

## Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations

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Bromochloromethane (BCM), a halogenated methane analogue, was evaluated for its anti-methanogenic activity in both batch and continuous fermentation systems. For batch fermentation, a roughage-based substrate was incubated with mixed rumen microflora for 24 h at two concentrations of BCM (5 and 10  $\mu\text{M}$ ). A 89–94% reduction in methane was obtained in terms of volume and truly degraded substrate (TDS;  $P < 0.05$ ). The partitioning factor (an index of efficiency of microbial protein production; expressed as mg TDS/ml net gas produced) increased from 3.55 to 3.73 ( $P < 0.05$ ), while the acetate:propionate proportion decreased from 2.80 to 2.22 ( $P < 0.05$ ). A complete inhibition of methanogens was associated with a 48% decrease in *Ruminococcus flavefaciens*, a 68% increase in *Fibrobacter succinogenes* and a 30% increase in rumen fungi when quantified using qPCR. The continuous fermentation was carried out using the roughage-based substrate in four fermenters, two fermenters being control and the other two fed with BCM (5  $\mu\text{M}$ ) once in a day, for nine consecutive days. A persistent effect of BCM on methane reduction (85–90%) was obtained throughout the study ( $P < 0.05$ ) with no effect on gas production, SCFA production, acetate:propionate proportion, true degradability and efficiency of microbial mass synthesis ( $P > 0.05$ ). The complete inhibition of methane resulted in a significant decrease in *R. flavefaciens* and methanogens ( $P < 0.05$ ) and an increase in fungal population ( $P < 0.05$ ), while there was no effect on total bacterial and *F. succinogenes* populations ( $P > 0.05$ ). The batch fermentation confirms the anti-methanogenic activity of BCM, while the continuous fermentation indicates the persistency of this effect under *in vitro* conditions.

### Bromochloromethane: Batch fermentation: Continuous rumen fermentation: Microbial communities: Fermentation characteristics

In recent years, methane production from the livestock has gained considerable attention due to the significant role of methane in global warming<sup>(1)</sup>. The methane generated from livestock contributes to 19% of the total methane production from anthropogenic sources<sup>(2)</sup>. Fermentation of feeds in the rumen is the largest source of methane from enteric fermentation. In the rumen, methane is produced by archaeal micro-organisms known as methanogens, which utilises predominantly CO<sub>2</sub> and hydrogen in the rumen to form methane, thereby maintaining the lower partial pressure of hydrogen in the rumen. This methane production from archaea in the rumen results in a 2–12% loss of metabolisable energy<sup>(1)</sup>. Therefore, decreasing methane production from ruminants is desirable for reducing the greenhouse gas emission with improved efficiency of digested energy utilisation. The strategies for methane reduction include chemical inhibitors (such as organic acids, oils, ionophores, etc.), feeding diets rich in unsaturated fatty acids and modifying feeding practices<sup>(3,4)</sup>. Among these chemical inhibitors, the halogenated methane analogue, bromochloromethane (BCM), has been used in a few studies to inhibit methanogenesis. This compound is believed to react with reduced vitamin B<sub>12</sub> and

results in the inhibition of cobamide-dependent methyl group transfer in methanogenesis<sup>(5,6)</sup>. Sustained reduction in methane, with improved feed efficiency has been reported with this compound when used in *in vivo* studies in cattle and sheep<sup>(7)</sup>; however, so far, no report is available on its effect on different microbial communities and accompanied rumen fermentation characteristics. Therefore, the present study aimed to analyse the effect of BCM on rumen fermentation and microbial community structure in *in vitro* batch fermentation and continuous fermentation systems. The batch incubation was used to optimise and evaluate the BCM concentration and the continuous fermentation system was used to analyse the persistency of the anti-methanogenic activity of the compound.

### Materials and methods

#### Rumen liquor

The rumen liquor from two rumen-fistulated non-lactating Friesian–Holstein cattle was collected manually by squeezing the feed mat into pre-warmed (approximately 39°C) thermos

**Abbreviations:** BCM, bromochloromethane; Ct, threshold cycle; PF, partitioning factor; TDS, truly degraded substrate; Tris, 2-amino-2-(hydroxymethyl) propane-1,3-diol.

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flasks. The cattle were maintained on a diet containing 2 kg of concentrate mixture and roughage was fed *ad libitum*, with free access to drinking-water. The concentrate mixture (in g/kg) comprised of rapeseed cake (360), maize (200), wheat (200), molasses (200), and vitamin and mineral mixture (40). The rumen liquor was collected from the cattle just before the morning feed and brought in warm (approximately 39°C) insulated flasks, strained through a 100 µm nylon net and used as the source of inoculum.

#### Bromochloromethane solution

A stock solution (0.1:0.9, v/v) of BCM (Sigma Aldrich, St Louis, MO, USA) in ethanol was prepared. The stock solution was further diluted with distilled water (0.005:0.995, v/v), and freshly diluted suspension was used in the experiment at concentrations of 5 µM (BCM<sub>L</sub>) and 10 µM (BCM<sub>H</sub>) in the incubation medium.

#### Batch fermentation

**Method.** The *in vitro* Hohenheim gas test apparatus<sup>(8)</sup> was used according to the protocol of Makkar *et al.*<sup>(9)</sup>. Three sets of syringes, i.e. blank, control and test, were prepared using hay (380 mg) as a substrate in the control and test syringes and not in the blank. BCM was added to the test syringes to give a final concentration of 5 and 10 µM. Each set had its respective blank syringes, with only buffered medium without any BCM and hay (for the control syringe), and one with buffered medium without the hay but containing BCM at 5 and 10 µM concentrations (for the test syringes). The buffered medium (40 ml) containing rumen microbes was dispensed into the syringes and incubated at 39°C for 24 h<sup>(9)</sup>. On a single day, each treatment was performed in three syringes and the experiment was repeated on three different days.

**Analysis of fermentation characteristics.** After incubation for 24 h, the batch fermentation was analysed for gas, methane, SCFA, protozoal numbers and truly degraded substrate (TDS), as described in Goel *et al.*<sup>(10)</sup>. The values of net gas, net methane and TDS were used for the calculation of methane reduction and the partitioning factor (PF; TDS/net gas produced), as described in Goel *et al.*<sup>(10)</sup>.

