Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis

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The interactions of lauric (C12) and myristic acid (C14) in suppressing ruminal methanogenesis and methanogens were investigated with the rumen simulation technique (Rusitec) using bovine ruminal fluid. The fatty acids were added to basal substrates (grass hay:concentrate, 1:1.5) at a level of 48 g/kg DM, provided in C12:C14 ratios of 5:0, 4:1, 3:2, 2.5:2.5, 2:3, 1:4 and 0:5. Additionally, an unsupplemented control consisting of the basal substrates only was employed. Incubation periods lasted for 15 (n = 4) and 25 (n = 2) d. CH4 formation was depressed by any fatty acid mixture containing at least 40 % C12, and effects persisted over the complete incubation periods. The greatest depression (70 % relative to control) occurred with a C12:C14 ratio of 4:1, whereas the second most effective treatment in suppressing CH4 production (60 % relative to control) was found with a ratio of 3:2. Total methanogenic counts were decreased by those mixtures of C12 and C14 also successful in suppressing methanogenesis, the 4:1 treatment being most efficient (60 % decline). With this treatment in particular, the composition of the methanogenic population was altered in such a way that the proportion of Methanococcales increased and Methanobacteriales decreased. Initially, CH4 suppression was associated with a decreased fibre degradation, which, however, was reversed after 10 d of incubation. The present study demonstrated a clear synergistic effect of mixtures of C12 and C14 in suppressing methanogenesis, mediated probably by direct inhibitory effects of the fatty acids on the methanogens.

Methane: Medium-chain fatty acids: Lipids: Rusitec

CH4 is the second most problematic greenhouse gas (Wuebbles & Hayhoe, 2002) and ruminant livestock are responsible for about 25 % of the total anthropogenic emission of CH4 (Khalil, 2000). Livestock generate CH4 via methanogenic archaea, which inhabit the rumen, i.e. microbes that utilise H2 in order to reduce CO2 (Miller, 1995). Fatty acids have been known to have antimicrobial properties against bacteria, yeasts, tumor cells and viruses for many decades (for example, Ababouch et al. 1992) and the use of dietary fats seems to be an effective method for suppressing ruminal methanogenesis (for example, Jouany, 1994). Among the saturated fatty acids, medium-chain fatty acids (MCFA) have been demonstrated to have the greatest inhibitory effect on ruminal methanogenesis (Blaxter & Czerkawski, 1966; Dohme et al. 2001a) and ruminal methanogens (Henderson, 1973). In a short-term in vitro study, Soliva et al. (2003b) showed the efficacy of lauric acid (C12) in that respect, while myristic acid (C14) decreased total counts of archaea but did not alter CH4 formation. However, methanogenesis was decreased with similar dietary proportions of C14 in vitro with the rumen simulation technique (Rusitec) system (46 g C14/kg DM; Dohme et al. 2001a) as well as in vivo (50 g C14/kg DM; Machmüller et al. 2003). Soliva et al. (2003b) showed that within 24 h of incubation some mixtures of C12 and C14 decreased CH4 formation to almost zero as did the supplementation of C12 alone, indicating a synergistic effect between C12 and C14. Koster & Cramer (1987) noted that mixtures of non-esterified C12 and C14, when added to granular sludge in batch-culture studies, could be even more efficient in suppressing CH4 production than when given alone. A major role of C14 in inhibiting ruminal methanogenesis could therefore be its synergism with C12, which would explain the known

Abbreviations: MCFA, medium-chain fatty acids; NDF, neutral-detergent fibre; OM, organic matter; Rusitec, rumen simulation technique; VFA, volatile fatty acids.

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efficacy of various feeds containing mixtures of C_{12} and C_{14} (Dohme et al. 2000).

The aim of the present investigation was to test, using the Rusitec (Czerkawski & Breckenridge, 1977), whether synergism of C_{12} and C_{14} in suppressing ruminal methanogenesis and methanogens occurs when supplemented to a mixture of grass hay and concentrate. In contrast to previous Rusitec studies, extended fermentation periods of up to 25 d were chosen. This should allow the development of possible adaptation processes in ruminal fermentation and methanogenesis. Furthermore, the still unclear relationship between the level of ruminal CH_{4} formation and the numbers of total archaea (Soliva et al. 2003a) was investigated in order to deepen our understanding of the CH_{4}-suppressing effect of MCFA.

Materials and methods

Experimental protocol: medium-chain fatty acid mixtures and time periods

The experiment was carried out with an eight-fermenter Rusitec system, modified as described by Machmüller et al. (2002). Seven mixtures with C_{12}:C_{14} ratios of 5:0, 2:1, 3:2, 2:5:2:5, 2:3, 1:4 and 0:5 were supplemented at 0·7 g/d (DM basis) to basal substrates and compared with the unsupplemented basal substrates (Table 1). The purity of the fatty acids was 97 % (Fluka Chemie AG, Buchs, Switzerland). The basal substrates consisted of grass hay and concentrate in a 1:1.5 ratio and were added in portions of 14 g DM/d. In order to simulate the chewing activity of the ruminant, the grass hay was minced in a regular food mixer (Moulinette™; GROUP Moulinex, Paris, France). Barley and soyabean meal, which represented the concentrate, were ground to a diameter of 1 mm. Each dietary treatment was tested in four replicates in subsequent experimental periods lasting for 15 d each (n = 4). The first 5 d were considered the minimal period necessary to obtain steady-state conditions in the Rusitec system (Czerkawski & Breckenridge, 1977). The following 10 d (days 6 to 15) were split into two measurement periods for later statistical calculations. The first sub-period (P1; days 6 to 10) complied with the incubation period applied in former Rusitec experiments (for example, Dohme et al. 1999; Abel et al. 2002; Machmüller et al. 2002). The second sub-period (P2; days 11 to 15) was a prolongation of the regularly conducted incubation period. P2 not only served as a control for the repeatability of the results but also tested whether adaptation processes to the experimental treatments took place and whether fermentation in the in vitro Rusitec system could be well maintained over a longer period of time. For the latter purpose, two of the four experimental runs were prolonged up to 25 d during which pH, NH_{3}, bacterial and protozoal counts, volatile fatty acids (VFA) and fermentation gases were measured.

