Changes in methane emission, rumen fermentation in response to diet and microbial interactions

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ARTICLE INFO

ABSTRACT

To evaluate relative contributions of different microbial groups in rumen, the mono-culture (i.e. bacteria, protozoa and fungi) and co-cultures (i.e. bacterial–protozoal, fungal–protozoal and bacterial–fungal) were tested *in vitro* using high and low roughage diets. Total gas and methane were higher in bacterial–fungal and bacterial–protozoal co-cultures, while lower in fungal–protozoal than controls (high and low roughage with complete rumen consortia; control 1 and 2, respectively). Digestibility and total volatile fatty acids were lower in bacterial–fungal co-culture with both high and low roughage diets. Methanogens decreased in bacterial–fungal co-culture with high roughage. With high roughage, counts were lower for bacteria with bacterial–protozoal, protozoa with fungal–protozoal, and fungi with the bacterial–fungal co-cultures. Total gas was higher in bacterial mono-culture with low roughage, but methane was not detected in any mono-culture. Digestibility and total volatile fatty acids were significantly lowered with protozoal mono-culture. Methanogens reduced significantly in mono-cultures with high roughage diet than control 1. Defaunation reduced methanogens without significantly affecting rumen fermentation.

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1. Introduction

Rumen is one of the most extensively studied gut community, characterized not only by high population density of anaerobes (10^{10-11} bacteria, 10^{8-9} methanogens, 10^6 ciliate protozoa and 10^6 anaerobic fungi/mL), but also by the diversity and complexity of micro-ecological interactions (Kumar et al., 2009, in press). On one side, bacteria and fungi are studied due to their remarkable genetic pool of hydrolytic enzymes (Krause et al., 2003); whereas, on the other hand, the interest in methanogens and protozoa is due to their role in methane production. As per Moss et al. (2000), ruminants lose between 2% and 15% of their ingested energy solely as methane, which is further influenced by the dietary and microbial interactions. However, despite negative effects, methanogens are crucial for the proper functioning of rumen, as these utilize reducing equivalents, routinely produced by hydrogen-producing microbes (Kumar et al., 2012), and thus act as hydrogen sinks. The physical association of methanogens with hydrogen producers is reported with ciliate protozoa (Kumar et al., 2009) and fungal mycelia (Jin et al., 2011). The symbiotic association of methanogens and protozoa generate 9–25% of methane (Newbold et al., 1995). Rumen fungi also produce substantial hydrogen and thus interact with methanogens that in turn alters their metabolism (Kumar et al., 2009). Besides, microbial interactions, diet also influence methanogens and methane production by altering the type of fermentation. Therefore, dietary changes may manipulate the rumen fermentation by inhibiting methanogens, while reducing methane emissions. Moreover, the synergism and antagonism among different microbial groups and even among different genera of the same group is quite complicated and thus, it is difficult to quantify the role of a particular microbial group in rumen. There are no known specific reports on the combined effect of varying diet and rumen co-culture/mono-culture on methane and rumen microflora. Therefore, an *in vitro* study to estimate the role of different microbial groups on rumen fermentation and methane emission was done in response to two different types of diet. This may help in understanding rumen microbial ecology and a strategy for methane mitigation.

2. Materials and methods

2.1. Diets

The diets prepared with different ratios of roughage (70% wheat straw and 30% berseem; *Trifolium alexandrinum*) and concentrate (maize 33%, groundnut cake 21%, mustard cake 12%), (wheat bran 20%, deoiled rice bran 11%, mineral mixture 2% and salt 1%).

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2.2. Source of rumen liquor

Rumen liquor from fistulated non-lactating Murrah buffaloes ($\approx$12 months of age, fed on standard diet of concentrate:roughage ratio; 40:60) was collected before morning feed by squeezing the collected feed mass into pre-warmed ($\approx$39 °C) thermos flasks and strained through 100 mm nylon net before being used as inoculums.

2.3. Separation of microbial groups

Different microbial groups from rumen contents were separated according to Lee et al. (2000) with the exceptions that potassium and chloramphenicol (inhibitory to methanogens) were replaced with vancomycin, and rumen liquor was not heat sterilized. All succeeding processes were conducted under anaerobic conditions as described by Bryant (1974). Strained rumen liquor was used for control 1 (with high roughage diet) and control 2 (with low roughage diet).

