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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 (C_{12}) and myristic acid (C_{14}) 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 C_{12}:C_{14} 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. CH_4 formation was depressed by any fatty acid mixture containing at least 40 % C_{12}, and effects persisted over the complete incubation periods. The greatest depression (70 % relative to control) occurred with a C_{12}:C_{14} ratio of 4:1, whereas the second most effective treatment in suppressing CH_4 production (60 % relative to control) was found with a ratio of 3:2. Total methanogenic counts were decreased by those mixtures of C_{12} and C_{14} 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, CH_4 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 C_{12} and C_{14} in suppressing methanogenesis, mediated probably by direct inhibitory effects of the fatty acids on the methanogens.

Methane: Medium-chain fatty acids: Lipids: Rusitec

CH_4 is the second most problematic greenhouse gas (Wuebbles & Hayhoe, 2002) and ruminant livestock are responsible for about 25 % of the total anthropogenic emission of CH_4 (Khalil, 2000). Livestock generate CH_4 via methanogenic archaea, which inhabit the rumen, i.e. microbes that utilise H_2 in order to reduce CO_2 (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.* 2001*a*) and ruminal methanogens (Henderson, 1973). In a short-term *in vitro* study, Soliva *et al.* (2003*b*) showed the efficacy of lauric acid (C_{12}) in that respect, while myristic acid (C_{14}) decreased total counts of archaea but did not alter CH_4 formation. However, methanogenesis was decreased with similar dietary proportions of C_{14} *in vitro* with the rumen simulation technique (Rusitec) system (46 g C_{14}/kg DM; Dohme *et al.* 2001*a*) as well as *in vivo* (50 g C_{14}/kg DM; Machmüller *et al.* 2003). Soliva *et al.* (2003*b*) showed that within 24 h of incubation some mixtures of C_{12} and C_{14} decreased CH_4 formation to almost zero as did the supplementation of C_{12} alone, indicating a synergistic effect between C_{12} and C_{14}. Koster & Cramer (1987) noted that mixtures of non-esterified C_{12} and C_{14}, when added to granular sludge in batch-culture studies, could be even more efficient in suppressing CH_4 production than when given alone. A major role of C_{14} in inhibiting ruminal methanogenesis could therefore be its synergism with C_{12}, 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.* 2003*a*) 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® S; 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 monitored.

Table 1. Composition of the dietary substrates

Basal diet...	Control	MCFA
Supply per fermenter (g DM/d)		
Grass hay	5·73	5·73
Concentrate	8·17	8·17
Barley	5·72	5·72
Soyabean meal	2·45	2·45
Mineral–vitamin premix*	0·07	0·07
Calcium carbonate	0·03	0·03
Lauric and myristic acid	–	0·70
Total DM supply	14·00	14·70
Analysed nutrient composition (g/kg DM)		
Organic matter	947	949
Diethyl ether extract	16	61
Crude protein	205	196
NDF	276	263
Non-NDF carbohydrates	450	430

MCFA, medium-chain fatty acids; NDF, neutral-detergent fibre.
* Contained (per kg): Ca, 140 g; P, 70 g; Na, 80 g; Mg, 30 g; Se, 15 mg; vitamin A, 150 mg; vitamin D_3, 3 mg; vitamin E, 1·67 g.

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 × 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 CO_2 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, diethyl ether 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 $6{\cdot}25 \times$ N. Contents of neutral-detergent fibre (NDF) were analysed after incubation with α-amylase (Termamyl 120L, Type S; Novo Nodirsk A/S, Bagsværd, Denmark), but without sodium sulfite, according to the protocol of Naumann & Bassler (1997). Non-NDF carbohydrates were defined as the organic matter (OM) not incorporated in diethyl ether extract, crude protein, and NDF. A hydrogen balance, comprising H_2 produced, utilised and recovered, was calculated by the equations of Demeyer (1991) considering VFA and CH_4.