Rusitec fermenter set-up

At the beginning of each experimental run the fermenters (1 litre volume each) were filled with 100 ml pre-warmed buffer solution (artificial saliva; Czerkawski & Breckenridge, 1977) and 900 ml fermenter fluid. Ruminal fluid was collected from a rumen-fistulated, non-lactating Brown Swiss cow fed a diet consisting of grass hay and concentrate in a ratio of 1:5:1:0. Before incubation, the ruminal fluid was strained through four layers of gauze. On the first day of each experimental run two nylon bags, one filled with solid ruminal content, the other filled with the respective dietary treatment, were put into each fermenter. The nylon bags (70 X 140 mm) had a pore size of 100 μm as was recommended by Carro et al. (1995). After 24 h of incubation the system was opened and the bag containing solid ruminal content was replaced by another bag containing the specific dietary treatment. Afterwards, each nylon bag was incubated in the fermenter for 48 h. Anaerobic conditions were re-established in the fermenters by rinsing the system with gaseous N_{2} for 3 min (3 litres/min) after the daily supply of substrate was completed. The buffer flow rate was kept at 500 ml/d.

Sampling procedures and analyses

Samples of fermenter fluid were analysed for pH, NH_{3} and redox potential (to monitor the anaerobic conditions) daily with the respective electrodes connected to a pH meter data-processing unit (model 713; Metrohm, Herisau, Switzerland). For the determination of VFA, 1·8 ml of the fermenter fluid samples was stabilised with 0·2 ml of a 46 mM-HgCl_{2} solution and frozen until analysis by GC (GC Star 3400 CX; Varian, Sugarland, TX, USA) as outlined by Tangerman & Nagengast (1996). Counts of ciliate protozoa (entodiniomorphs and holotrichs) and bacteria were obtained daily with Bürker counting chambers (0·1 mm and 0·02 mm depth, respectively; Blau Brand®, Wertheim, Germany). Fermentation gases were collected over 24 h in gas-tight bags (TECOBAG 81; Tesseraux Container GmbH, Bürstadt, Germany) connected to the fermenters. The fermentation gases were collected completely by flushing the Rusitec system with gaseous N_{2} before uncoupling the bags from the fermenters. Fermentation gases were analysed for the concentrations of CH_{4}, H_{2},
and CO₂ by a Hewlett Packard gas chromatograph (model 5890 Series II; Avondale, PA, USA) equipped with a flame ionisation detector, a thermal conductivity detector and a Carboxen-1000 column (mesh size 60/80; Fluka Chemie AG). The volume of the fermentation gases collected was quantified by pressing the gas into a closed tube filled with water and measuring the amount of water displaced. Substrates and substrate residues after 48 h of incubation were lyophilised and analysed for the amounts of DM, total ash, diethylether extract (only the substrates) and N (Dumas method, Leco-Analyser Type FP-2000; Leco Corporation, St Joseph, MI, USA), following standard procedures (Naumann & Bassler, 1997). Crude protein was calculated as 6.25 × N. Contents of neutral-detergent fibre (NDF) were analysed after incubation with α-amylase (Termamyl 120L, Type S; Novo Nordisk A/S, Bagsværd, Denmark), but without sodium sulphite, according to the protocol of Naumann & Bassler (1997). Non-NDF carbohydrates were defined as the organic matter (OM) not incorporated in diethylether extract, crude protein, and NDF. A hydrogen balance, comprising H₂ produced, utilised and recovered, was calculated by the equations of Demeyer (1991) considering VFA and CH₄.

Quantification of the methanogens

The quantification of the CH₄-producing archaea was carried out with the fluorescence in situ hybridisation technique, according to the laboratory manual of Stahl et al. (1995) with modifications briefly described by Machmüller et al. (2003). The fluorescence in situ hybridisation technique is based on the hybridisation of specific oligonucleotide probes complementary to the ribosomal RNA of the target microbes and can now routinely be used to identify and quantify microbes without cultural isolation. Based on Lin et al. (1997), five 16S rRNA oligonucleotide probes (Microsynth GmbH, Balgach, Switzerland) were used, one domain-specific probe targeting all methanogens (S-S-Arc-0915-a-A-20) and four order-specific probes, targeting Methanococcales (S-F-Mcoc-1109-a-A-20), Methanosarcinales (S-O-Msar-0860-a-A-21), Methanomicrobiales (S-O-Mmic-1200-a-A-21) and Methanobacteriales (S-F-Mbac-0310-a-A-22). While the probe Arch-0915 has been described by Stahl & Amann (1991), the four probes targeting the different methanogenic orders were designed and found to be specific for the respective target methanogens by Raskin et al. (1994). The hybridisation temperatures for the respective probes were 56°C for Arch-0915, 55°C for Mcoc-1109, 60°C for Msrar-0860, 53°C for Mmic-1200 and 57°C for Mbac-0310. Samples were viewed with an epifluorescence microscope (BX-60; Olympus Optical AG, Volketswil, Switzerland). Images of the fluorescent signals were taken with a SCCD colour video camera (DXC-950P; Sony Corporation, Tokyo, Japan) and counted automatically with a software program (analySIS, version 3.1; Soft Imagine System GmbH, Uster, Switzerland).