2.4. Batch fermentation

In vitro Hohenheim gas test apparatus (Menke and Steingass, 1988) was used according to Makkar et al. (1995). Twelve sets of syringes (Table 1) and controls were prepared using high and low roughage diet (200 mg) as substrates. The buffered medium (30 mL) containing rumen microbes was dispensed into syringes and incubated at 39 °C for 24 h and the contribution of each rumen microbial group in mono-culture (i.e. bacteria, fungi and protozoa) and co-culture (bacterial–fungal, fungal–protozoal and bacterial–protozoal) were evaluated on methane emission and other rumen fermentation parameters in triplicate, and the experiment was repeated thrice.

2.5. Analysis

Total gas was recorded from the calibrated scale on syringes. For methane, the gas (10 mL) was withdrawn using a leak-proof syringe and analyzed according to Kumar et al. (2012). The methane produced from the substrate was calculated as follows:

Methane production (mL) = Total gas (mL)

$\qquad \times$ Methane content (%) in sample

The methane values were converted in mmol as:

Methane (mmol) = Volume of methane in ml/(Gas constant

$\qquad \times$ Temperature in Kelvin/Atmospheric pressure)

In vitro dry matter digestibility was calculated as per Tilley and Terry (1963) and total volatile fatty acids as per Barnett and Reid (1957).

2.6. Microbial population

After incubation, 1.0 mL of spiked syringe content from each dietary trial was immediately poured in anaerobic diluents (Joblin, 2005) and serially diluted. For methanogens, 10^{-4}–10^{-11} dilutions were inoculated into serum bottles containing 'BY' medium (Joblin, 2005; Kumar et al., in press). Each tube was flushed with 80% H_2 and 20% CO_2 to attain 200 kPa of pressure. The tubes were incubated at 37 °C and mixed manually each day. After 20 days, the level of methane in the headspace gas was determined with gas chromatograph. Tubes with methane >100 ppm were regarded as positive for the determination of methanogens by 'most probable number (MPN)' method. The MPN values were calculated from the methane positive cultures (controls) as per Clarke and Owens (1983). Fungal and bacterial counts were taken as thallus forming units (TFU/mL) and colony forming units (CFU/mL), respectively, using roll-tube method on media as described by (Joblin, 1981). For protozoal counts, uniform aliquots from the syringes were taken and mixed with equal volumes of preservative [bromocresol green/ formalin (30–40 w/v HCHO in water)/saline, 0.06/0.14/0.8, w/v/v] and maintained at 4 °C until analyzed. The protozoa were enumerated on Fuchs Rosenthal counting chamber (depth 0.2 mm, 0.0125 µL/grid).

2.7. Statistical analysis

Data from the experiments were analyzed as a randomized complete design using General Linear Model procedure (Wilkinson, 1997). The statistical model used was:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where, Y_{ij} is the response variable, μ the overall mean, T_i the effect of treatment, and e_{ij} the random residual error. Following a significant F test ($P < 0.05$), differences among means were examined by Duncan multiple range test.

3. Results

3.1. Methane emission and rumen fermentation

Total gas production was higher in controls 1 and 2 (whole rumen microbial consortia with high and low roughage diet, respectively) followed by bacterial–fungal and bacterial–protozoal co-cultures in comparison to fungal–protozoal co-culture with high and low roughage diets. Methane emissions (mmol/g substrate) decreased significantly ($P < 0.05$) when high and low roughage diet were fermented with bacterial–protozoal (4.28 and 2.22), bacterial–fungal, (3.96 and 2.45) and fungal–protozoal co-cultures (2.48 and 1.54), whereas controls 1 produced highest methane (6.28) followed by control 2 (4.11) as shown in Fig. 1A.

A significant decrease in pH from strained rumen liquor (6.65) to control 1 (6.36) and 2 (6.02) was observed. The decrease in pH was also observed when high and low roughage diet were fermented with bacterial–protozoal (6.41 and 6.25) and

Table 1
The experimental design; HRD: high roughage (80%) diet; LRD: low roughage (20%) diet.