Quantification of the methanogens

The quantification of the CH_4-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 Arc-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 Arc-0915, 55°C for Mcoc-1109, 60°C for Msar-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 3CCD 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

CH_4 emission was depressed ($P<0{\cdot}001$) by MCFA mixtures containing C_{12} in proportions of 40 % or more (Table 2). The highest CH_4-suppressing effect compared with the unsupplemented control was obtained with C_{12}:C_{14} 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). CH_4 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 CO_2 was affected ($P<0{\cdot}05$) by the MCFA treatments but comparisons among means showed no specific significant differences. The overall average value for the CO_2 emission was 66·5 mmol/l. The amount of H_2 in the gaseous phase was increased ($P<0{\cdot}001$) by the supply of those MCFA mixtures that were high in C_{12} proportion, whereas the MCFA mixtures low in C_{12} proportion had smaller effects relative to the control treatment. However, a significant increase in H_2 release compared with control was achieved only with C_{12}:C_{14} in a 4:1 ratio, leading to an increase in the amount of H_2 found in the gaseous phase by nearly 12-fold. The calculated total amounts of H_2 produced from various H_2-producing processes did not differ significantly between the individual MCFA treatments. Compared with control, lower ($P<0{\cdot}001$) values for H_2 utilised and recovered were found with C_{12}:C_{14} ratios of 5:0, 4:1, 3:2, 2·5:2·5 and 2:3, thus being affected inversely to the gaseous H_2 release measured. The lowest H_2 recovery rate was found with the C_{12}:C_{14} 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)

	C_{12}:C_{14} ratio									*P* values (model 1)		
Trait	0:0	5:0	4:1	3:2	2·5:2·5	2:3	1:4	0:5	SEM†	TRT	PER	TRT × PER
Gaseous emissions												
CH_4 (mmol/d)	8·72^{a}	5·01^{c}	2·60^{d}	3·45^{cd}	4·52^{cd}	5·95^{bc}	7·76^{ab}	8·35^{a}	0·492	0·0001	0·1744	0·2266
CO_2 (mmol/d)	69·0	66·0	63·9	61·0	65·6	68·0	66·1	69·9	1·66	0·0489	0·1984	0·1322
H_2 (mmol/d)	0·30^{b}	2·05^{ab}	3·58^{a}	2·16^{ab}	2·24^{ab}	1·28^{b}	0·40^{b}	0·36^{b}	0·413	0·0001	0·9643	0·7084
Hydrogen balance												
Produced (mmol/d)	115·1	106·6	105·3	100·8	108·3	113·2	112·9	120·2	4·24	0·2112	0·2815	0·3684
Utilised (mmol/d)	78·0^{a}	57·2^{cd}	49·8^{d}	46·1^{d}	58·1^{cd}	64·7^{bc}	73·6^{ab}	77·7^{a}	2·12	0·0001	0·0780	0·4117
Recovered (%)	68·3^{a}	54·9^{cd}	47·5^{d}	45·8^{d}	55·0^{cd}	57·5^{cd}	65·3^{ab}	65·3^{ab}	1·23	0·0001	0·8091	0·8310
Fermenter fluid properties												
pH	6·81	6·83	6·82	6·83	6·82	6·81	6·76	6·76	0·026	0·1198	0·5575	0·1560
NH_3 (mmol/l)	11·58^{ab}	6·79^{cd}	4·83^{d}	5·34^{d}	6·72^{cd}	9·47^{bc}	12·18^{ab}	13·01^{a}	0·691	0·0001	0·0005	0·6782
VFA (mmol/l)	101·0	100·9	102·8	100·5	103·3	107·0	110·0	118·0	4·00	0·1884	0·9750	0·5787
Acetate (mol %)	53·0	50·7	50·0	48·6	49·7	49·4	51·3	52·7	0·77	0·0328	0·0060	0·9998
Propionate (mol %)	17·4	16·0	19·5	17·8	16·7	16·0	16·7	17·2	0·81	0·4328	0·5375	0·6341
Butyrate (mol %)	20·0	21·3	20·3	21·4	21·4	22·1	20·7	19·7	0·91	0·1945	0·0001	0·2793
Isobutyrate (mol %)	0·43	0·40	0·35	0·29	0·33	0·45	0·44	0·46	0·045	0·2235	0·0726	0·4920
Valerate (mol %)	6·83^{c}	8·86^{a}	8·82^{a}	9·21^{a}	9·33^{a}	8·94^{a}	8·32^{ab}	7·37^{bc}	0·197	0·0001	0·6048	0·9997
Isovalerate (mol %)	2·32^{ab}	2·69^{ab}	1·11^{b}	2·72^{ab}	2·50^{ab}	3·05^{a}	2·59^{ab}	2·57^{ab}	0·307	0·0045	0·0007	0·7063
Acetate:propionate ratio *x*:1	3·24	3·26	2·81	2·91	3·17	3·24	3·24	3·26	0·135	0·0914	0·0150	0·7817