Quantifications of the archaea and the different methanogenic orders were done for the incubation days 10 and 15, which in the following are attributed to the two sub-periods P1 and P2, respectively.

Calculations and statistical analysis

Two statistical models were applied to analyse the variance of the data in the present experiment. Model 1 was based on the mixed procedure of the SAS program (version 8.2; SAS Institute Inc., Cary, NC, USA) with random and repeated statements as recommended by Littell et al. (1998). This model was applied to all data to evaluate the effects of treatment, sub-periods (P1 and P2), and the interaction among them. Additionally, experimental run was included in the model. Model 2 evaluated data for P1 (days 6 to 10) and P2 (days 11 to 15) separately with the general linear model procedure of SAS, regarding dietary treatment and experimental run as effects. All multiple comparisons among means were carried out with the Tukey method. In both models daily observations for each fermenter were averaged within each sub-period (P1 and P2). In all calculations treatment was the main effect and the sub-periods were considered as repeated observations obtained from the same fermenter. The subsequent experimental periods were considered as a blocking factor in the experimental design. Data are presented separately in the Tables for days 6 to 10 (P1) and days 11 to 15 (P2) whenever the group of traits showed some significant interactions in model 1.

Results

Effects of medium-chain fatty acids on fermentation gases

CH₄ emission was depressed (P<0.001) by MCFA mixtures containing C₁₂ in proportions of 40 % or more (Table 2). The highest CH₄-suppressing effect compared with the unsupplemented control was obtained with C₁₂:C₁₄ in a 4:1 ratio (decline of 70 %), followed by ratios of 3:2, 2:5:2:5, 5:0, and 2:3 (declines of 60, 48, 43 and 32 %, respectively) for days 6 to 15 (Table 2). CH₄ release over 25 d (Fig. 1) showed that stable conditions were achieved from day 6 onwards. Differences developed among treatments were widely consistent up to day 21, after which irregular developments occurred. The gaseous emission of CO₂ was affected (P<0.05) by the MCFA treatments but comparisons among means showed no specific significant differences. The overall average value for the CO₂ emission was 66.5 mmol/l. The amount of H₂ in the gaseous phase was increased (P<0.001) by the supply of those MCFA mixtures that were high in C₁₂ proportion, whereas the MCFA mixtures low in C₁₂ proportion had smaller effects relative to the control treatment. However, a significant increase in H₂ release compared with control was achieved only with C₁₂:C₁₄ in a 4:1 ratio, leading to an increase in the amount of H₂ found in the gaseous phase by nearly 12-fold. The calculated total amounts of H₂ produced from various H₂-producing processes did not differ significantly between the individual MCFA treatments. Compared with control, lower (P<0.001) values for H₂ utilised and recovered were found with C₁₂:C₁₄ ratios of 5:0, 4:1, 3:2, 2:5:2:5 and 2:3, thus being affected inversely to the gaseous H₂ release measured. The lowest H₂ recovery rate was found with the C₁₂:C₁₄ ratio of 3:2. This rate was lower by 22.5 % than that found in the unsupplemented control. There were no significant
Table 2. Effects of medium-chain fatty acids on fermentation gas formation and fermenter fluid properties averaged over days 6 to 15

(Mean values and standard errors of the means)

<table>
<thead>
<tr>
<th>Trait</th>
<th>C12:C14 ratio</th>
<th>SEM†</th>
<th>P values (model 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>5.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Gaseous emissions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH4 (mmol/d)</td>
<td>8.72</td>
<td>5.01</td>
<td>2.60</td>
</tr>
<tr>
<td>CO2 (mmol/d)</td>
<td>69.0</td>
<td>66.0</td>
<td>63.9</td>
</tr>
<tr>
<td>CH4 (mmol/d)</td>
<td>0.30</td>
<td>2.05</td>
<td>3.58</td>
</tr>
<tr>
<td>Hydrogen balance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Produced (mmol/d)</td>
<td>115.1</td>
<td>106.6</td>
<td>105.3</td>
</tr>
<tr>
<td>Utilised (mmol/d)</td>
<td>78.0</td>
<td>57.2</td>
<td>49.8</td>
</tr>
<tr>
<td>Recovered (%)</td>
<td>68.3</td>
<td>54.5</td>
<td>47.5</td>
</tr>
<tr>
<td>Fermenter fluid properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.81</td>
<td>6.83</td>
<td>6.82</td>
</tr>
<tr>
<td>NH3 (mmol/l)</td>
<td>11.58</td>
<td>6.79</td>
<td>4.83</td>
</tr>
<tr>
<td>VFA (mmol/l)</td>
<td>101.0</td>
<td>100.9</td>
<td>102.8</td>
</tr>
<tr>
<td>Acetate (mol %)</td>
<td>53.0</td>
<td>50.7</td>
<td>50.0</td>
</tr>
<tr>
<td>Propionate (mol %)</td>
<td>17.4</td>
<td>16.0</td>
<td>19.5</td>
</tr>
<tr>
<td>Butyrate (mol %)</td>
<td>20.0</td>
<td>21.3</td>
<td>20.3</td>
</tr>
<tr>
<td>Isobutyrate (mol %)</td>
<td>0.43</td>
<td>0.40</td>
<td>0.35</td>
</tr>
<tr>
<td>Valerate (mol %)</td>
<td>6.83</td>
<td>8.86</td>
<td>8.82</td>
</tr>
<tr>
<td>Isovalerate (mol %)</td>
<td>2.30</td>
<td>2.69</td>
<td>1.11</td>
</tr>
<tr>
<td>Acetate:propionate ratio x:1</td>
<td>3.24</td>
<td>3.26</td>
<td>2.81</td>
</tr>
</tbody>
</table>

TRT, fatty acid treatment; PER, sub-period of total measurement period; VFA, volatile fatty acids.