Trials	Diet composition (% roughage) and fermenting microbial group(s)		
Control 1	HRD + whole rumen liquor + buffer	Trial 6	HRD + protozoa + buffer
Control 2	LRD + whole rumen liquor + buffer	Trial 7	LRD + bacteria + fungi + buffer
Trial 1	HRD + bacteria + fungi + buffer	Trial 8	LRD + bacteria + protozoa + buffer
Trial 2	HRD + bacteria + protozoa + buffer	Trial 9	LRD + fungi + protozoa + buffer
Trial 3	HRD + fungi + protozoa + buffer	Trial 10	LRD + bacteria + buffer
Trial 4	HRD + bacteria + buffer	Trial 11	LRD + fungi + buffer
Trial 5	HRD + fungi + buffer	Trial 12	LRD + protozoa + buffer
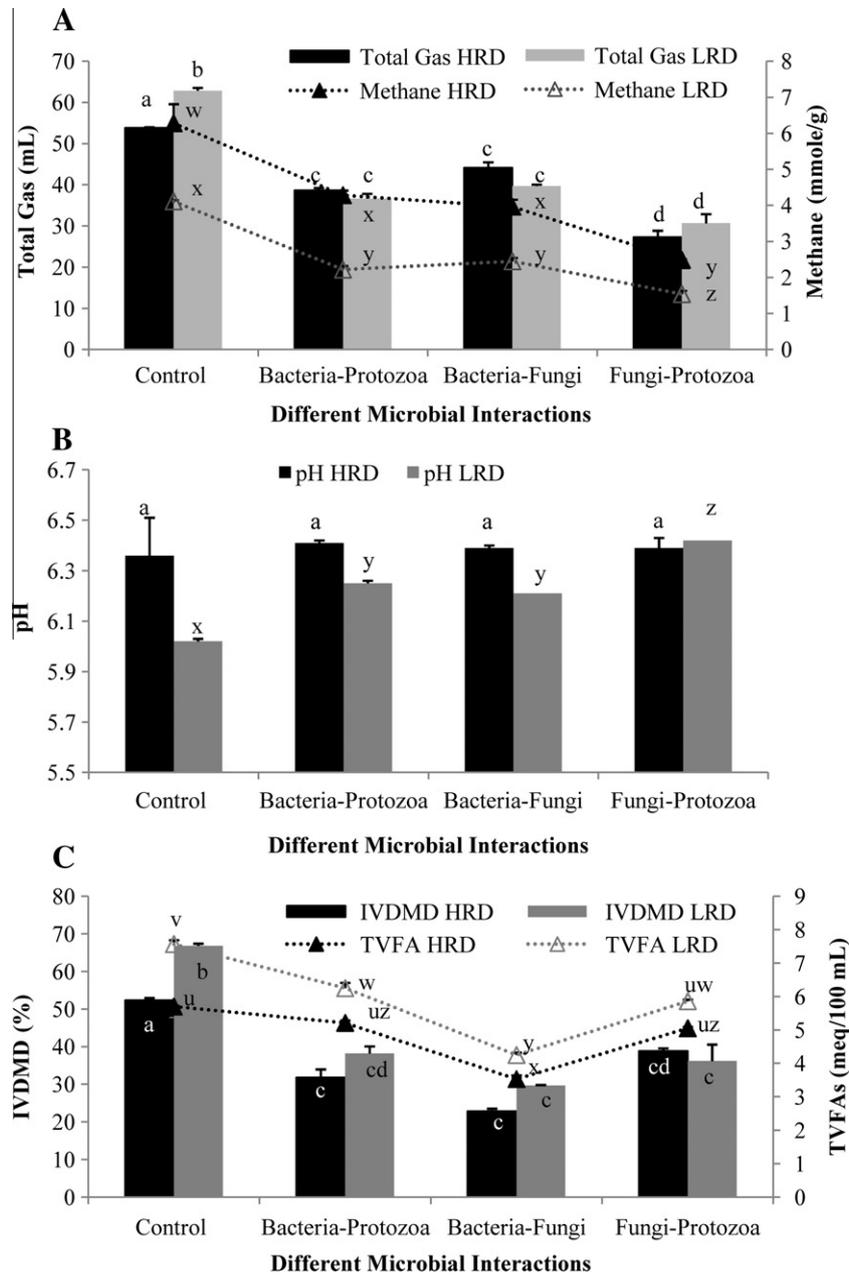
Fig. 1. Effect of co-cultures of rumen microbial groups and diets (A) total gas and methane (B) pH (C) IVDMD (*in vitro* dry matter digestibility) and TVFAs (total volatile fatty acids). HRD: high roughage diet (80%): LRD: low roughage diet (20%); different superscripts: differ significantly (*P* < 0.05); error bar: standard error of three replicates.

