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Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion

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The efficiency of myristic acid (14 : 0) as a feed additive to suppress CH_4 emissions of ruminants was evaluated under different dietary conditions. Six sheep were subjected to a 6 × 6 Latin square arrangement. A supplement of non-esterified 14 : 0 (50 g/kg DM) was added to two basal diets differing in their forage:concentrate values (1 : 1·5 and 1 : 0·5), which were adjusted to dietary Ca contents of 4·2 and 9·0 g/kg DM, respectively. Comparisons were made with the unsupplemented basal diets (4·2 g Ca/kg DM). The 14 : 0 supplementation decreased ($P<0·001$) total tract CH_4 release depending on basal diet type (interaction, $P<0·001$) and dietary Ca level ($P<0·05$, *post hoc* test). In the concentrate-based diet, 14 : 0 suppressed CH_4 emission by 58 and 47 % with 4·2 and 9·0 g Ca/kg DM, respectively. The 14 : 0 effect was lower (22 %) in the forage-based diet and became insignificant with additional Ca. Myristic acid inhibited ($P<0·05$) rumen archaea without significantly altering proportions of individual methanogen orders. Ciliate protozoa concentration was decreased ($P<0·05$, *post hoc* test) by 14 : 0 only in combination with 9·0 g Ca/kg DM. Rumen fluid NH_3 concentration and acetate:propionate were decreased ($P<0·05$) and water consumption was lower ($P<0·01$) with 14 : 0. The use of 14 : 0 had no clear effects on total tract organic matter and fibre digestion; this further illustrates that the suppressed methanogenesis resulted from direct effects against methanogens. The present study demonstrated that 14 : 0 is a potent CH_4 inhibitor but, to be effective in CH_4 mitigation feeding strategies, interactions with other diet ingredients have to be considered.

Methane: Myristic acid: Forage: Calcium

CH_4 is currently recognised as the second most important greenhouse gas emitted from anthropogenic sources (Wuebbles & Hayhoe, 2002). With the Kyoto protocol, there is a renewed interest to address mitigation of CH_4 in animal agriculture. Annually worldwide, approximately 81 Tg CH_4 are emitted from the gastrointestinal tracts of domestic ruminants and an additional 7 Tg CH_4 originate annually from the manure of these animals (Johnson *et al.* 2000). Related to total anthropogenic CH_4 emissions, this means that domestic ruminants are responsible for 25 % of total anthropogenic CH_4 emission (Khalil, 2000).

Concerning the CH_4 produced within the rumen, one possible mitigation strategy is the use of dietary fats (Moss *et al.* 2000). It is known that medium-chain fatty acids (MCFA) have the potential to suppress rumen methanogenesis (Blaxter & Czerkawski, 1966) and methanogens (Henderson, 1973). Ruminant diets containing coconut oil, a fat rich in the MCFA lauric acid (12 : 0) and myristic acid (14 : 0), decreased daily CH_4 emissions *in vitro* (up to 88 % suppression; Dong *et al.* 1997) and *in vivo* (up to 73 % suppression; Machmüller & Kreuzer, 1999); *in vivo*, coconut oil had no major adverse effects on nutrient digestion and utilisation of dietary energy or protein. However, as *in vitro* evaluations show, the extent of the CH_4-suppressing effect of MCFA seems to depend on the kind of diet used. Coconut oil had a lower efficacy in suppressing CH_4 when the fibrous proportion of the diet was increased (Machmüller *et al.* 2001). Additionally, the CH_4-suppressing effect of non-esterified MCFA was lower when Ca was added to the diet (Machmüller *et al.* 2002). Comparing the effects of various non-esterified MCFA (8 : 0, 10 : 0, 12 : 0, 14 : 0) *in vitro*, 12 : 0 and 14 : 0 were identified to be most effective against rumen methanogens and methanogenesis (Dohme *et al.* 2001*a*).