* Treatment mean values within a row with unlike superscript letters were significantly different (P<0.05).

† For details of dietary substrates and procedures, see Table 1 and p. 690.

† Calculated by model 1. Means by treatment (df 21), n=8.
interactions of treatment and sub-period in gaseous emissions and H₂ balance.

Effects of medium-chain fatty acids on microbial counts

The MCFA treatments affected the ruminal microbial populations differently (Table 3). Total archaeal counts were affected by the MCFA supply in both sub-periods (P<0.001). While in P1 archaeal counts were depressed (P<0.05) by C₁₂:C₁₄ ratios of 5:0, 4:1 and 3:2 with decreases of 34, 61 and 47 % relative to the control, in P2 only the 4:1 and 3:2 ratios led to a significant decrease (57 and 47 %, respectively). Generally, in P2 total numbers of archaea were lower (P<0.001) compared with P1, and interactions with the MCFA treatment occurred (P<0.05). All individual methanogenic orders, except Methanococcales, were significantly suppressed by the effective MCFA treatments. However, the four orders were affected to a different extent as a result of the MCFA supplementation, thus leading to changes in the population composition of the archaea. Out of all MCFA treatments, the counts of Methanobacteria were decreased most with the C₁₂:C₁₄ ratio of 4:1 (−46 and −59 % compared with the control in P1 and P2, respectively). For the counts of Methanomicrobiales similar effects were found, but here counts were significantly lower with this ratio compared with counts found with a C₁₂:C₁₄ ratio of 0:5. The counts of Methanosarcinales were lowest in P1 with the 4:1 treatment, followed by the C₁₂:C₁₄ ratios 5:0 and 3:2, all of them significantly different from the counts in the control. In P2 the largest decrease (P<0.0001) in counts of Methanosarcinales compared with the counts found in the control was observed with C₁₂:C₁₄ ratios of 3:2, 4:1 and 2.5:2.5 (−67, −64 and −56 %, respectively). Regarding the proportions of the different methanogenic orders (data not shown), Methanococcales was found to increase (P<0.05) by up to 17.2 % (C₁₂:C₁₄ ratio of 4:1; P1) relative to control and Methanosarcinales to decrease (P<0.005) by up to 11.2 % (C₁₂:C₁₄ ratio of 5:0; P1) and 9.4 % (C₁₂:C₁₄ ratio of 3:2; P2). Proportions of Methanobacteriales and Methanomicrobiales were not significantly affected due to any MCFA supplementation.

Bacterial counts were not affected as a result of the MCFA treatments and amounted to 2.7×10⁸/ml and 2.2×10⁹/ml in the sub-periods P1 and P2, respectively, across all treatments. The same was found in the extended periods of days 16 to 25 with an average count of 2.1×10⁹ bacteria/ml. Counts of total ciliate protozoa (data not shown), entodiniomorphs and holotrichs were significantly affected in P1 due to the MCFA treatment. This effect was not significant in P2. However, complete defaunation took place when supplying C₁₂:C₁₄ in ratios of 4:1, 3:2, 2:5:2.5 and 2:3 from day 7 onwards, resulting in the low average protozoal counts in P1. Interactions between the MCFA treatments and sub-periods occurred for both entodiniomorph (P<0.001) and holotrich (P<0.01) ciliate protozoal counts. In the unsupplemented control, no holotrich ciliate protozoa were found in the period of days 16 to 25, whereas entodiniomorph ciliate protozoa were present until day 25 with counts of 0.29×10⁹/ml on the last day.

Effects of medium-chain fatty acids on fermenter fluid properties

Supplementation with mixtures of C₁₂ and C₁₄ did not alter fermenter fluid pH, which was 6.8 on average across all treatments during the whole measurement time (Table 2). Redox potential (data not shown) did not differ among MCFA treatments and averaged −323 and −320 mV in P1 and P2, respectively. Compared with the unsupplemented control, NH₃ concentration in fermenter fluid was depressed (P<0.001) due to the supplementation of C₁₂:C₁₄ in ratios of 5:0, 4:1, 3:2 and 2:5:2:5, with the highest decreases of 58 and 54 % found with the ratios 4:1 and 3:2, respectively. Mixtures high in C₁₄ proportions did not decrease NH₃ concentration. No significant effects of MCFA treatments were found in the concentration of total VFA (mean value 106.7 mmol/l) and in the molar proportions of propionate, butyrate and isobutyrate (mean values 17, 21 and 0.4 %, respectively) during the measurement period of 15 d. The molar proportion of acetate was affected by the MCFA treatment (P<0.05), but comparison among means did not show specific significant differences (mean value of acetate, 52 % of the total VFA). The molar proportion of valerate was enhanced (P<0.001) with each MCFA treatment, except for the treatment with C₁₄ supplied alone. The proportion of isovalerate was also affected (P<0.01) because of the fatty acid treatments but not in a systematic way. The acetate:propionate ratio remained unaffected and amounted to 3:1 on average over all treatments. Treatment effects on fermenter fluid properties did not depend on sub-period, and in the extended period from days 16 to 25, pH, NH₃ concentration, and VFA concentrations and composition showed the same trends as in the two sub-periods P1 and P2 (data not shown).
### Table 3. Effects of medium-chain fatty acids on microbial counts in fermenter fluid in sub-periods P1 (days 6 to 10) and P2 (days 11 to 15)*