**Hydrogen recovery.** The hydrogen recovery was calculated as hydrogen consumed/hydrogen produced<sup>(11)</sup>. The hydrogen produced as fermentation end products and hydrogen consumed to form methane were determined from the amount and molar proportions of acetate (C2), propionate (C3), butyrate (C4), isovalerate (Ci5) and valerate (C5), and methane produced as described in the following:

$$\text{H}_2 \text{ produced (HP)} = 2 \times \text{C2} + \text{C3} + 4 \times \text{C4} + 2 \times \text{Ci5} + 2 \times \text{C5},$$

$$\text{H}_2 \text{ utilised (HU)} = 2 \times \text{C3} + 2 \times \text{C4} + 4 \times \text{methane} + \text{C5},$$

$$\text{H}_2 \text{ recovery} = (\text{HU}/\text{HP}) \times 100.$$

For example, as in the present study, the net production of SCFA in 24 h was 1.8 mmol with a molar proportion of 0.66 (acetate), 0.22 (propionate), 0.08 (butyrate), 0.0037 (isovalerate) and 0.01 (valerate), and a methane volume of 12 ml (0.4684 mmol with a molar concentration of 0.99).

The methane values were converted in mmol as

$$\text{mmol methane} = \text{volume of methane (in ml)} / (\text{gas constant} \\ \times \text{temperature in Kelvin/atmospheric pressure}).$$

The values for hydrogen produced and utilised in the fermentation, using the afore-mentioned example, are 1.94 and 1.63, respectively, resulting in 84 % recovery of hydrogen.

**Analysis of microbial population.** For DNA isolation, an aliquot (1.8 ml) of fermentation fluid from the syringe was taken using a wide-bore pipette, while the contents were being stirred to obtain a homogeneous sample containing plant particles and liquid. The sample aliquots were preserved at -70°C until analysed. The DNA isolation was carried out using a modified method of Denman & McSweeney<sup>(12)</sup>. Briefly, an aliquot (1.5 ml) from the preserved sample was placed in the 2 ml capacity screw-capped microcentrifuge tube containing 1 g of zirconium beads (0.1 mm; BioSpec Products, Inc., Roth, Karlsruhe, Germany). One millilitre of lysis buffer (0.2 % SDS, 100 mM-Tris-HCl, 5 mM-EDTA, 200 mM-NaCl) was then added and the tubes were placed in liquid nitrogen (-196°C) for 1 min. The sample tubes were then thawed at room temperature and vortexed for 2 min. The freeze-thawing step was repeated twice to enhance the efficiency of DNA extraction. Thereafter, the samples were incubated at 4°C for 10 min and then centrifuged at 14 000g for 15 min at 4°C. The supernatants (0.3 ml in duplicate) were transferred to 1.5 ml capacity Eppendorf tubes and an equal volume of the glass milk (5 g silica in 30 ml guanidine isothiocyanate (3 M), pH 6.0-6.5) was added to it. The tubes were shaken gently for 5 min for allowing the DNA to bind. The glass milk suspension was then centrifuged at 10 000g for 1 min and then washed with aqueous cold ethanol (70:30, ethanol:nanopore water, v/v). In the final step, the DNA was eluted with 100 µl of PCR grade water (Sigma) and stored at -20°C until analysis. The purity and concentration of DNA in the extracted sample was determined as A<sub>260</sub>/A<sub>280</sub>.

The different microbial groups such as total bacteria, total fungi, methanogens, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were determined in the samples using a SYBR green quantitative PCR assay. The PCR machine used was from Biorad (Veenendaal, The Netherlands; iQ5 Real-Time PCR detection system) with a 96-well plate. The PCR conditions and primers for different communities used were from Denman & McSweeney<sup>(12)</sup> and Denman *et al.*<sup>(13)</sup>. The reaction mixture (25 µl) comprised of a SYBR green mix (12.5 µl), primers (forward and reverse, 0.71 µl each), PCR grade water (8.58 µl) and the template (2.5 µl). Total microbial rumen DNA was diluted to a concentration of 10 ng/ml before use in the quantitative PCR assays. The assay was conducted with the following cycle conditions: one cycle at 50°C for 2 min and 95°C for 2 min for initial denaturation; 40 cycles at 95°C for 15 s and 60°C for 1 min for primer annealing and product elongation. The dissociation curve analysis of PCR end products was performed with 71 cycles at 95°C for 1 min, followed by 60°C for 10 s. A negative blank (without the DNA template) was also run for each primer pair. For batch incubations, relative population sizes of *R. flavefaciens*, *F. succinogenes*, total rumen fungi and methanogens were expressed as a proportion of total

rumen bacterial 16S rDNA at 24 h. The threshold cycle ( $\Delta Ct$ ) values were calculated by subtracting the Ct value of the target gene from the Ct value of the reference gene (16S rDNA of total bacteria at 24 h). The relative expression<sup>(12)</sup> of different groups has been calculated from the  $\Delta Ct$  values as  $2^{-\Delta Ct}$ . The shifts in microbial communities<sup>(14)</sup> due to the addition of BCM in the batch system were determined by taking the respective microbial population in the substrate (control) as 100.

### Continuous fermentation

**System description.** The continuous fermenter system<sup>(15)</sup> consisted of six water-jacketed glass vessels (HWS Labortechnik, Mainz, Germany), each having a capacity of 2 litres, with a working volume of nearly 950 ml. The vessels were provided with an outlet at a 45° angle, which allowed the outflow from the fermenter to a 2 litre capacity bottle placed in cold water (4°C). The fermenters were equipped with combined probes for monitoring temperature and pH (Jumo GmbH & Co., Fulda, Germany), stainless steel stirrers and a buffer-providing system. Temperature was adjusted to 39°C by a circulating water-bath linked to a water jacket. Stirring speed and period and buffer inflow rate were controlled individually via a computer program. The pH measurement was carried out with the software Autoferm, provided with the fermenter.

**Incubation conditions.** The fermentation was carried out for 15 d with a daily addition of hay as a substrate (20 g/d; 10 g each at 09.00 and 17.00 hours). Before inoculation, four fermenters were pre-filled with 600 ml of buffer and a half-day ration of substrate (10 g), and were flushed with CO<sub>2</sub>. Rumen fluid was collected and processed as described in Section 'Rumen liquor' and used as a source of inoculum (300 ml in each fermenter). Rumen buffer solution<sup>(15)</sup> was added continuously at a rate of 1 litre in 24 h, controlled through a computer program. The fermenter liquid was stirred at a slow speed of 60 rpm for 12 min during feeding periods, with an intermittent (after every cycle of 12 min) fast shaking (500 rpm) for 3 s. After a cycle of slow and fast shaking, the fermenter contents were stirred slowly for 1 min in every 30 s. The outflow collected in the 2 litre capacity bottle kept in cold water-bath was sampled and the bottle was emptied every 24 h.