Based on these *in vitro* observations, the objective of the present study was to evaluate *in vivo* the effects of the supplementation of a non-esterified MCFA on total tract CH_4 emissions and digestion at varying dietary proportions of forage and Ca. Non-esterified MCFA 14 : 0 was chosen since 12 : 0 could result in a depression of feed intake (Blaxter & Czerkawski, 1966; Dohme *et al.* 2001*b*). The present investigation contributes knowledge useful in developing effective feeding strategies to mitigate CH_4 emissions from domestic ruminants.

Abbreviations: ADF, acid-detergent fibre; MCFA, medium-chain fatty acids; NDF, neutral-detergent fibre; OM, organic matter; T_d, dissociation temperature; VFA, volatile fatty acids.

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Materials and methods

Animals and experimental design

Castrated male sheep of the Swiss White Hill breed (*n* 6), aged 9 months with a mean live weight of 41·1 (SD 2·2) kg at the start of the experiment, were assigned to six dietary treatments in a 6 × 6 Latin square arrangement. In six experimental periods, each sheep received a different sequence of diets. Two basal diet types were fed, which differed in forage : concentrate value (1 : 1·5 and 1 : 0·5). The basal diets were calculated to cover 1·3 times maintenance requirements (Swiss Federal Research Station of Animal Production, 1999). This meant daily quantities of either 290 g DM of hay and 413 g DM of concentrate (concentrate-based diet type, Table 1) or 559 g DM of hay and 265 g DM of concentrate (forage-based diet type). The meadow hay was chopped (type SS110; Gruber Maschinen GmbH, Gaspoltshofen, Austria) to an average particle size of 67 (SD 43) mm. The two basal diets were combined with two levels of pure non-esterified 14 : 0 (0 and 50 g/kg DM) and two levels of dietary Ca (4·2 and 9·0 g/kg DM, the latter only in combination with the 14 : 0-supplemented diets). The purity of the 14 : 0 used was ≥98 % (Edenor C 14 98–100; Cognis Deutschland GmbH, Düsseldorf, Germany) and the level of supplementation was the same as in the *in vitro* study of Dohme *et al.* (2001*a*). The proportion of dietary Ca was chosen to obtain a Ca:14 : 0 molar value of either 1 : 2 (4·2 g Ca/kg DM) or 1 : 1 (9·0 g Ca/kg DM). To increase the dietary Ca level, calcium carbonate was used. The dietary Ca content of 4·2 g/kg DM was within the range recommended for sheep (2·4 to 3·0 g Ca/d; Agricultural Research Council, 1980; Swiss Federal Research Station of Animal Production, 1999). With the increased dietary Ca content of 9·0 g/kg DM, the Ca : P was 2·4 : 1 on average. The diets were offered in two equal portions at 08.00 and 16.00 hours. In each experimental period, the sheep were adapted to the respective experimental diet for 14 d, and then subjected to 8 d of complete and separate collection of faeces and urine, and 2 d of quantitative measurement of CH_4 emission in respiratory chambers. Animals were housed individually in pens (1·3 m × 1·9 m) fitted with automatic drinking bowls except in the 10 d measurement periods, when they were kept in metabolism cages (0·6 m × 1·1 m). In the cages, water troughs were filled twice daily with 5 litres fresh tap water. In the barn there was daylight and additional artificial light from 08.00 until 18.00 hours. At the end of the experiment, the mean live weight of the sheep was 49·1 (SD 1·9) kg. The experiment was carried out in accordance with Swiss guidelines for animal welfare.