(Mean values and standard errors of the means)

<table>
<thead>
<tr>
<th>Trait</th>
<th>Days</th>
<th>0.0</th>
<th>5.0</th>
<th>4.1</th>
<th>3.2</th>
<th>2.5:2.5</th>
<th>2.3</th>
<th>1:4</th>
<th>0.5</th>
<th>SEM †</th>
<th>TRT-2</th>
<th>TRT-1</th>
<th>PER</th>
<th>TRT × PER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbial counts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacteria (10⁹/ml)</strong></td>
<td>6–10</td>
<td>2.71</td>
<td>2.84</td>
<td>2.74</td>
<td>2.80</td>
<td>2.65</td>
<td>2.72</td>
<td>2.70</td>
<td>2.76</td>
<td>0.063</td>
<td>0.5058</td>
<td>0.3896</td>
<td>0.0001</td>
<td>0.9633</td>
</tr>
<tr>
<td></td>
<td>11–15</td>
<td>2.24</td>
<td>2.27</td>
<td>2.21</td>
<td>2.19</td>
<td>2.21</td>
<td>2.18</td>
<td>2.27</td>
<td>2.19</td>
<td>0.040</td>
<td>0.6067</td>
<td>0.4895</td>
<td>0.0001</td>
<td>0.5361</td>
</tr>
<tr>
<td><strong>Entodiniomorphs (10³/ml)</strong></td>
<td>6–10</td>
<td>8.88a</td>
<td>0.19c</td>
<td>0.00c</td>
<td>0.10c</td>
<td>0.00c</td>
<td>4.20b</td>
<td>5.63ab</td>
<td>0.822</td>
<td></td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>11–15</td>
<td>2.10</td>
<td>0.19</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>1.15</td>
<td>1.91</td>
<td>0.454</td>
<td></td>
<td>0.0070</td>
<td>0.0011</td>
<td>0.0088</td>
<td>0.0346</td>
</tr>
<tr>
<td><strong>Holotrichs (10³/ml)</strong></td>
<td>6–10</td>
<td>1.91a</td>
<td>0.00b</td>
<td>0.00b</td>
<td>0.00b</td>
<td>0.00b</td>
<td>2.27a</td>
<td>2.49a</td>
<td>0.182</td>
<td></td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0348</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>11–15</td>
<td>0.19</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.10</td>
<td>0.10</td>
<td>0.071</td>
<td></td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Archaea (10⁸/ml)</strong></td>
<td>6–10</td>
<td>3.09a</td>
<td>2.03bc</td>
<td>1.20c</td>
<td>1.65c</td>
<td>2.10bc</td>
<td>2.67ab</td>
<td>2.93ab</td>
<td>2.79bc</td>
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<td>1.79ab</td>
<td>1.09c</td>
<td>1.33c</td>
<td>1.70bc</td>
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<td><strong>Methanogenic orders (10⁷/ml)</strong></td>
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<tr>
<td><strong>Methanococcales</strong></td>
<td>6–10</td>
<td>5.8</td>
<td>6.1</td>
<td>6.4</td>
<td>6.7</td>
<td>6.3</td>
<td>6.6</td>
<td>6.0</td>
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<td></td>
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<td>5.1</td>
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<td>6.2</td>
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<td>2.6</td>
<td>2.0</td>
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<tr>
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<tr>
<td><strong>Methanobacteriales</strong></td>
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<td>6.5</td>
<td>5.6</td>
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<td></td>
<td>11–15</td>
<td>10.1</td>
<td>7.9</td>
<td>3.9</td>
<td>6.3</td>
<td>7.4</td>
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<td>8.9</td>
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<tr>
<td><strong>Methanosarcinales</strong></td>
<td>6–10</td>
<td>5.2</td>
<td>2.0</td>
<td>1.9</td>
<td>2.4</td>
<td>2.3</td>
<td>3.8</td>
<td>4.2</td>
<td>4.1</td>
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<td>0.0441</td>
<td>0.0048</td>
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<tr>
<td></td>
<td>11–15</td>
<td>4.5</td>
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<td>4.8</td>
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<td>0.44</td>
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</table>

TRT, fatty acid treatment, evaluated by model 1 (TRT-1) and model 2 (TRT-2) evaluating individual sub-periods; PER, sub-period of total measurement period (means of days 6 to 10 and means of days 11 to 15; model 1); TRT × PER, interaction of TRT and PER (model 1).

- Treatment mean values within a row with unlike superscript letters were significantly different (P<0.05).
- * For details of dietary substrates and procedures, see Table 1 and p. 690.
- † Calculated by model 2. Means by treatment (df 21), n=4.
Effects of medium-chain fatty acids on nutrient degradation