TRT, fatty acid treatment; PER, sub-period of total measurement period; VFA, volatile fatty acids.
^{a,b,c,d} 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.

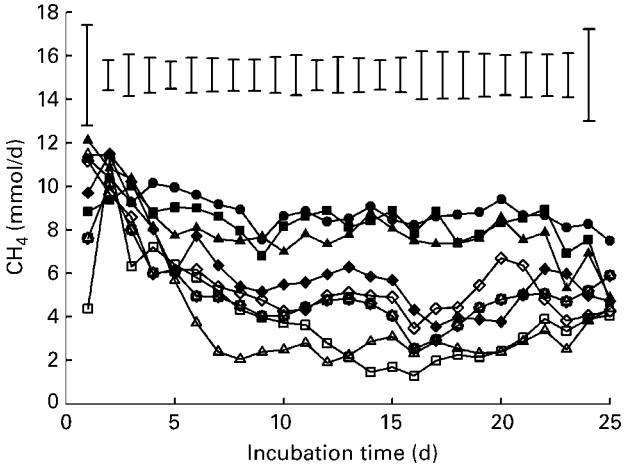

Fig. 1. *In vitro* CH_4 release (mmol/d) from fermenter fluid, when incubating a control diet (−•−) and diets supplemented with mixtures of C_{12} and C_{14} in ratios of 0:5 (−■−), 1:4 (−▲−), 2:3 (−◆−), 2·5:2·5 (−○−), 3:2 (−□−), 4:1 (−△−) and 5:0 (−◇−). The incubation period was 25 d (days 1 to 15, *n* 4; days 16 to 25, *n* 2). Mean values are shown, with vertical bars representing standard errors of the mean.

interactions of treatment and sub-period in gaseous emissions and H_2 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 C_{12}:C_{14} 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 *Methanobacteriales* were decreased most with the C_{12}:C_{14} 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 C_{12}:C_{14} ratio of 0:5. The counts of *Methanosarcinales* were lowest in P1 with the 4:1 treatment, followed by the C_{12}:C_{14} ratios 5:0 and 3:2, all of them significantly different from the counts in the control. In P2 the largest decrease ($P<0·001$) in counts of *Methanosarcinales* compared with the counts found in the control was observed with C_{12}:C_{14} 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 % (C_{12}:C_{14} ratio of 4:1; P1) relative to control and *Methanosarcinales* to decrease ($P<0·05$) by up to 11·2 % (C_{12}:C_{14} ratio of 5:0; P1) and 9·4 % (C_{12}:C_{14} 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 $2·7 \times 10^9$/ml and $2·2 \times 10^9$/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 $2·1 \times 10^9$ 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 C_{12}:C_{14} 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 $0·29 \times 10^3$/ml on the last day.