Sampling procedures and measurements

The sheep were weighed before the morning feeding at the beginning of each collection period and after each respiratory measurement. Feed intake was recorded daily and refusals were removed before the next respective feeding time. Daily water consumption was measured when the animals were in the metabolism cages. Samples of hay and concentrates were collected during each experimental period. For subsequent chemical analysis, feed refusals

Table 1. Allowance and composition of the experimental diets

	Concentrate-based diet type*			Forage-based diet type*		
Myristic acid (g/kg DM)…	0	50	50	0	50	50
Ca (g/kg DM)…	4·2	4·2	9·0	4·2	4·2	9·0
Allowance (g DM/d)						
Hay	290	290	290	559	559	559
Concentrate	413	413	413	265	265	265
Barley	289	289	289	186	186	186
Soyabean meal	124	124	124	80	80	80
Vitamin–trace element premix†	3·7	3·7	3·7	3·7	3·7	3·7
Myristic acid	–	35·2	35·2	–	40·9	40·9
Calcium carbonate	1·5	1·5	10·5	–	–	10·4
Analysed nutrient composition (g/kg DM)						
OM	947	949	938	942	944	933
Crude protein (6·25 × N)	176	168	166	147	140	138
Petrol ether extract	21	61	60	19	59	58
Cell-wall fractions						
NDF	391	372	368	491	468	462
ADF	189	180	178	262	250	247
Hemicellulose	202	192	190	229	218	215
Non-NDF carbohydrates	360	349	345	285	278	275
Ash	53	51	62	58	56	67
Ca	4·2	4·0	8·4	4·2	4·0	8·4
P	3·9	3·7	3·7	3·5	3·3	3·3
Gross energy (MJ/kg DM)	19·0	19·9	19·9	18·9	19·8	19·8

OM, organic matter; NDF, neutral-detergent fibre; ADF, acid-detergent fibre.

* The diet types differed in forage:concentrate. Value 1:1·5 for the concentrate-based diet type and 1:0·5 for the forage-based diet type.

† Contained (per kg) Na 100 g; Cu 1·5 g; Se 15 mg; lysine 5 g; vitamin A 600 mg; vitamin D_3 5 mg; vitamin E 2·5 g.

and samples of all dietary components were milled through a 0·75 mm screen. Complete faeces and urine were collected separately in the 8 d periods twice daily and stored at −20°C immediately after collection. In the metabolism cages, urine was acidified by 3 M-H_2SO_4 to avoid N losses. At the end of the collection periods, proportionate samples of faeces and urine were taken and stored at −20°C until chemical analysis. Part of the faeces was lyophilised for 48 h and milled through a 0·75 mm screen.

Contents of DM, ash, neutral-detergent fibre (NDF), acid-detergent fibre (ADF) and, subsequent to hydrolysis with 4 M-HCl, petrol ether extract in feed and lyophilised faeces were determined according to standard methods (Naumann & Bassler, 1997). For DM and ash, an automatic analyser was used (Thermogravimetric determinator, TGA-500; Leco Corporation, St Joseph, MI, USA). Values of NDF and ADF were corrected for ash content, and α-amylase was used for NDF determination as recommended by Van Soest *et al.* (1991). The DM content of the urine was estimated from the urine density (DM (kg/l) = (density − 1) × 2·6) as suggested by Hinsberg (1953). N content was measured in feed, non-lyophilised faeces and acidified urine with an automatic C/N analyser (type FP-2000; Leco Instrumente GmbH, Kirchheim, Germany) by the Dumas method. Ca and P contents were determined photometrically according to standard methods (Naumann & Bassler, 1997) using an autoanalyser (Skalar SANplus; Skalar, Breda, The Netherlands). Gross energy content of feed and lyophilised faeces was assessed by an adiabatic combustion calorimeter (C 7000 Calorimeter; IKA-Werke GmbH & Co. KG, Staufen, Germany).