The apparent degree of OM degradation was decreased \((P<0.001)\) by the MCFA treatment in P1 (Table 4). In P2, differences to the control became insignificant in most MCFA treatments, except for the C\(_{12}:C\)\(_{14}\) ratio of 3:2 (\(\sim 9\%\)). Interactions between the MCFA treatment and sub-periods \((P<0.05)\) also occurred for this trait. Compared with the unsupplemented control, the apparent rate of crude protein degradation was decreased \((P<0.001)\) by 13, 10 and 10% in P1 with treatments containing C\(_{12}\) and C\(_{14}\) in ratios of 4:1, 3:2 and 2:5:2:5, respectively. In P2, crude protein degradation was decreased \((-12\%; P<0.01)\) with the 3:2 treatment. The apparent degree of NDF degradation was decreased \((P<0.001)\) due to the supplementation of all fatty acid mixtures in P1, except for the treatment with C\(_{14}\) only. In P2 no effect of MCFA supplementation occurred. CH\(_{4}\) release related to the amount of apparently degraded OM was decreased \((P<0.001)\) in both sub-periods with all the C\(_{12}:C\)\(_{14}\) treatments except for 1:4 and C\(_{14}\) alone. CH\(_{4}\) release related to the amount of apparently degraded NDF was affected by the MCFA supplementation in P1 \((P<0.01)\) as well as in P2 \((P<0.001)\). However, compared with the unsupplemented control, only the C\(_{12}:C\)\(_{14}\) ratio of 4:1 decreased CH\(_{4}\) per unit of NDF degraded \((48\%; P<0.01)\) in P1. In P2, on the other hand, the treatments with C\(_{12}:C\)\(_{14}\) in ratios of 5:0, 4:1, 3:2 and 2:5:2:5 affected this trait (decreases of 39, 59, 57 and 35% respectively; \(P<0.001)\).

Discussion

Suitability of the techniques used

One major purpose of the present study was to confirm previous short-term in vitro findings (Soliva et al. 2003b) on the interactions of the two MCFA C\(_{12}\) and C\(_{14}\) in suppressing ruminal methanogenesis; results that were found in the absence of feed particles. Harfoot et al. (1974) showed that the presence of feed particles may diminish the efficacy of MCFA since it decreases the proportion of fatty acids attached to ruminal microbes, a factor crucial for the inhibitory effect of MCFA on methanogens (Henderson, 1973). In the present study, a concentrate-based feed substrate was used, since Machmüller et al. (2003) showed that C\(_{14}\) alone can be effective in inhibiting CH\(_{4}\) release than C\(_{14}\) in sheep, and there are two studies, one in vitro (Soliva et al. 2003b) and another in vivo (Dohme et al. 2001b), in which C\(_{14}\) was ineffective. In contrast, others (Dohme et al. 2001a; Machmüller et al. 2003) showed in vitro and in vivo that C\(_{14}\) alone can be effective in inhibiting methanogenesis. Overall, the efficacy of C\(_{14}\) seems variable and may depend on a combination of factors such as the dietary proportion of C\(_{14}\), being low in the study of Dohme et al. (2001b), the composition of the basal diet (Machmüller et al. 2003), the proportion of fermentable matter shifted from the rumen to the hindgut and, in case of in vitro studies, the origin of ruminal fluid (sheep or cattle).

Culture studies with granular sludge have indicated that mixtures of non-esterified C\(_{12}\) and C\(_{14}\) might be more efficient in inhibiting methanogenesis than one of these fatty acids alone (Koster & Cramer, 1987). Incubating ruminal fluid and MCFA for 24 h in vitro (Soliva et al. 2003b) neither proved nor disproved a synergistic effect of mixtures of C\(_{12}\) and C\(_{14}\) on ruminal CH\(_{4}\) production since the sole supply of C\(_{12}\) nearly abolished CH\(_{4}\) formation. The slope of the regression curve in the in vitro study of Soliva et al. (2003b), however, suggested a certain synergistic effect between the two MCFA. In the present in vitro study, the synergistic effect between C\(_{12}\) and C\(_{14}\) in suppressing CH\(_{4}\) emission was clearly demonstrated. The use of the C\(_{12}:C\)\(_{14}\) ratios of 4:1, 3:2 and 2:5:2:5 resulted in a more pronounced CH\(_{4}\)-suppressing effect than the supply of C\(_{12}\) alone. Coconut oil and palm-kernel oil, feedstuffs rich in MCFA, have C\(_{12}:C\)\(_{14}\) ratios of 2:6:10 to 3:1, which is in the range of the most efficient mixture of C\(_{12}\).
Table 4. Effects of medium-chain fatty acids on the apparent degree of nutrient degradation and methane release per unit of apparently degraded organic matter and fibre in sub-periods P1 (days 6 to 10) and P2 (days 11 to 15)*

(Mean values and standard errors of the means)

<table>
<thead>
<tr>
<th>Traits</th>
<th>Days 6–10</th>
<th>6–10</th>
<th>4:1</th>
<th>3:2</th>
<th>2:5–2:5</th>
<th>2:3</th>
<th>1:4</th>
<th>0:5</th>
<th>SEM †</th>
<th>TRT-2</th>
<th>TRT-1</th>
<th>PER</th>
<th>TRT × PER</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM</td>
<td>6–10</td>
<td>0·696a</td>
<td>0·645b</td>
<td>0·634b</td>
<td>0·649b</td>
<td>0·645b</td>
<td>0·651b</td>
<td>0·659b</td>
<td>0·663b</td>
<td>0·0063</td>
<td>0·0022</td>
<td>0·0001</td>
<td>0·0203</td>
</tr>
<tr>
<td>Crude protein</td>
<td>6–10</td>
<td>0·670a</td>
<td>0·618abc</td>
<td>0·583</td>
<td>0·600abc</td>
<td>0·602abc</td>
<td>0·630abc</td>
<td>0·645abc</td>
<td>0·670a</td>
<td>0·0115</td>
<td>0·0002</td>
<td>0·0001</td>
<td>0·0066</td>
</tr>
<tr>
<td>NDF</td>
<td>6–10</td>
<td>0·396a</td>
<td>0·274cd</td>
<td>0·238d</td>
<td>0·286cd</td>
<td>0·296cd</td>
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<td>0·362ab</td>
<td>0·0126</td>
<td>0·0001</td>
<td>0·3259</td>
<td>0·0892</td>
</tr>
<tr>
<td>CH4 (mmol/g OM degraded)</td>
<td>6–10</td>
<td>0·95a</td>
<td>0·57cde</td>
<td>0·30de</td>
<td>0·51de</td>
<td>0·50de</td>
<td>0·66bcd</td>
<td>0·82abc</td>
<td>0·87ab</td>
<td>0·015</td>
<td>0·0001</td>
<td>0·1313</td>
<td>0·2273</td>
</tr>
<tr>
<td>CH4 (mmol/g NDF degraded)</td>
<td>6–10</td>
<td>4·90a</td>
<td>4·23ab</td>
<td>2·53a</td>
<td>3·64ab</td>
<td>3·41ab</td>
<td>4·86a</td>
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<td>4·96a</td>
<td>0·442</td>
<td>0·0028</td>
<td>0·0001</td>
<td>0·0229</td>
</tr>
</tbody>
</table>

