically (Vogels et al. 1980). The symbiotic relationship between methanogens and ciliates can generate up to 37 % of rumen methane (Finlay et

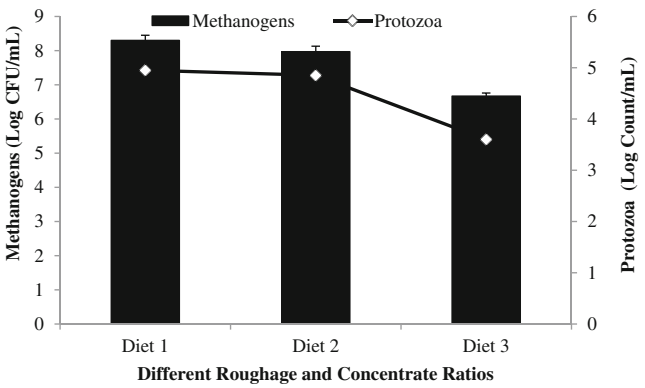

Fig. 1 Effect of different roughage to concentrate ratios on rumen methanogens and protozoa (for diets 1, 2 and 3, see Table 1)

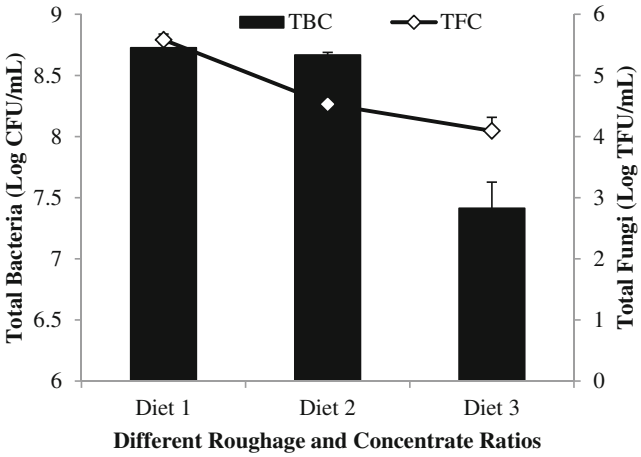

Fig. 2 Effect of different roughage to concentrate ratios on total bacteria and fungi (for diets 1, 2 and 3, see Table 1)

al. 1994). Therefore, low roughage feeding has the potential to reduce methane by reducing protozoal counts (Van Soest 1994), and thus methanogens. In addition, Ohene-Adjei et al. (2007) also suggested association of different archaeal phylotypes with specific groups of protozoa. Therefore, it is important to count these organisms as there are many direct and indirect effects of protozoa, not only on rumen fermentation but also on other rumen microflora. A significant reduction ($P<0.05$) in protozoal counts was observed when diets 1 and 2 were compared to diet 3; however, no noticeable differences were observed in protozoa counts between diets 1 and 2 (Fig. 1). The results obtained are in accordance with those of Van Soest (1994), who stated that concentrate feeding reduces methane by reducing protozoa.

Total bacterial counts were lower ($P<0.05$) on diet 3 compared to diets 1 and 2 (Fig. 2). Singh and Singh (1997) reported a decrease in methanogens and cellulolytic bacteria when a concentrate: roughage diet (75:25) was given to cattle. Yanez-Ruiz et al. (2008) also reported more cellulolytic bacteria in lamb groups fed with higher roughage than in a group fed with a low roughage diet.

Anaerobic fungi found in the rumen and other parts of the gastro-intestinal tract of herbivorous animals have a positive role to play in fiber degradation, as evidenced by the presence of different fibrolytic enzymes (Paul et al. 2003). Fungal counts were lower ($P<0.05$) in diet 3 than in diets 1 and 2 (Fig. 2). Kamra (2005) also documented that fiber-based diets stimulate fungal growth in the rumen of buffalo in comparison to diets rich in easily fermentable carbohydrates, thus supports the present findings.

The present study concludes that a diet low in roughage content may not only have a positive impact on animal environment sustainability but may also enhance rumen performance.

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