On the last day of each experimental period, rumen fluid samples were taken from each sheep 5 h after the morning feeding, using a flexible stomach tube inserted via the oesophagus. Immediately after collection, rumen fluid samples were strained through four layers of compress gauze (average pore size of 1000 μm, type 17; MedPro Novamed AG, Flawil, Switzerland). Thereafter, rumen fluid pH and NH_3 concentration were measured using a pH meter (model 632; Metrohm, Herisau, Switzerland) equipped with the respective electrodes. Ciliate protozoa and bacteria were enumerated microscopically in rumen fluid samples using 0·1 mm and 0·02 mm depth Bürker counting chambers (Blau Brand®, Wertheim, Germany), respectively. Holotrich and entodiniomorphid ciliate protozoa were counted separately. Samples for determination of volatile fatty acids (VFA) were stabilised with 0·046 M mm-$HgCl_2$ solution (200 μl/1·8 ml rumen fluid) and frozen (−20°C) until analysis. Concentrations of VFA were determined by GC using a GC Star 3400 CX (Varian, Sugarland, TX, USA) equipped with a glass column (2 m × 2 mm, 10 % SP-1200/1 % H_3PO_4 on 80/100 Chromosorb W AW, Cat. no. 11965; Supelco, Bellefonte, PA, USA) at the following conditions: N_2 carrier (30 ml/min); injector temperature 250°C; oven temperature 120°C; detector temperature 300°C. Rumen fluid osmolality was measured using a freezing point osmometer (Multi-Osmette; Precision Systems Inc., Natwick, MA, USA). For the enumeration of methanogens, samples of rumen fluid were frozen in liquid N_2 and stored at −70°C. The fluorescence *in situ* hybridisation technique was applied in general as outlined by Stahl *et al.* (1995). Cells were fixed with 4 % (w/v) sterile paraformaldehyde–PBS (pH 7·2) for 3 h at 4°C. Mild sonication (two times for 30 s) was done to avoid the formation of clusters and to optimise homogenisation of the samples (Sandaa *et al.* 1999). Thereafter, 3 μl of the suspensions were spotted onto gelatine-coated slides, air-dried and dehydrated sequentially in 50, 80 and 100 % (v/v) ethanol for 3 min each. Oligonucleotide probes were purchased from Microsynth (Microsynth GmbH, Balgach, Switzerland) with reactive fluorescent dye Cy3 at the 5′ end. The spotted slides were hybridised in 8 μl hybridisation buffer, containing 0·1 % (w/v) sodium dodecyl sulfate and 25 ng of the respective probe. Formamide was used to standardise the hybridisation temperature at 37°C. For 1°C decrease of dissociation temperature (T_d) of the oligonucleotide, 2 % (v/v) formamide was added to the hybridisation buffer. After hybridisation, the slides were washed for 20 min at 37°C with the hybridisation buffer and mounted with an antifade reagent (Fluoro Guard™; Bio Rad, Hercules, CA, USA). Labelled samples were examined with an epifluorescence microscope (BX-60; Olympus Optical AG, Volketswil, Switzerland) and pictures were recorded by a 3CCD colour video camera. Individual cells were counted using image analysis software (analySIS, version 3.1; Soft Imagine System GmbH, Uster, Switzerland). Two replicates were prepared per sample, and twenty fields of view were counted per replicate. To characterise the methanogens inhabiting the rumen, five oligonucleotide probes were used as suggested by Raskin *et al.* (1994) and Lin *et al.* (1997): one domain-specific probe targeting total rumen archaea, i.e. all methanogens (S-D-Arch-0915-a-A-20; T_d 56°C); four order-specific probes (S-F-Mbac-0310-a-A-22 (T_d 57°C) for Methanobacteriales, S-F-Mcoc-1109-a-A-20 (T_d 55°C) for Methanococcales, S-O-Mmic-1200-a-A-21 (T_d 53°C) for Methanomicrobiales, S-O-Msar-0860-a-A-21 (T_d 60°C) for Methanosarcinales).