TRT, fatty acid treatment, evaluated by model 1 (TRT-1) and model 2 (TRT-2) evaluating individual sub-periods; PER, sub-period of total measurement period (means of days 6 to 10 and means of days 11 to 15; model 1); TRT × PER, interaction of TRT and PER (model 1); OM, organic matter; NDF, neutral-detergent fibre.

* For details of dietary substrates and procedures, see Table 1 and p. 690.

† Calculated by model 2. Means by treatment (df 21), n 4.
and C_{14} used in the present study, and which explains the high efficacy of these oils in suppressing CH_{4} formation \textit{in vitro} (Dong et al. 1997; Dohme et al. 2000) and \textit{in vivo} (Machmüller & Kreuzer, 1999).

In the present study, free H_{2} accumulated in treatments with high CH_{4}-suppressing effects (particularly with a C_{12}:C_{14} ratio of 4:1). This is consistent with previous Rusitec-derived results (Machmüller et al. 2002) where MCFA supplementation significantly increased the amount of H_{2} in the gaseous phase. This accumulation probably resulted from a persistent H_{2} production of ruminal microbes while CH_{4} formation, as an H_{2}- and CO_{2}-consuming process, was suppressed. As seen in previous studies (Machmüller et al. 1998, 2002), the accumulation of H_{2} was not stoichiometric with the inhibition of CH_{4} formation, illustrating that an increasing amount of H_{2} produced must have been used elsewhere. Other possible H_{2}-consuming processes are, for instance, reductive acetogenesis (Van Nevel & Demeyer, 1995; Le Van et al. 1998) and propionate formation, which is known for its inverse relationship to CH_{4} formation (Whitelaw et al. 1984). However, neither VFA was correspondingly affected in the present study. Propionate production requires interactions between microbial species that produce succinate, an H_{2}-demanding process, and other microbial species that decarboxylate succinate to propionate (Wolin et al. 1997). Since an accumulation of succinate in the rumen is improbable (Blackburn & Hungate, 1963), it might be possible that some of the succinate-producing microbes were inhibited by certain MCFA treatments. Finally, H_{2} could also have been utilised for the reduction of sulfate to sulfides (Morvan et al. 1996; Hino & Asanuma, 2003), but this product was not determined in the present study.

Effects on ruminal microbial populations

It is well documented that MCFA have the potential to adversely affect ruminal ciliate protozoa, several bacterial species and methanogens (Henderson, 1973; Matsumoto et al. 1991; Dohme et al. 2001a; Soliva et al. 2003b). Supplementing 46 g C_{12} or C_{14}/kg total substrate \textit{in vitro} suppressed ciliate protozoal counts only with C_{12} but not with C_{14} (Dohme et al. 2001a). Likewise, in the present study neither entodiniomorph nor holotrich ciliate protozoa were affected by the supplementation of C_{14} alone or a C_{12}:C_{14} ratio of 1:4. All other treatments decreased counts of entodiniomorph as well as holotrich ciliate protozoa in the first sub-period (days 6 to 10), and all these treatments, except C_{12} alone, resulted in a complete defaunation of the fermenter fluid from experimental day 7 onwards. Defaunation, or a large decrease in the ciliate protozoal population, represents the loss of an important H_{2} donor for the ruminal archaea. Some archaea are associated with the ciliate protozoa ecto- and endosymbiotically (Finlay et al. 1994) and a decrease in ciliate protozoal counts might therefore decrease archaeal counts as well. However, limitations set by a lack of H_{2} were not the main reason for the suppressed CH_{4} formation found in those treatments, since gaseous H_{2} accumulated slightly in the treatments successful in suppressing CH_{4} formation. Furthermore, Dohme et al. (1999) showed that MCFA supplementation via coconut oil suppressed methanogenesis in both faunated and defaunated ruminal fluid. The significant decrease in ruminal fluid NH_{3} concentration and in apparent protein degradation found in treatments inhibiting ruminal ciliate protozoal population might be explained by the associated inhibition of the nutrient- and bacteria-degrading activity of the protozoa (Jouany, 1994). Similar to the findings of Dohme et al. (2001a), a suppression of the ciliate protozoal population did not result in the expected compensatory increase of the bacterial population (Jouany, 1994), which suggests that there might have been adverse MCFA effects on some bacterial species as well.