After an adaptation period of 6 d, the fermenters were run for the next 9 d. During this period, two fermenters (F1 and F3) were taken as a control, while in the other two fermenters (F2 and F4), BCM was added once in a day during morning feeding to bring its concentration to 5  $\mu$ M.

**Sampling and analysis.** For the analysis of fermentation characteristics after the adaptation period, the samples were withdrawn from the outflow and the fermenter vessel. The sampling was done for 9 d of the experimental period. Some disturbance in the buffer flow was recorded on 2 d and hence the samples collected were not analysed for these 2 d. The protocol followed for different sampling ports is as follows:

- (a) *Samples from fermenter vessels.* On a daily basis, a uniform sample/aliquot (10 ml) from the fermentation liquor was taken directly from the fermenter at 08.45 hours just before the morning feed with a 20 ml wide-bore pipette. Just before taking the aliquot, the contents in the fermenters were shaken thoroughly

using the inbuilt stirrers at a fast speed for 15 s. Different aliquots from the withdrawn sample were processed for the determination of SCFA, protozoa and microbial communities as described previously. The sampling was done daily for the whole experimental run of 9 d.

- (b) *Methane production in fermenters using syringes.* It was not possible to measure gas production directly from the fermenters; therefore, the contents were taken from the fermenters and incubated in 100 ml capacity calibrated syringes used in the batch fermentation. After 2 h of feeding, two 20 ml uniform aliquots of the fermented liquid from the fermenters were taken in pre-warmed (39°C) syringes for gas and methane measurements. An aliquot of 20 ml was incubated along with 10 ml of fresh carbonated bicarbonate buffer for 24 h, while the other was analysed immediately as a 0 h sample (SCFA and substrate incubated at the start of the incubation). The substrate in the syringes comprised of a portion of the feed which came with 20 ml of the aliquot from the fermenters. In order to keep the volume of the fermenter to 1 litre, 40 ml of fresh carbonated bicarbonate buffer was added to each fermenter. After 24 h, the incubated syringe sample was analysed for gas and methane as described in Goel *et al.*<sup>(14)</sup>.

- (c) *Samples from the overflow.* The volume of the overflow was recorded and the vessel was emptied daily. An aliquot of 30 ml taken from overflow vessels was centrifuged (17 000 g, 20 min) to obtain a pellet. This pellet (undigested feed plus microbial mass) was freeze-dried and weighed. The pellet was digested with neutral detergent solution for 1 h with heating and refluxing to dissolve microbial mass. The undigested residue obtained on the crucible, after thoroughly washing with distilled water, was dried and weight recorded. The weight of this pellet after subtraction from the weight of the pellet obtained after freeze-drying gave the weight of microbial mass in 30 ml of the outflow. The weights of microbial mass and undigested feed so obtained were converted to the total volume of the overflow obtained in 24 h. From these values, the following characteristics were determined:

The per cent true degradability of the feed = ((hay DM added to the fermenter in 24 h minus weight of the undigested feed in 24 h)  $\times$  100)/hay DM added to the fermenter in 24 h.

### Efficiency of microbial protein synthesis

= (g microbial mass in 24 h/g feed truly degraded in 24 h).

**Microbial interactions.** The DNA extraction and the quantitative PCR assay was performed as described previously. The effect of BCM on microbial population was evaluated using relative quantification as described under batch incubation. The shifts in microbial communities<sup>(14)</sup> due to the addition of BCM in the continuous fermentation system were determined by taking the respective microbial population in the control fermenter as 100. The values for the relative expression of different microbial communities for the fermenters running under similar conditions were pooled and subjected to statistical analysis as discussed in the following.

**Table 1.** Methane production and partitioning factor values on the addition of bromochloromethane in the batch system

Substrate	Partition factor (x)	Methane (y)	MR (%)	M/TDS (z)	MR <sub>TDS</sub> %
Control	3.55 <sup>a</sup>	15.8 <sup>b</sup>		0.0444 <sup>b</sup>	
BCM <sub>L</sub> *	3.73 <sup>b</sup>	3.5 <sup>a</sup>	89.1	0.0058 <sup>a</sup>	88.7
BCM <sub>H</sub> *	3.64 <sup>ab</sup>	1.1 <sup>a</sup>	94.3	0.0031 <sup>a</sup>	94.3
SEM	0.021	2.11	3.45	0.01211	1.23

x, mg of truly degraded substrate (TDS)/ml gas; z, ml methane produced (m)/mg of truly digested substrate; y, ml net methane/100 ml net gas; MR, methane reduction on a volume basis; MR<sub>TDS</sub>, methane reduction on the basis of TDS.

<sup>a,b</sup> Values in the same column with different superscript letters are significantly different at  $P < 0.05$ .

\*BCM<sub>L</sub> = 5 μM; BCM<sub>H</sub> = 10 μM.

### Statistical analysis

The results for gas, methane, SCFA, true degradability and microbial mass efficiency were analysed by a one-way ANOVA using Statistica<sup>®</sup> software (version 5.1; Statsoft Inc., Tulsa, OK, USA). The differences between means were compared using Duncan's multiple range test ( $P < 0.05$ ). Dunnett's test was used to compare the microbial population from batch (control syringe *v.* syringe with BCM) and continuous fermentations (control fermenter *v.* fermenter with BCM). Significant effects of the addition of BCM on microbial communities were reported at  $P < 0.05$ .

## Results

### Batch fermentation

**Gas, methane and true substrate degradability.** The results on the effect of BCM on the partitioning of nutrients into gases and microbial mass (PF) are presented in Table 1. There was an increase in the PF value from 3.55 to 3.73 ( $P < 0.05$ ) in the syringes, where BCM was added at the lower concentration when compared with the control. Similarly, the TDS also did not change significantly ( $P > 0.05$ ; data not shown).

Bromochloromethane significantly ( $P < 0.05$ ) reduced methane production values when calculated as the proportion of total gas as well as when expressed as methane per unit of TDS when compared with the control. A reduction of 89–94% in methane was observed on both the volume and TDS basis at both concentrations (Table 1). The methane on a volume basis shows the concentration of methane in the gas released, and the methane on a truly degraded basis is a

measure of channelisation of substrate carbon to methane production.