Effects of medium-chain fatty acids on fermenter fluid properties

Supplementation with mixtures of C_{12} and C_{14} 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, NH_3 concentration in fermenter fluid was depressed ($P<0·001$) due to the supplementation of C_{12}:C_{14} 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 C_{14} proportions did not decrease NH_3 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 C_{14} 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, NH_3 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)

		C_{12}:C_{14} ratio									*P* values			
Trait	Days	0:0	5:0	4:1	3:2	2·5:2·5	2:3	1:4	0:5	SEM†	TRT-2	TRT-1	PER	TRT × PER
Microbial counts														
Bacteria (10^9/ml)	6–10	2·71	2·84	2·74	2·80	2·65	2·72	2·70	2·76	0·063	0·5058	0·3896	0·0001	0·9633
	11–15	2·24	2·27	2·21	2·19	2·21	2·18	2·27	2·19	0·040	0·6067			
Entodiniomorphs (10^3/ml)	6–10	$8·88^{a}$	$0·19^{c}$	$0·00^{c}$	$0·10^{c}$	$0·00^{c}$	$0·10^{c}$	$4·20^{b}$	$5·63^{ab}$	0·822	0·0001	0·0001	0·0001	0·0001
	11–15	2·10	0·19	0·00	0·00	0·00	0·00	1·15	1·91	0·454	0·0070			
Holotrichs (10^3/ml)	6–10	$1·91^{a}$	$0·00^{b}$	$0·10^{b}$	$0·00^{b}$	$0·10^{b}$	$0·00^{b}$	$0·76^{ab}$	$1·53^{ab}$	0·346	0·0019	0·0001	0·0011	0·0088
	11–15	0·19	0·00	0·00	0·00	0·00	0·00	0·00	0·10	0·071	0·4586			
Archaea (10^8/ml)	6–10	$3·09^{a}$	$2·03^{bc}$	$1·20^{c}$	$1·65^{c}$	$2·10^{abc}$	$2·67^{ab}$	$2·93^{ab}$	$2·79^{ab}$	0·214	0·0001	0·0001	0·0001	0·0348
	11–15	$2·53^{a}$	$1·79^{ab}$	$1·09^{b}$	$1·33^{b}$	$1·70^{ab}$	$2·27^{a}$	$2·49^{a}$	$2·37^{a}$	0·182	0·0001			
Methanogenic orders (10^7/ml)														
Methanococcales	6–10	5·8	6·1	6·4	6·7	6·3	6·6	7·0	6·8	0·72	0·9397	0·4895	0·0001	0·0690
	11–15	6·1	5·1	4·3	5·6	4·4	6·2	6·7	5·9	0·74	0·2818			
Methanomicrobiales	6–10	$3·3^{ab}$	$2·4^{ab}$	$1·8^{b}$	$2·5^{ab}$	$3·1^{ab}$	$3·2^{ab}$	$3·7^{a}$	$3·3^{a}$	0·32	0·0082	0·0001	0·0169	0·5361
	11–15	$2·9^{ab}$	$2·6^{ab}$	$1·2^{b}$	$1·4^{ab}$	$2·4^{ab}$	$3·1^{ab}$	$3·1^{ab}$	$3·1^{a}$	0·40	0·0103			
Methanobacteriales	6–10	$9·5^{ab}$	$8·3^{ab}$	$5·6^{b}$	$7·8^{ab}$	$8·6^{ab}$	$9·6^{ab}$	$9·6^{ab}$	$9·7^{a}$	0·84	0·0312	0·0003	0·0001	0·1037
	11–15	$10·1^{a}$	$7·98^{a}$	$3·9^{b}$	$6·3^{ab}$	$7·1^{ab}$	$8·3^{a}$	$8·9^{a}$	$9·0^{a}$	0·80	0·0008			
Methanosarcinales	6–10	$5·2^{a}$	$2·0^{cd}$	$1·9^{d}$	$2·4^{bcd}$	$3·5^{abcd}$	$3·8^{abcd}$	$4·2^{ab}$	$4·1^{abc}$	0·45	0·0003	0·0001	0·0441	0·0048
	11–15	$4·5^{ab}$	$2·8^{bc}$	$1·6^{c}$	$1·5^{c}$	$2·0^{c}$	$4·2^{ab}$	$4·8^{a}$	$3·1^{abc}$	0·44	0·0001			

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).
[a,b,c,d] 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 ($P<0{\cdot}001$) 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 (−9 %). Interactions between the MCFA treatment and sub-periods ($P<0{\cdot}05$) also occurred for this trait. Compared with the unsupplemented control, the apparent rate of crude protein degradation was decreased ($P<0{\cdot}001$) 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 %; $P<0{\cdot}01$) with the 3:2 treatment. The apparent degree of NDF degradation was decreased ($P<0{\cdot}001$) 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 ($P<0{\cdot}001$) 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 ($P<0{\cdot}01$) as well as in P2 ($P<0{\cdot}001$). 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 %; $P<0{\cdot}01$) 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; $P<0{\cdot}001$).