bacterial–fungal (6.39 and 6.21) co-cultures. Compared to the controls, no significant (*P* < 0.05) change in pH was observed within bacterial–protozoal and bacterial–fungal co-cultures with high and low roughage diet. The pH shift in fungal–protozoal co-culture (6.39 and 6.42) with high and low roughage diet was not significant (*P* < 0.05) and was similar to Control 1, bacterial–protozoal and bacterial–fungal co-cultures fermenting high roughage diet. However, bacterial–protozoal and bacterial–fungal co-cultures fermenting low roughage diet showed significant (*P* < 0.05) pH change compared to control 2 (Fig. 1B).

Percent digestibility was significantly (*P* < 0.05) higher in control 2 (66.83%) followed by control 1 (52.33%), whereas bacterial–protozoal and fungal–protozoal co-cultures with low roughage diet showed maximum digestibility of 38.16% and 36.16%, respectively. With high roughage diet, both the groups showed similar digestibility. The bacterial–fungal group showed

lowest digestibility of 22.83 and 29.66 with high and low roughage diet, respectively. Total volatile fatty acids (meq/100 mL) were higher in control 2 (7.57), followed by bacterial–protozoal co-culture with low roughage diet (6.25). Control 1 and fungal–protozoal co-culture with low roughage diet produced a similar amount of total volatile fatty acids. No significant (*P* < 0.05) difference in total volatile fatty acids amount was found with bacterial–protozoal and fungal–protozoal co-cultures with high roughage diet, however bacterial–fungal co-culture produced significantly (*P* < 0.05) lower total volatile fatty acids (3.53 and 4.25) with high and low roughage diet, respectively. The patterns obtained for total volatile fatty acids were similar to digestibility (Fig. 1C).

In mono-cultures, no significant differences (*P* < 0.05) in total gas production (mL) were observed among bacterial (28.16 with high roughage diet), fungal (28.16 and 28.33) and protozoal groups (28.16 and 26.83) with high and low roughage diet, respectively

except bacterial group with low roughage diet (35.50). However, significant decrease ($P < 0.05$) in total gas production was observed when individual groups were compared with controls. Methane emissions were even lower than the detectable limit in each group with both the diets (Fig. 2A).

The pH changes were lowest in protozoal group (6.55 and 6.52) with high and low roughage diet, respectively, whereas significant differences ($P < 0.05$) were observed in pH of bacterial and fungal group fermenting high and low roughage diet, compared to controls (Fig. 2B). Percent digestibility was also significantly low ($P < 0.05$) in protozoal group (17.66 and 15.16) with high and low roughage diet, respectively, supporting the results obtained for pH. However, no significant ($P < 0.05$) change in digestibility was observed between bacterial and fungal group. A similar pattern in total volatile fatty acids results was also observed. The total

volatile fatty acids were higher in bacterial group with low roughage diet (4.16) than high roughage diet (3.60), followed by fungal and protozoal group (Fig. 2C).