**SCFA.** The addition of BCM did not influence the total SCFA content (1.77–1.79 mmol/syringe;  $P > 0.05$ ; Table 2). A significant decrease in the acetate:propionate (C2:C3) values, from 2.80 to 2.21 was observed on the addition of BCM ( $P < 0.05$ ). The molar proportion of different SCFA varied among the treatments, and an unexceptional high concentration of isovalerate was observed in the samples where methane was inhibited using BCM. The molar proportion of isovalerate increased from 0.0019 in the control to 0.021 in the syringes with BCM ( $P < 0.05$ ).

A 92–94% of hydrogen recovery was observed in the control syringes, while these values were only 58–64% and 38–40% in the syringes containing BCM at a concentration of 5 and 10 μM, respectively (Table 2).

**Microbial communities.** The addition of BCM resulted in complete inhibition of methanogens (Fig. 1) as indicated by their higher Ct values comparable with the negative blank (data not shown). This change was accompanied by a decrease in the *R. flavefaciens* population by 48% ( $P < 0.05$ ). However, a 68% increase in *F. succinogenes* ( $P < 0.05$ ) and a 30% increase in rumen fungi ( $P > 0.05$ ), relative to the total bacterial population, were observed. There was no change in the protozoal population ( $49.9 \times 10^3$ – $52.1 \times 10^3$ /ml, data not shown;  $P > 0.05$ ).

### Continuous fermentation

The effect of BCM addition on rumen fermentation was studied for 9 d, excluding the 6-d adaptation period. An adaptation time was indicated by irregular fluctuations in the pH, just before the morning feed (09.00 hours), during the first 4 d of incubation (from 6.05 to 7.05). Thereafter, the pH stabilised (between 6.7 and 6.8 just before the morning feed) in all fermenters, indicating the stabilised fermentation conditions (Fig. 2). Daily fluctuations were obtained according to the feeding schedules (09.00 and 17.00 hours) as reflected in pH values. pH dropped steeply (approximately from 6.5 to 6.1) after the morning and afternoon feeding periods with a slight increase in between (approximately 6.0–6.1), and an increase during the night-time to a maximum just before the next morning feed (approximately 6.5–6.6). The outflow turnover from all the fermenters was 1 litre/d.

**Gas and methane production.** Gas and methane values were recorded from the contents of fermenters incubated in the syringes (Figs. 3 and 4). The first two values in the figures represent the gas and methane before the addition of BCM.

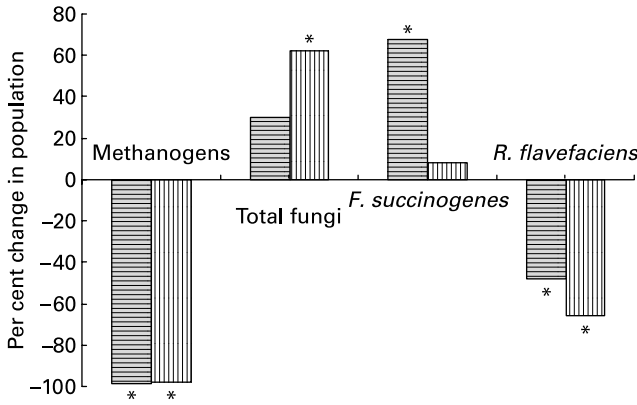
**Table 2.** SCFA production and molar proportion of individual fatty acids in the batch system

Treatment	Total SCFA produced (mmol/syringe)	Molar proportions					C2:C3	Hydrogen recovery (%)
		C2	C3	C4	Ci5	C5		
Control	1.79	0.658 <sup>b</sup>	0.234	0.0089 <sup>a</sup>	0.0019 <sup>a</sup>	0.0108	2.80 <sup>b</sup>	92–94
BCM <sub>L</sub> *	1.77	0.577 <sup>a</sup>	0.261	0.0125 <sup>b</sup>	0.0212 <sup>b</sup>	0.0114	2.22 <sup>a</sup>	58–64
BCM <sub>H</sub> *	1.78	0.579 <sup>a</sup>	0.262	0.0124 <sup>b</sup>	0.0182 <sup>b</sup>	0.0110	2.21 <sup>a</sup>	38–40
SEM	0.011	0.0023	0.0011	0.0122	0.0023	0.0015	0.017	

<sup>a,b</sup> Values in the same column with different superscript letters are significantly different at  $P < 0.05$ .

\*BCM<sub>L</sub> = 5 μM; BCM<sub>H</sub> = 10 μM.





**Fig. 1.** Per cent changes in microbial population on the addition of bromochloromethane (BCM) in batch (□) and continuous (▤) fermentation systems. \*Significant difference in the treatment from their respective controls ( $P < 0.05$ ).

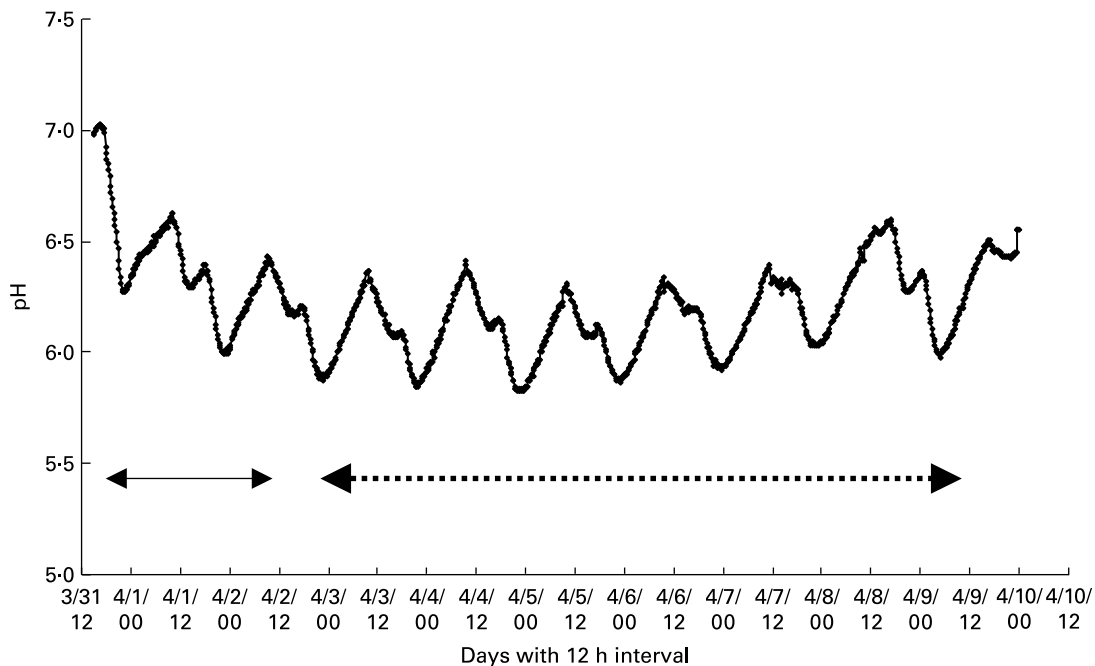
One day before the addition of BCM, the total gas in the syringes from the contents of all fermenters varied between 50 and 52 ml, with methane production values ranging from 10 to 11 ml of total gas in 24 h. After 2 d of the addition of BCM, the gas values declined slightly to 45 ml and thereafter decreased to 35 ml. After 2 d, a decrease of 30% in the total gas was observed in the syringes with contents of fermenters from F2 and F4. A complete inhibition of methane production (from 12 ml to negligible) was observed in the syringes incubated from contents of F2 and F4 on the following day of the addition of BCM and the effect was persistent during the rest of the fermentation study ( $P < 0.05$ ). A 9–10 ml methane per ml of the total gas was obtained in the syringes incubated with the content of control fermenters F1 and F3 throughout