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.* 2003*b*) 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 a more pronounced inhibition of total-tract CH_4 release in sheep fed on a concentrate-based diet supplemented with C_{14} rather than when fed a forage-based diet. The incubation period of 10 d applied previously in Rusitec experiments was extended to 15 d in the present study, and, for some variables, to 25 d. Fermentation parameters such as pH, bacterial counts and redox potential were consistent for at least 15 to 20 d. In all fermenters subjected to the MCFA unsupplemented control, there were still entodiniomorph ciliate protozoa present on day 25. Most treatment effects did not change between sub-periods. This shows that the Rusitec system used was appropriate to investigate a prolonged incubation time and also that the commonly used measurement period of days 6 to 10 satisfactorily reflected most treatment effects.

Previous assumptions of Raskin *et al.* (1994) and Soliva *et al.* (2003*b*) that the four hybridisation probes chosen to quantify distinct taxonomic orders of methanogens cover all methanogens occurring in the rumen were confirmed. Summing the counts of all methanogenic orders on days 10 and 15 across all treatments accounted for 97 (SD 16) and 98 (SD 6) %, respectively, of total archaea quantified with the Arc-0915 nucleotide probe. The methanogenic order that is most abundant, however, seems to vary widely among different studies. In the study of Lin *et al.* (1997), *Methanobacteriales* had the highest proportion of all orders in steers, cows and goats, whereas *Methanomicrobiales* predominated in sheep. In our previous investigations with sheep (Machmüller *et al.* 2003) and with ruminal fluid of a donor cow (Soliva *et al.* 2003*b*), *Methanococcales* dominated, while in the present study *Methanobacteriales* were most abundant, followed by *Methanococcales* (Table 3).

Effects on ruminal methanogenesis

MCFA are known to have the potential to suppress ruminal CH_4 formation (for example, Blaxter & Czerkawski, 1966). *In vitro*, Dohme *et al.* (2001*a*) identified C_{12} and C_{14} as the only two MCFA being effective in suppressing ruminal CH_4 formation and methanogens. However, although C_{12} supplied alone suppressed CH_4 release by 43 % in the present study, C_{14} did not affect CH_4 release at any time during the whole measurement period. Blaxter & Czerkawski (1966) demonstrated that non-esterified C_{12} is more potent in inhibiting CH_4 release than C_{14} in sheep, and there are two studies, one *in vitro* (Soliva *et al.* 2003*b*) and another *in vivo* (Dohme *et al.* 2001*b*), in which C_{14} was ineffective. In contrast, others (Dohme *et al.* 2001*a*; 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.* (2001*b*), 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.* 2003*b*) 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.* (2003*b*), 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 2·6:1·0 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)