CH_4 release was measured continuously with a dual chamber as elements of an open-circuit indirect respiration calorimetry system. The chambers (5·44 m^3 volume each) were aluminium–glass constructions, allowing sight contact between sheep, and were air-conditioned (ambient temperature 17·4 (SD 0·5)°C, relative humidity 60·8 (SD 11·1) %, air flow 8·2 (SD 0·1) m^3/h, atmospheric pressure 959 (SD 9) hPa). In-line electronic flow meters (type 8GD-LRM; Fluid Inventor AB, Stockholm, Sweden) continuously recorded the air volume leaving the chambers. Daily CH_4 emission data were based on two consecutive days measuring 22·5 h each. The CH_4 detector used was a Binos 1001 (Fisher-Rosemount, Baar-Walterswil, Switzerland). The detector was calibrated manually before each measurement period. Within each interval of 90 min, one automatic calibration, four measurements of the CH_4 concentrations in the air flowing into the chambers and twenty-four measurements of the CH_4 concentrations in the outgoing air from each chamber were performed.

Calculations and statistical analysis

Crude protein content was calculated as 6·25 × N content. Hemicellulose was computed by the difference between NDF and ADF (Van Soest *et al.* 1991). Non-NDF carbohydrates were defined as the organic matter (OM)

not incorporated in crude protein, petrol ether extract and NDF. The apparent total tract digestibility of OM and nutrients was calculated from the total dietary intake and the total excretion via faeces during the 8 d collection periods (intake minus excretion in relation to intake). The formula of Brouwer (1965) was used for calculation of CH_4 energy loss.

Data were statistically analysed using the general linear model procedure of SAS (version 6.12; SAS Institute Inc., Cary, NC, USA). ANOVA was carried out regarding diet type (*n* 2), supplementation (*n* 3), diet type × supplementation, animal (*n* 6) and experimental period (*n* 6). The tables give the mean values for the six experimental diets (*n* 6), the overall mean values for the three supplementation groups (*n* 12), the standard errors of means and the *P* values for treatment effects and the interaction. All multiple comparisons among means were performed with Tukey's studentised range *post hoc* test considering $P \leq 0{\cdot}05$ to be significant. Pearson's correlation coefficients were calculated between rumen microbial counts. Fig. 1 gives the mean diurnal pattern of CH_4 release for the six experimental diets (*n* 6) and, at every 4 h, standard deviations.

Results

The different forage:concentrate values of the two basal diets resulted in differences in the analysed contents of crude protein, cell-wall fractions and non-NDF carbohydrates (Table 1). The contents of petrol ether extract and gross energy were increased with 14 : 0 supplementation and, likewise, the addition of calcium carbonate increased the contents of ash and Ca.

Supplementing the basal diets with 14 : 0 decreased ($P<0{\cdot}001$) rumen fluid pH from 7·0 to 6·6 (Table 2). The NH_3 concentration in rumen fluid was also decreased by 14 : 0 supplementation ($P<0{\cdot}01$) but this effect was more pronounced in the concentrate-based diet type (interaction, $P<0{\cdot}05$). The basal diet type and extra Ca had no effects on pH and NH_3 concentration. In VFA concentrations and profile, no significant treatment interaction between diet type and supplementation occurred. Supplementing 14 : 0 resulted in an increase ($P<0{\cdot}01$) in the concentrations of acetate, propionate and total VFA. This was not reflected in acetate proportion but in propionate proportion ($P<0{\cdot}01$) of total VFA. Acetate : propionate was decreased ($P<0{\cdot}05$, *post hoc* test) with 14 : 0 supplementation when the dietary Ca level was 4·2 g/kg. Additionally, with 14 : 0 supplementation the proportions of butyrate ($P<0{\cdot}001$) and isovalerate ($P<0{\cdot}01$) were depressed. Compared with the concentrate-based diet, the forage-based diet resulted in decreased ($P<0{\cdot}01$) concentrations of total VFA, acetate, propionate and valerate ($P<0{\cdot}05$). This did not affect the molar VFA proportions. Extra Ca