3.2. Microbial count

Methanogens (log count/mL) did not decrease considerably in bacterial–protozoal (8.72 and 6.55), and fungal–protozoal (8.74 and 6.37) co-cultures in comparison to control 1 (8.63) and 2 (6.66). However, a significant ($P < 0.05$) decrease in counts was observed in bacterial–fungal co-culture with high roughage diet (7.66), whereas, with low roughage diet, no changes were observed over control 2, bacterial–protozoal and fungal–protozoal co-cultures with low roughage diet (Table 2).
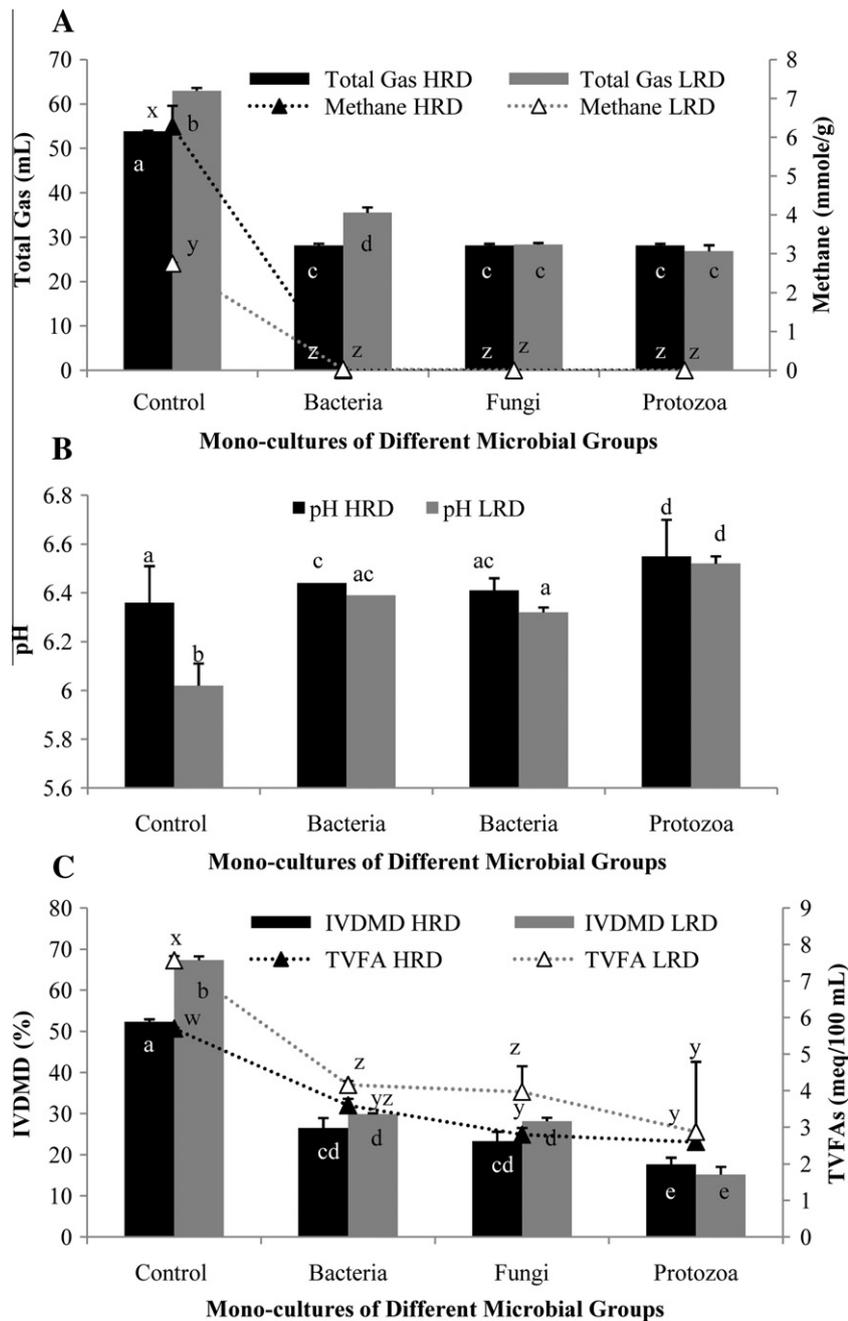

Fig. 2. Effect of mono-cultures of rumen microbial groups and diets (A) total gas and methane (B) pH (C) IVDMD (*in vitro* dry matter digestibility) and TVFAs (total volatile fatty acids). For details see Fig. 1.

Total bacterial counts with high and low roughage diet decreased significantly ($P < 0.05$) in bacterial–protozoal co-culture compared to control 1 and 2, whereas no change in bacterial counts was observed in bacterial–fungal co-culture compared to controls (Table 2). In bacterial–fungal co-culture, significant ($P < 0.05$) decrease (2.45) in fungal counts was observed with high roughage diet, whereas no difference in fungal counts was observed in other experiments. No change in protozoal count was observed in bacterial–protozoal co-culture (5.43 and 5.11) in comparison to controls (5.37 and 4.97) with high and low roughage diet, respectively, whereas in fungal–protozoal co-culture, protozoal counts were 4.81 and 3.65; significantly ($P < 0.05$) lower than both controls and bacterial–protozoal co-culture (Table 2).