the run, with a slight change in the total gas from 52 to 48 ml and 52 to 46 ml in F1 and F3, respectively.

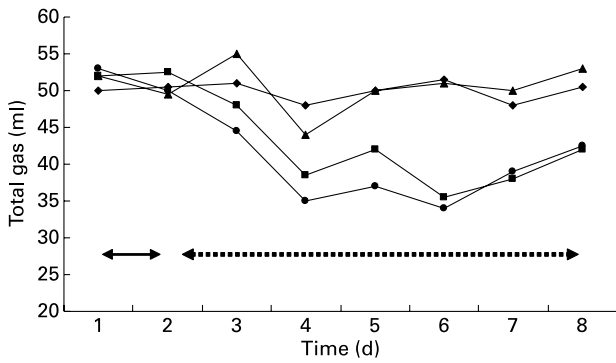
**Feed degradability and efficiency of microbial mass synthesis.** The values for true degradability of substrate obtained from the outflow of the fermenters ranged from 64 to 67%, 71 to 74%, 63 to 70% and 69 to 72% for the fermenters F1, F2, F3 and F4, respectively, throughout the study. No difference was observed in the true degradability of substrate in all the fermenters in terms of days as well as in terms of the addition of BCM ( $P > 0.05$ ; Fig. 5). The values for the efficiency of microbial mass produced per unit of digested feed ranged from 0.22 to 0.29, 0.20 to 0.28, 0.27 to 0.29 and 0.22 to 0.27 in the fermenters F1, F2, F3 and F4, respectively (Fig. 6). BCM addition did not affect the efficiency of microbial protein synthesis per unit feed digested ( $P > 0.05$ ).

**SCFA.** The total SCFA content ranged from 65 to 87  $\mu\text{mol/ml}$  in the outflow from all the fermenters. There was no difference in the overall content of SCFA in the fermenters as a function of days of incubation or the addition of BCM ( $P > 0.05$ ). The fermenters supplemented with BCM had slightly lower total SCFA contents (Fig. 7). Table 3 represents the mean values for the overall molar proportion of different SCFA in the outflow from all the fermenters in 9 d. There was no effect on the C2:C3 ratio; however, an unexpected increase in the isovalerate concentration was also observed, as was observed in the batch incubation ( $P < 0.05$ ).

**Microbial interactions in the continuous system.** Similar to that in the batch culture system, a complete inhibition of methanogens was observed ( $P < 0.05$ ). The absolute Ct values obtained for total bacterial population from each fermenter at different days was similar (data not shown), suggesting that there was no change in the bacterial population. The inhibition of methanogens was accompanied by a 66% decrease in the population of *R. flavefaciens* ( $P < 0.05$ ),



**Fig. 2.** Daily fluctuations in the pH at 12 h interval in one fermenter. Arrows with the solid line represent the pH values during the adaptation period, while arrows with the dashed line represent the pH values after adaptation conditions.



**Fig. 3.** Gas production in the fermenters (F1 (—◆—) and F3 (—▲—), control; F2 (—■—) and F4 (—●—), added with 5 μM-bromochloromethane (BCM). Arrows with the solid line represent the gas values 1 d before the addition of BCM, while arrows with the dashed line represent the values after the addition of BCM.

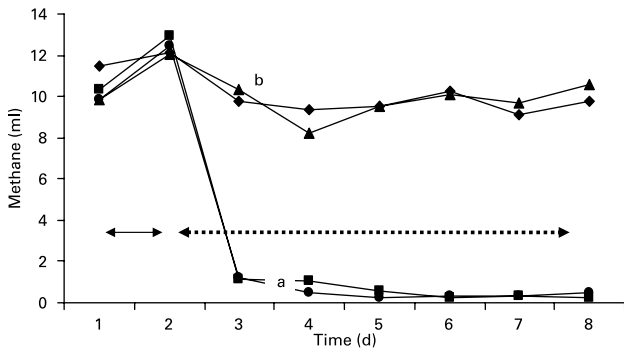
a 62% increase in the total fungal population ( $P < 0.05$ ) and an 8% increase in *F. succinogenes* ( $P > 0.05$ ), when compared with that in the control fermenters (Fig. 1). However, the increase in the *Fibrobacter* population was much lower than that observed under the batch culture system. Similar to that in the batch incubations, there was no effect of BCM on the protozoal population. The protozoal population ranged between  $29.13 \times 10^3$  and  $33.2 \times 10^3$ /ml in all the fermenters (data not shown;  $P < 0.05$ ).

**Discussion**

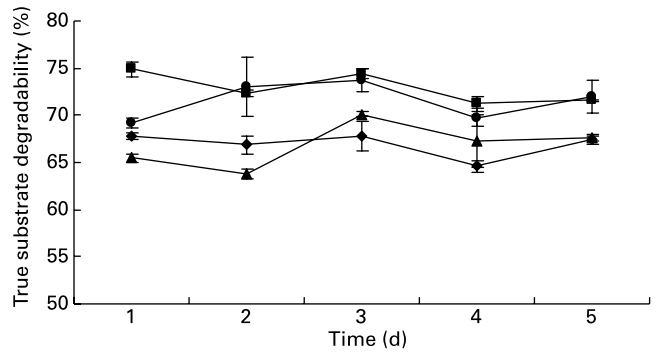
The results of the present study confirm the anti-methanogenic activity of BCM under the batch and continuous fermentation systems. The novelty of the present study is the effect of BCM on the microbial populations and the persistency of its effects in the continuous fermentation system.