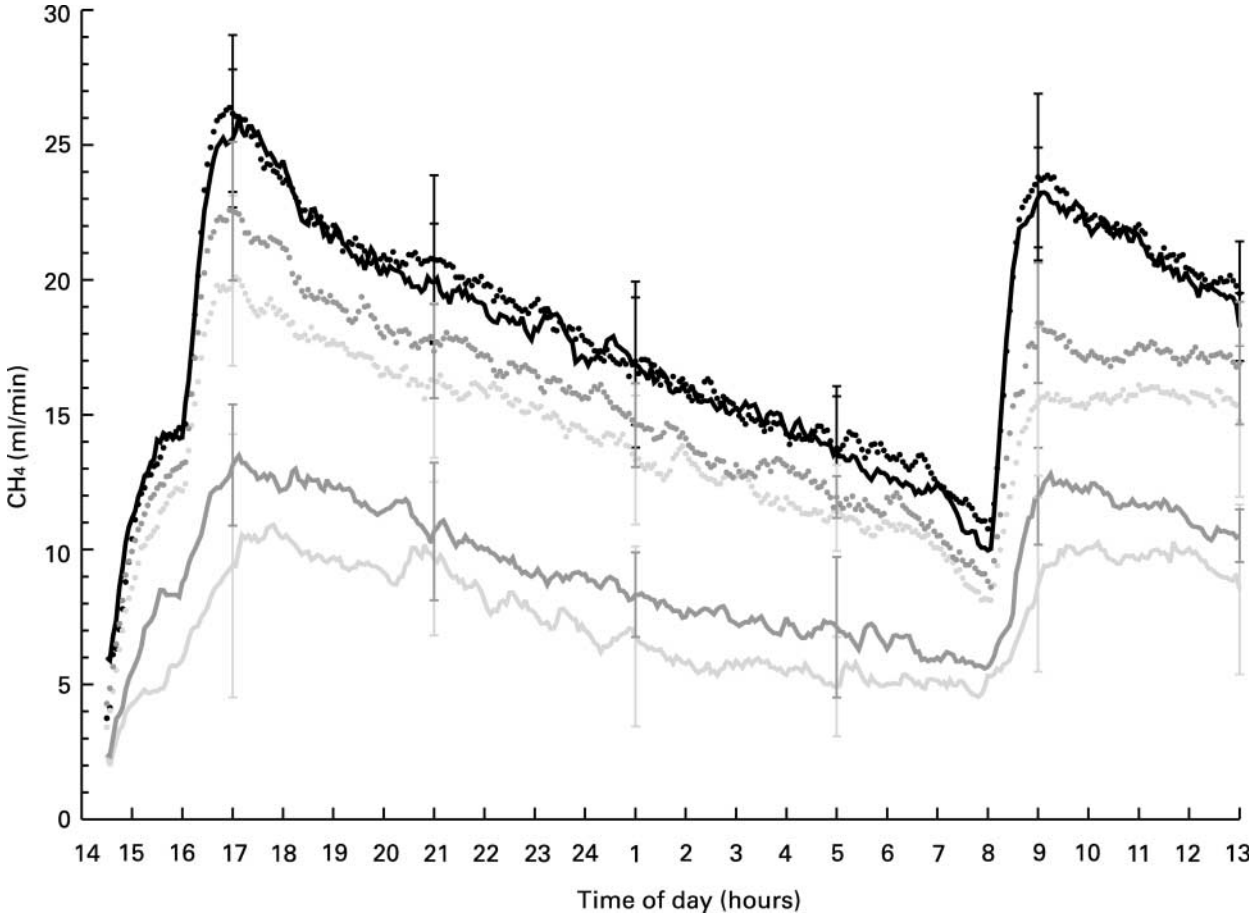

Fig. 1. Diurnal pattern of CH_4 release from sheep fed either a concentrate- (-, -, -) or a forage- (•, •, -) based diet type combined with either 0 g 14 : 0/kg DM and 4·2 g Ca/kg DM (-, •) or 50 g 14 : 0/kg DM and 4·2 g Ca/kg DM (-, •) or 50 g 14 : 0/kg DM and 9·0 g Ca/kg DM (-, •). The vertical bars indicate standard deviations at selected times.