		C_{12}:C_{14} ratio									*P* values			
Traits	Days	0:0	5:0	4:1	3:2	2·5:2·5	2:3	1:4	0:5	SEM†	TRT-2	TRT-1	PER	TRT × PER
Nutrient degradation														
OM	6–10	0·696^{a}	0·645^{b}	0·634^{b}	0·649^{b}	0·645^{b}	0·651^{b}	0·659^{b}	0·663^{b}	0·0063	0·0001	0·0001	0·6203	0·0309
	11–15	0·686^{a}	0·649^{ab}	0·640^{ab}	0·626^{b}	0·650^{ab}	0·659^{ab}	0·656^{ab}	0·673^{ab}	0·0105	0·0222			
Crude protein	6–10	0·670^{a}	0·618^{abc}	0·583^{c}	0·600^{bc}	0·602^{bc}	0·630^{abc}	0·645^{ab}	0·670^{a}	0·0123	0·0002	0·0001	0·0066	0·1739
	11–15	0·647^{ab}	0·585^{bc}	0·592^{bc}	0·568^{c}	0·605^{abc}	0·628^{abc}	0·632^{abc}	0·673^{a}	0·0153	0·0014			
NDF	6–10	0·396^{a}	0·274^{cd}	0·238^{d}	0·286^{cd}	0·296^{cd}	0·273^{cd}	0·320^{bc}	0·362^{ab}	0·0126	0·0001	0·3259	0·2361	0·3558
	11–15	0·373	0·358	0·257	0·259	0·294	0·313	0·318	0·374	0·0325	0·0892			
CH_4 per unit of degraded nutrients														
CH_4 (mmol/g $OM_{degraded}$)	6–10	0·95^{a}	0·57^{cde}	0·30de	0·51de	0·50de	0·66^{bcd}	0·82^{abc}	0·87^{ab}	0·015	0·0001	0·0001	0·1313	0·2273
	11–15	0·95^{a}	0·53^{cd}	0·28^{d}	0·30^{cd}	0·50^{cd}	0·64^{bc}	0·86^{ab}	0·91^{ab}	0·060	0·0001			
CH_4 (mmol/g $NDF_{degraded}$)	6–10	4·90^{a}	4·23^{ab}	2·53^{b}	3·64^{ab}	3·41^{ab}	4·86^{a}	5·27^{a}	4·96^{a}	0·442	0·0028	0·0001	0·0229	0·1117
	11–15	5·16^{a}	3·14^{cd}	2·10^{d}	2·22^{cd}	3·36^{bcd}	4·22^{abc}	5·58^{a}	5·10^{ab}	0·372	0·0001			

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 mattter; NDF, neutral-detergent fibre.

^{a,b,c,d} 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.

and C_{14} used in the present study, and which explains the high efficacy of these oils in suppressing CH_4 formation *in vitro* (Dong *et al.* 1997; Dohme *et al.* 2000) and *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.* 2001*a*; Soliva *et al.* 2003*b*). Supplementing 46 g C_{12} or C_{14}/kg total substrate *in vitro* suppressed ciliate protozoal counts only with C_{12} but not with C_{14} (Dohme *et al.* 2001*a*). 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.* (2001*a*), 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.* 2001*a*; Soliva *et al.* 2003*b*), 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 *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; Thormar *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 monocaprin 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.* 2003*a*). Generally, these variations could be explained by different activities of the individual methanogenic cells (Soliva *et al.* 2003*a*,*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.* 2003*b*) in which, as a result of the supplementation of C_{12} and mixtures of C_{12} and C_{14}, *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 *Methanosarcinales*. 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 C_{12}:C_{14} ratios of 4:1 and 3:2. This change in archaeal population seems, however, not large enough to explain the particularly high CH_4-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 & Palmquist, 1984). A decrease in ruminal degradation of fibre, but not of OM, due to C_{12} supplementation was also observed in the Rusitec studies of Dohme *et al.* (2001*a*) and Machmüller *et al.* (2002) within experimental periods of 10 d. C_{14} was ineffective in that respect (Dohme *et al.* 2001*a*; present results) as was the case for fibre digestion in sheep fed a C_{14}-supplemented diet (Machmüller *et al.* 2003). In the present study, the C_{12}-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 C_{12} and C_{14}, once more underlining the presence of synergistic effects between both fatty acids. Initially decreased fibre degradation could have been caused by H_2 accumulation, which might have inhibited activity of the fibre-degrading bacteria (Miller, 1995). However, these microbes must have adapted to C_{12} and C_{14} 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 C_{12} and C_{14} for the purpose of suppressing CH_4 emission of domestic ruminants is superior to using either of these fatty acids alone. The most effective mixture of C_{12} and C_{14}, when supplemented to a complete ruminant diet and incubated for up to 20 d, was the 4:1 treatment, which decreased CH_4 release by about 70 %. Additionally, this treatment only impaired ruminal fibre degradation at the beginning of the measurement period, demonstrating that the CH_4-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 C_{12}:C_{14} 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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