Corresponding to the variations found in CH_{4} release, clear effects of C_{12} on methanogenic counts, as described earlier (Dohme et al. 2001a; Soliva et al. 2003b), were found, and again the effects of C_{14} were smaller than that of C_{12} and not significant. In sheep, C_{14} did not clearly affect methanogenic counts either (Machmüller et al. 2003). Nevertheless, Henderson (1973) found more pronounced effects of C_{14} than of C_{12} on pure cultures of \textit{Methanobrevibacter ruminantium}. Despite the uncertain effect of C_{14} alone, C_{12} and C_{14} acted synergistically in suppressing total archaeal counts when supplied in ratios of 4:1 and 3:2. In both sub-periods, the depressions were numerically larger with these ratios by about 40 and 20 \%, respectively, than with C_{12} alone. This indicates a cooperative action of these two fatty acids and, therefore, a highly effective mechanism in inhibiting ruminal methanogens. Thus the present results confirm previous assumptions that MCFA are directly toxic to methanogens (Dong et al. 1997; Dohme et al. 1999). However, the mechanisms of how MCFA affect methanogens are still unclear. Koster & Cramer (1987) suspected that C_{12} and C_{14} inhibit the metabolic activity of the methanogens by adsorption on to the microbial cell wall. Many studies were carried out investigating the effects of non-esterified MCFA and MCFA-monoacylglycerols on different microbes (Kabara & Vrable, 1977; Thornar et al. 1987; Bergsson et al. 1998). According to these studies and other evidence (McLay et al. 2002), the cell membrane is thought to be the primary target of each agent and an enhanced inhibition would be the result of a multiple attack on the membrane. Lipophilic acids, including monolaurin (C_{12} monoacylglycerol), are thought to disrupt the membrane integrity, which in turn interferes with membrane activities such as the transport of amino acids, resulting in cell starvation (Kabara, 1993). In the study of Bergsson et al. (2001), Gram-positive cocci were viewed by transmission electron microscopy after incubation with monopracin for 30 min. Disappearance of the plasma membrane and electron-transport granules was evident and the cell wall of some of the microbial cells appeared to be affected, which led to cell death.

Inconsistencies between CH_{4} formation and on methanogenic counts were smaller in the present study than found in other studies (Soliva et al. 2003a). Generally, these variations could be explained by different activities of the individual methanogenic cells (Soliva et al. 2003a,b) or by population changes resulting from the different susceptibility of individual methanogenic orders with differing CH_{4}-producing activities to these MCFA. In the present
study, certain shifts in the proportion of methanogenic orders caused by the MCFA treatments were found, as was also reported for a short-term incubation study (Soliva et al. 2003b) in which, as a result of the supplementation of C12 and mixtures of C12 and C14, Methanococcales were depressed to a larger extent than the other methanogenic orders. However, in the present study, the MCFA treatments predominantly depressed the counts of Methanosaarcinales. Counts of Methanococcales were not significantly affected by any MCFA treatment and therefore increased in proportion to total archaea as all others were depressed, especially with C12:C14 ratios of 4:1 and 3:2. This change in archaeal population seems, however, not large enough to explain the particularly high CH4-suppressing effect of these two MCFA mixtures, particularly since the number of all archaeal orders declined numerically. Sharp et al. (1998) showed that representatives of the family Methanobacteriaceae are the methanogens most frequently associated with ciliate protozoa. In the present study, counts of Methanobacteriales were significantly suppressed due to the 4:1 MCFA treatment on incubation day 15 while ciliate protozoa had already been completely eliminated from the fermenters by day 10.

**Effects on apparent organic matter and fibre degradation**

Lipids added to ruminant diets can greatly disturb ruminal fermentation by impairing the degradation of non-lipid energy sources (Jenkins, 1993). In particular, the ruminal digestion of structural carbohydrates can be decreased by 50% or more, even when less than 100 g fat/kg are added (Jenkins & Palnquist, 1984). A decrease in ruminal degradation of fibre, but not of OM, due to C12 supplementation was also observed in the Rusitec studies of Doehme et al. (2001a) and Machmüller et al. (2002) within experimental periods of 10 d. C14 was ineffective in that respect (Doehme et al. 2001a; present results) as was the case for fibre digestion in sheep fed a C14-supplemented diet (Machmüller et al. 2003). In the present study, the C12-alone treatment also caused a decreased fibre degradation, but only in the first sub-period, while incubations for 11 to 15 d mostly reversed these effects. The same was true for most mixtures of C12 and C14, once more underlining the presence of synergistic effects between both fatty acids. Initially decreased fibre degradation could have been caused by H2 accumulation, which might have inhibited activity of the fibre-degrading bacteria (Miller, 1995). However, these microbes must have adapted to C12 and C14 in contrast to other microbes such as the methanogens and the ciliate protozoa. This observation is of special importance since the degradation of plant cell walls by ruminants has a major economic value for developing countries and also for developed countries applying certain feeding strategies (Krause et al. 2003).

**Conclusions**

The present results demonstrate that the use of mixtures of C12 and C14 for the purpose of suppressing CH4 emission of domestic ruminants is superior to using either of these fatty acids alone. The most effective mixture of C12 and C14, when supplemented to a complete ruminant diet and incubated for up to 20 d, was the 4:1 treatment, which decreased CH4 release by about 70%. Additionally, this treatment only impaired ruminal fibre degradation at the beginning of the measurement period, demonstrating that the CH4-suppressing effect was not mainly caused by a depression in fibre degradation. This suggests that the desired effect of a lower methanogenesis persists while the unfavourable depression in ruminal fibre degradation seems to be reversible after some time. A C12:C14 ratio of 4:1 is reasonably close to that found in fats such as coconut oil and palm-kernel oil, thus explaining the high efficacy of such feedstuffs that are available in farm practice. Reasons for the high efficacy of the MCFA mixtures seem to be a synergism of the two fatty acids in suppressing the total archaeal population and, to a certain extent, the associated changes in composition of the methanogenic population and metabolic activity of the individual methanogens.

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Fatty acid combinations and ruminal methane


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