*Effect of bromochloromethane on rumen fermentation*

The batch incubation was done for 24 h and the continuous fermentation was carried out for 9 d, excluding the days of the adaptation period. In a continuous fermenter, a stable fermentation is normally achieved in 5–7 d<sup>(16)</sup>. In the present study,



**Fig. 4.** Methane production values in the fermenters (F1 (—◆—) and F3 (—▲—), control; F2 (—■—) and F4 (—●—), added with 5 μM-bromochloromethane (BCM); the unlike letters represent the statistical differences among the values. Arrows with the solid line represent the value of methane 1 d before the addition of BCM, while arrows with the dashed line represent the methane values after the addition of BCM.

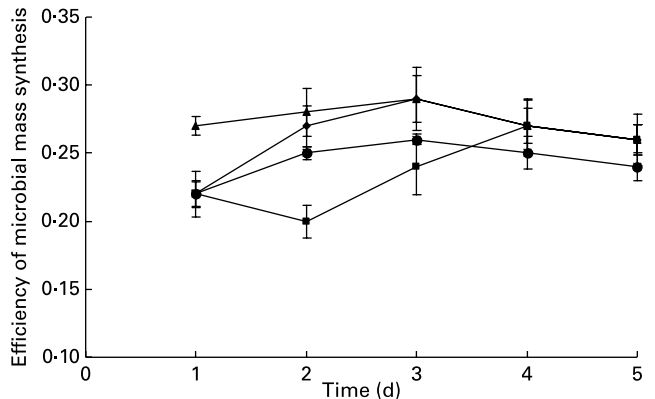


**Fig. 5.** Effect of bromochloromethane (BCM) on true substrate degradability (%) (F1 (—◆—) and F3 (—▲—), control; F2 (—■—) and F4 (—●—), added with BCM). The error bars represent the standard deviation.

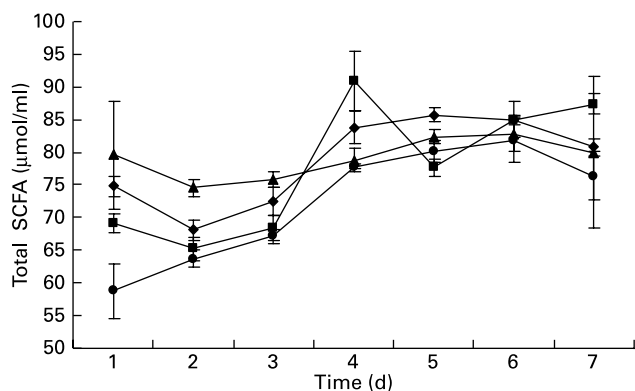
stable pH values in the fermenters were obtained within 6 d of the start of the fermentation.

*Gas and methane production.* The effect of BCM on methane production in the *in vitro* fermentation was similar to those reported in other studies<sup>(17–19)</sup>. In the present study, using the batch incubation, similar total gas production was observed in the control syringes and the syringes containing BCM; however, in the continuous system, the total gas production decreased after 2 d of the addition of BCM when compared with the control fermenters. This indicates that BCM has a slight negative effect on rumen fermentation under long-term incubation when studied *in vitro*. Sawyer *et al.*<sup>(17)</sup> reported a significant reduction (85%) in methane when BCM was fed to lambs. McCrabb *et al.*<sup>(19)</sup> did not observe any adverse effect on rumen fermentation when they administered BCM complexed with cyclodextrin *in vivo* in steers; however, they observed reduced methane emissions ( $0 \pm 2.4$  ml/min compared with  $205 \pm 5.2$  ml/min for the control) for 28 d, leading to the patenting of the compound.

Similar methane reduction values (89–94%) were obtained in the batch as well as in the continuous fermentation, which clearly indicates that BCM is a potent methane inhibitor at the concentrations tested in the present study. In the present study, we did not observe adaptive response of the methanogens to BCM unlike bromoethanesulfonate when tested against pure cultures of methanogens<sup>(20)</sup>.



**Fig. 6.** Effect of the addition of 5 μM-bromochloromethane (BCM) on microbial mass synthesis in continuous fermentation (F1 (—◆—) and F3 (—▲—), control; F2 (—■—) and F4 (—●—), added with BCM). The error bars represent the standard deviation.



**Fig. 7.** Total SCFA ( $\mu\text{mol/ml}$ ) in the fermenters (F1 (♦) and F3 (▲), control; F2 (■) and F4 (●), added with  $5\ \mu\text{M}$ -bromochloromethane). The error bars represent the standard deviation.

SCFA. There was no effect of complete inhibition of methane on the total SCFA content on the addition of BCM in the batch and continuous systems. A reduction in the acetate:propionate molar ratio in the rumen fluid has been described as a common feature of several anti-methanogens<sup>(21,22)</sup>. This indicates a concurrent reduction of methane formation and redirection of hydrogen from methane to reduced SCFA. Similar observations for total SCFA were made by Denman *et al.*<sup>(13)</sup> when they fed BCM to steers, resulting in a methane reduction of 59%. The total mean SCFA concentration was unaffected by the treatments but the acetate:propionate ratio was significantly higher ( $P < 0.01$ ) in the BCM-treated animals. The branched-chain SCFA, isobutyrate and isovalerate as a percentage of total SCFA were higher ( $P < 0.01$ ) for the BCM treatment<sup>(13)</sup>.

The imbalance in the relocation of electrons in methane could be suggested as a reason for higher individual molar proportion of butyrate as obtained in the present study. The electrons, which were not consumed in methane, were available for the reduction of NADH that mediates the conversion of acetoacetyl CoA to butyryl CoA and finally to butyrate<sup>(23)</sup>.

A comparative high concentration of isovalerate was observed on the addition of BCM in both batch and continuous fermentation studies. This increase in isovalerate production may reflect higher degradation of amino acids, as isovalerate is a product of leucine catabolism<sup>(24)</sup>. The increased degradation of amino acids should result in higher ammonia production from the incubations. In the present study, we did

not observe increased ammonia values when compared with the control (data not shown). The possible reasons for this could be the improved ammonia incorporation by rumen microbes (i.e. increased microbial protein synthesis, which is also reflected in higher PF in the batch incubations).