It is evident from Table 3 that methanogens decreased from controls 1 (8.63) and 2 (6.67) to bacterial (7.11 and 6.34), followed by fungal (7.23 and 6.02) and protozoal group (5.57 and 5.23) on high and low roughage diet, respectively. However, no significant decreases in bacterial counts were observed in bacterial group and controls (Table 3). No changes in fungal counts were recorded but for protozoa, counts were significantly ($P < 0.05$) lower (4.81 and 3.46) than controls 1 (5.37) and 2 (4.97) (Table 3).

4. Discussion

Different researchers have reviewed the interactions involved in fibre degradation by rumen microbial groups (Jouany, 1989; Wolin et al., 1997; Lee et al., 2000; Foroozandeh et al., 2009; Hook et al., 2011), yet, a shift in methane emission, rumen fermentation and response of bacteria, fungi, and protozoa in context to diet and inter group interactions is unclear. In present study, the differences in total gas production in response to diet provide a comparison of the role of microbial groups in degradation of fibre in high (80%) and low (20%) roughage diets. Higher total gas production with bacterial–protozoal, bacterial–fungal co-cultures and mono-culture of bacteria with low roughage diets, clearly indicate the roles of bacteria in feed digestion due to their predominance and metabolic diversity (Cheng et al., 1991). In co-cultures, methane emissions were similar to total gas, and the results are in agreement with Kamra et al. (2006), who reported significant ($P < 0.01$) positive correlation ($r = 0.57$) between total gas and methane production. However, in mono-cultures, interspecies hydrogen transfer must not have occurred thus methane emissions were not detected despite total gas production.

Low roughage diet produced lower methane with bacterial–protozoal, bacterial–fungal and fungal–protozoal co-cultures compared to high roughage diet. Yan et al. (2000) also reported the negative correlation between proportion of concentrate in diet and methane emissions. Influence of forage:concentrate ratio on acetate:propionate ratio is well documented, and Johnson and Johnson (1995) reported a drastic decrease in methane emission from 6–12% (forage-based diet) to 2–3% on high concentrate (90%) diet. Similarly, Eun et al. (2004) reported highest methane

production on high forage (70%) diet over medium (50%) and low (30%) forage diet. Although pH is expected to decrease after fermentation (Lee et al., 2000), but fermentation of diet with different microbial groups in either mono- or co-culture did not follow a similar pattern. This is because the fermentation capacity of each group differs individually or in combination. The protozoal group alone and in combination with fungi was associated with poor fermentation and thus, a non significant change in pH occurred, as compared to strained rumen liquor. However, pH changes with bacterial–protozoal, bacterial–fungal co-cultures, and bacterial and fungal mono-cultures were comparable to controls, suggesting a role of bacteria and fungi in fibre digestion.

The digestibility did not indicate any significant differences in the fermentation patterns of bacteria and fungi individually. In co-culture, bacterial–protozoal, as well as bacterial–fungal co-cultures showed similar digestibility results. Since, fungi are regarded as the primary colonizers their role becomes critical in the digestion of poor quality forages (Dagar et al., 2011). The possible reason for their poor in vitro degradation of high roughage diet might be explained on the basis of their life cycle that lasts for 72 and 24 h incubation might be an insufficient time for the production of hydrolytic enzymes. Furthermore, the rapid growth of bacteria might affect the fungal growth (Dehority and Tirabasso, 2000). Mono-culture also supports co-culture's results, as no significant differences in digestibility were observed with bacterial and fungal groups. However, protozoal group showed least digestibility among all groups, as also observed by Lee et al. (2000). The results of total volatile fatty acids were similar to digestibility, and thus in agreement with Kamra (2005).

Rumen microbes interact to improve digestion beyond the capacity of individual species, provide nutrients and also remove inhibitory products. Therefore, we tried to assess the dynamic nature of rumen microbial groups, as the population changes with diet and other microbes in the close proximity. Kamra (2005) reported that though the methane emission was reduced to an undetectable level and methanogens reduced significantly, but not eliminated completely, as in our study. In co-cultures, no significant change in methanogens was observed except bacterial–fungal co-culture with high roughage diet. The endo- and exo-symbiotic relationship of protozoa and methanogens has been well reported (Finlay et al., 1994). The count obtained with mono-culture groups are also in agreement with Kamra et al. (2006). The lack of protozoa in co-culture might have reduced the counts.

Total bacterial counts significantly reduced in bacterial–protozoal co-culture compared to controls, whereas in bacterial–fungal co-culture, there was no change in counts. Kamra (2005) mentioned that protozoa feed on bacteria, thus decreasing the counts, whereas in their absence, bacteria and fungi counts increase in rumen. However, no significant change in count was observed with mono-culture. Total fungi remained unchanged in all the co-culture and mono-culture fermentation except high roughage diet with bacterial–fungal co-culture. This might be due to – (a) the predominance and higher metabolic activity of

Table 2

Effect of co-cultures of rumen microbial groups and diets on rumen microbial groups (log count). For details see Table 1; means in the same column bearing different superscripts differ significantly ($P < 0.05$); ±: standard error of three replicates.

Trials	Methanogens (log count/mL)	Total bacteria (CFU/mL)	Total fungi (TFU/mL)	Protozoa (log count/mL)
HRD (Control 1)	8.63[b]	9.85 ± 0.02[b]	5.49 ± 0.17[a]	5.37 ± 0.07[a]
LRD (Control 2)	6.66 ± 0.02[c]	8.70 ± 0.05[a]	4.90 ± 0.02[a]	4.97 ± 0.05[ab]
HRD + bacteria–protozoa	8.72[b]	9.01 ± 0.02[a]	Nil	5.43 ± 0.03[a]
LRD + bacteria–protozoa	6.55 ± 0.03[c]	7.90 ± 0.06[c]	Nil	5.11 ± 0.03[ab]
HRD + bacteria–fungi	7.66[a]	9.66 ± 0.10[b]	2.45 ± 0.37[b]	Nil
LRD + bacteria–fungi	6.51 ± 0.04[c]	8.89 ± 0.06[a]	4.53 ± 0.17[a]	Nil
HRD + fungi–protozoa	8.74[b]	Nil	5.26 ± 0.27[a]	4.81 ± 0.03[b]
LRD + fungi–protozoa	6.37 ± 0.07[c]	Nil	4.90 ± 0.04[a]	3.65 ± 0.03[c]

Table 3
Effect of mono-cultures and different diets on other rumen microbial groups (log count); for details see Tables 1 and 2.

Trials	Methanogens (log count/mL)	Total bacteria (CFU/mL)	Total fungi (TFU/mL)[*]	Protozoa (log count/mL)
HRD (Control 1)	8.63 ± 0.04[b]	9.85 ± 0.01[b]	5.46 ± 0.01	5.37 ± 0.01[a]
LRD (Control 2)	6.67 ± 0.09[c]	8.70 ± 0.05[a]	5.76 ± 0.02	4.97 ± 0.05[ac]
HRD + bacteria	7.11 ± 0.09[c]	9.61 ± 0.11[b]	Nil	Nil
LRD + bacteria	6.43 ± 0.05[ce]	8.67 ± 0.10[a]	Nil	Nil
HRD + fungi	7.23 ± 0.05[c]	Nil	5.26 ± 0.02	Nil
LRD + fungi	6.02 ± 0.06[cde]	Nil	5.69 ± 0.17	Nil
HRD + protozoa	5.57 ± 0.15[cd]	Nil	Nil	4.81 ± 0.71[c]
LRD + protozoa	5.23 ± 0.05[cd]	Nil	Nil	3.46 ± 0.08[b]

[*] Non Significant.

bacteria in response to high roughage diet that is supported by rumen fermentation results, and (b) antibiosis between the two groups. The lower count of protozoa high and low roughage diet might be due to lack of bacteria and fungi, and improper feed utilization and thus poor adaptation of the protozoa to such an environment. In case of co-culture, low roughage diet with fungi and protozoa also leads to a lower protozoal count; probably due to the concentrate-rich diet that affects protozoa and thus, is in accordance with Van Soest (1994).

5. Conclusion

With high roughage, the fungal group was more active than bacterial group, whereas bacterial group was more active with low roughage. Protozoa alone did not perform better in rumen fermentation with high and low roughage diets. In high roughage diet, methanogens decreased significantly with co-culture of bacteria and fungi, as well as mono-culture of these, showing a combined effect of diet and defaunation. However, methane emissions did not decrease to a greater extent, therefore, removal of protozoa decreases methanogens without affecting rumen fermentation. The diet along with defaunation would be better for methane mitigation and proper rumen function both.

Acknowledgements

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