In the present study, the reduction in methane production and the increase in the molar proportion of branched-chain SCFA due to BCM treatment are consistent with those reported by McCrabb *et al.*<sup>(19)</sup>. The lowered hydrogen recovery in the syringes with BCM has also been observed by other workers<sup>(25–27)</sup>.

**Efficiency of microbial protein synthesis.** A significant increase in the PF (partitioning of nutrients into gases and microbial mass) from 3.55 to 3.73 in the batch incubation on the addition of BCM indicates improved efficiency of microbial mass synthesis. However, there was no effect on the substrate degradability and the microbial mass produced per unit feed degraded ( $P > 0.05$ ) when BCM was added in continuous fermenters. Sawyer *et al.*<sup>(17)</sup> reported increased digestibility of DM, protein and nitrogen-free extract, when BCM was fed to lambs. The increased energy from these fractions, and that resulting from the decreased methane production, provided a significantly greater quantity of metabolisable energy for all animals when BCM was fed. McSweeney & McCrabb<sup>(28)</sup> suggested that a reduction in methanogenesis in the rumen may affect the feed intake by 0–13% depending on the dose of the inhibitor, diet and physiological state of the animal. There are contradictory reports regarding the substrate digestibility and feed conversion efficiency when a methane inhibitor was used in *in vivo* trials. The studies using BCM as an anti-methanogenic agent predicted an increase in the feed conversion efficiency by 0–11%, as reviewed by McSweeney & McCrabb<sup>(28)</sup>. On the other hand, Sawyer *et al.*<sup>(17)</sup> reported a significant reduction in the feed conversion efficiency by 5% when BCM was fed to lambs. Although no improvements in daily gain were observed when the BCM complex was fed, there were improved efficiencies in feed conversion<sup>(19)</sup>. No difference in the digestibility of nutrients was observed between the control and BCM (0.04 ml of BCM capsules) when fed to Muzaffarnagari rams<sup>(29)</sup>. Denman *et al.*<sup>(13)</sup> also reported that BCM fed to steers did not affect the DM intake and mean live-weight gains.

#### Effect on rumen microbial ecology

The interactions among microbes in complex microbial habitats are often unpredictable and complex. Studies on

**Table 3.** Molar proportions of individual SCFA from the outflow of continuous fermenters (Mean values and standard deviations)

Fermenter	Molar proportions													
	C2		C3		Ci4		C4		Ci5		C5		C2:C3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F1 (control)	0.664	0.006	0.200	0.024	0.002	0.003	0.116	0.016	0.002	0.002	0.013	0.006	3.30	0.097
F2 (+BCM)	0.569	0.014	0.174	0.031	0.003	0.002	0.176	0.020	0.055	0.010*	0.020	0.004	3.32	0.474
F3 (control)	0.647	0.008	0.203	0.025	0.002	0.005	0.130	0.014	0.002	0.003	0.013	0.004	3.18	0.112
F4 (+BCM)	0.576	0.021	0.170	0.034	0.003	0.004	0.171	0.028	0.058	0.014*	0.020	0.004	3.29	0.428

BCM, bromochloromethane.

\* Significant differences in the values of control (F1 and F3) and fermenters with  $5\ \mu\text{M}$ -BCM (F2 and F4) ( $P < 0.05$ ).

interactions of methanogens (hydrogen consumers) and other microbial groups (hydrogen producers) have been highly productive, leading to the understanding of the mechanisms behind interspecies hydrogen transfer in some ecosystems<sup>(30,31)</sup>, although for the rumen system such studies are lacking. In the present study, the inhibition of methane by BCM was accompanied by a complete inhibition of methanogen population. However, these changes did not affect the total bacterial and protozoal populations. A significant negative effect of inhibition of methane production and methanogens was observed only for *R. flavefaciens* populations in both the batch and continuous fermentations. On the other hand, the BCM induced the proliferation of the Fibrobacter and the total fungal populations. The ruminal methanogens are often associated with protozoa; however, in the present study, we did not observe the inhibition of protozoal numbers, which supports our previous study that the relationship between protozoal numbers and methanogenesis is not obligatory<sup>(14)</sup>.

Guo *et al.*<sup>(32)</sup> used bromoethanesulfonate as a methane inhibitor and observed that a 10.4% decrease in the methanogen population resulted in a 50% increase in *F. succinogenes* and a 61.9% decrease in the total fungal population, with no effect on total bacteria and *R. flavefaciens*. We observed a significant negative effect on the *R. flavefaciens* population, which might be due to the increased partial pressure of hydrogen accumulated in the system. This partial pressure of hydrogen has been reported to influence the metabolism of the two important fibre-degrading species<sup>(28)</sup> *R. flavefaciens* and *R. albus*, inhibiting NADH oxidation in both species and diverting hydrogen to form other products such as succinate and ethanol<sup>(33)</sup>. The adaptive changes in rumen microbial ecology due to a complete inhibition of methanogens are relatively unknown, although the increase in propionate production has been observed in most studies. We observed an increased isovalerate production as a consequence of disruption of interspecies hydrogen transfer. The role of branched-chain fatty acids (isovalerate and isobutyrate) as growth promoters for cellulolytic bacteria is well documented. Allison *et al.*<sup>(34)</sup> observed that single branched-chain fatty acids can support the growth of certain strains of *R. flavefaciens* and *R. albus*, but *F. succinogenes* requires both a branched- and a straight-chain fatty acid.

### Conclusion

In conclusion, BCM is a potent methane inhibitor. In both the batch and continuous fermentation systems, it reduced methane production without affecting the substrate degradability. The complete inhibition of methane resulted in substantial accumulation of hydrogen; therefore, a closer look on the association of methanogens with other microbial communities and interspecies hydrogen-transfer mechanisms among different microbial communities (such as reductive acetogens, not studied here) could explain the mechanism behind these complex microbial interactions. The persistent effects obtained using continuous fermenters in the present study suggest that BCM has the potential for decreasing methane production from ruminants and enhancing productivity. The results of the present study also highlight the importance of the continuous fermenters as an intermediate between batch and *in vivo*

studies. Potential methane inhibitors could be evaluated for persistency of their effects using continuous fermenters, decreasing the cost and time of evaluation for the successful introduction of methane inhibitors in livestock diet.

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### References

1. Johnson KA & Johnson DE (1995) Methane emissions from cattle. *J Anim Sci* **73**, 2483–2492.
2. Jouany JP & Martin C (2003) Enteric methane (and CO<sub>2</sub>) emissions by ruminants CarboEurope-GHG, September, 4th and 5th 2003, Clermont-Ferrand. <http://gaia.agraria.unitus.it/ceuroghg/cftalk9.pdf>
3. Hegarty RS (1999) Reducing rumen methane emissions through elimination of rumen protozoa. *Aust J Agr Res* **50**, 1321.
4. Sharma RK (2005) Nutritional strategies for reducing methane production by ruminants – a review. *J Res SKUAST-J* **4**, 1–12.
5. Wood JM, Kennedy FS & Wolfe RS (1968) The reaction of multi-halogenated hydrocarbons with free and bound reduced vitamin B12. *Biochemistry* **7**, 1707–1713.
6. Chalupa W (1977) Manipulating rumen fermentation. *J Anim Sci* **46**, 585–599.
7. May C, Payne AL, Stewart PL, *et al.* (1995) A delivery system for agents International Patent Application No. PCT/AU95/00733.
8. Menke KH & Steingass H (1988) Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim Res Dev* **28**, 7–55.
9. Makkar HPS, Blümmel M & Becker K (1995) Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in *in vitro* techniques. *Br J Nutr* **73**, 897–913.
10. Goel G, Makkar HPS & Becker K (2008) Effects of *Sesbania sesban* and *Carduus pycnocephalus* leaves and Fenugreek (*Trigonella foenum-graecum* L.) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. *Anim Feed Sci Technol* (In the Press).
11. Demeyer DI (1991) Quantitative aspects of microbial metabolism in the rumen and hindgut. In *Rumen Microbial Metabolism and Ruminant Digestion*, pp. 217–237 [JP Jouany, editor]. Paris: INRA.
12. Denman SE & McSweeney CS (2006) Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol* **58**, 572–582.
13. Denman SE, Tomkins NW & McSweeney CS (2007) Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol* **62**, 313–322.
14. Goel G, Makkar HPS & Becker K (2008) Changes in microbial population, methanogenesis and rumen fermentation in response to saponin rich fractions of different plant materials. *J Appl Microbiol* **105**, 770–777.
15. Selje N, Hoffmann EM & Becker K (2006) Fermentation parameters and maintenance of rumen protozoa under different



- feeding regimes in a continuous fermenter system. *Proc Soc Nutr Physiol* **15**, 95.
16. Stanier G & Davies A (1981) Effects of the antibiotic monensin and an inhibitor of methanogenesis on *in vitro* continuous rumen fermentations. *Br J Nutr* **45**, 567–578.
  17. Sawyer MS, Hoover WH & Sniffen CJ (1974) Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. *J Anim Sci* **38**, 908–914.
  18. Horton GM (1980) Use of feed additives to reduce ruminal methane production and deaminase activity in steers. *J Anim Sci* **50**, 1160–1164.
  19. McCrabb GJ, Berger KT, Magner T, *et al.* (1997) Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects of growth. *Aust J Agric Res* **48**, 323–329.
  20. Ungerfeld EM, Rust SR, Boone DR, *et al.* (2004) Effects of several inhibitors on pure cultures of ruminal methanogens. *J Appl Microbiol* **97**, 520–526.
  21. Mohammed N, Ajisaka N, Lila Z, *et al.* (2004) Effect of Japanese horseradish oil on methane production and ruminal fermentation *in vitro* and in steers. *J Anim Sci* **82**, 1839–1846.
  22. Mwenya B, Santoso B, Sar C, *et al.* (2004) Effects of including  $\beta$ 1-4 galacto-oligosaccharides, lactic acid bacteria or yeast culture on methanogenesis as well as energy and nitrogen metabolism in sheep. *Anim Feed Sci Technol* **115**, 313–326.
  23. Macfarlane S & Macfarlane GT (2003) Regulation of short-chain fatty acid production. *Proc Nutr Soc* **62**, 67–72.
  24. Voet D & Voet JG (1995) *Biochemistry*. New York: John Wiley and Sons, Inc.
  25. Rufener WH & Wolin MJ (1968) Effect of  $\text{CCl}_4$  on  $\text{CH}_4$  volatile acid production in continuous culture of rumen organisms and sheep rumen. *Appl Microbiol* **16**, 1955–1956.
  26. van Nevel CJ, Henderickx HK, Demeyer DI, *et al.* (1969) Effect of chloral hydrate on methane and propionic acid in the rumen. *Appl Microbiol* **17**, 695.
  27. Trei JE, Scott GC & Parish RC (1972) Influence of methane inhibition on energetic efficiency of lambs. *J Anim Sci* **34**, 510.
  28. McSweeney CS & McCrabb GJ (2002) Inhibition of rumen methanogenesis and its effects on feed intake, digestion, and animal production. In *Greenhouse Gases and Animal Agriculture: Proceedings of the 1st International Conference on Greenhouse Gases and Animal Agriculture*, pp. 129–138 [J Takashaki, B Young, M Kreuzer and CR Solvia, editors]. New York: Elsevier Health Sciences.
  29. Lalu K, Bhar R & Mandal AB (2007) Effect of dietary addition of bromochloromethane on nutrient utilization and nitrogen balance in Muzaffarnagari rams. *Anim Nutr Feed Technol* **7**, 73–78.
  30. Stieb M & Schink B (1986) Anaerobic degradation of isovalerate by a defined methanogenic coculture. *Arch Microbiol* **144**, 291–295.
  31. Muralidharan V, Rinker KD, Hirsh IS, *et al.* (1997) Hydrogen transfer between methanogens and fermentative heterotrophs in hyperthermophilic cocultures. *Biotechnol Bioeng* **56**, 268–278.
  32. Guo YQ, Liu JX, Zhu WY, *et al.* (2007) Shifts of rumen microbial population detected by real-time PCR when methanogenesis is inhibited. *J Anim Feed Sci* **16**, 107–112.
  33. Wolin MJ, Miller TL & Stewart CS (1997) Microbe–microbe interactions. In *The Rumen Microbial Ecosystem*, 2nd ed. pp. 467–491 [PN Hobson and CS Stewart, editors]. New York: Blackie Academic and Professional.
  34. Allison MJ, Bryant MP & Doetsch RN (1962) Studies on the metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. *J Bacteriol* **83**, 523–532.