Table 2. Effect of myristic acid on rumen fluid properties*
(Mean values and standard errors of the means)

	Concentrate-based diet type			Forage-based diet type			Supplementation						
Myristic acid (g/kg DM)…	0	50	50	0	50	50	0	50	50		*P* values		
Ca (g/kg DM)…	4·2	4·2	9·0	4·2	4·2	9·0	4·2	4·2	9·0				
n…	6	6	6	6	6	6	12	12	12	SEM	D	S	D×S
pH	7·00^{a}	6·62^{ab}	6·37^{b}	7·04^{a}	6·56^{ab}	6·70^{ab}	7·02^{A}	6·59^{B}	6·53^{B}	0·109	0·27	0·0004	0·21
NH_3 (mmol/l)	10·99^{a}	5·36^{b}	4·90^{b}	7·87^{ab}	5·85^{b}	7·12^{ab}	9·43^{A}	6·01^{B}	5·60^{B}	1·030	0·87	0·0024	0·050
VFA concentration (mmol/l)													
Acetate	47·4^{ab}	60·4^{a}	61·3^{a}	41·2^{b}	53·1^{ab}	49·6^{ab}	44·3^{B}	56·7^{A}	55·5^{A}	3·20	0·0045	0·0015	0·67
Propionate	13·0^{bc}	25·2^{a}	23·1^{ab}	10·4^{c}	18·3^{abc}	15·9^{abc}	11·7^{B}	21·8^{A}	19·5^{A}	2·36	0·0093	0·0010	0·56
Butyrate	6·7	5·9	6·4	6·5	5·1	5·7	6·6	5·5	6·1	0·54	0·21	0·17	0·89
Isobutyrate	0·4	0·1	0·2	0·4	0·2	0·3	0·4	0·2	0·3	0·12	0·61	0·25	0·77
Valerate	0·9	1·0	1·0	0·6	0·8	0·8	0·7	0·9	0·9	0·12	0·030	0·30	0·97
Isovalerate	0·7	0·4	0·4	0·5	0·5	0·6	0·6	0·5	0·6	0·08	0·87	0·20	0·14
Total VFA	69·0^{b}	93·0^{a}	92·4^{a}	59·5^{b}	78·0^{ab}	73·0^{ab}	64·3^{B}	85·5^{A}	82·7^{A}	5·22	0·0026	0·0011	0·64
Molar proportion (%)													
Acetate	68·4	64·9	66·4	68·6	68·2	68·3	68·5	66·6	67·4	1·53	0·16	0·45	0·60
Propionate	19·0^{b}	26·9^{a}	25·0^{ab}	18·1^{b}	23·2^{ab}	21·0^{ab}	18·5^{B}	25·0^{A}	23·0^{A}	1·73	0·054	0·0040	0·62
Butyrate	9·8^{ab}	6·5^{b}	6·8^{b}	10·9^{a}	6·7^{b}	8·2^{ab}	10·3^{A}	6·6^{B}	7·5^{B}	0·85	0·21	0·0008	0·75
Isobutyrate	0·6	0·1	0·3	0·5	0·3	0·4	0·5	0·2	0·3	0·15	0·47	0·10	0·79
Valerate	1·3	1·1	1·1	1·1	1·1	1·2	1·2	1·1	1·1	0·15	0·79	0·77	0·51
Isovalerate	1·0^{a}	0·5^{b}	0·4^{b}	0·9^{ab}	0·6^{ab}	0·8^{ab}	0·9^{A}	0·5^{B}	0·6^{B}	0·11	0·15	0·0058	0·073
A:P ratio	3·9	2·4	2·8	4·0	3·0	3·4	3·9^{A}	2·7^{B}	3·1^{AB}	0·37	0·18	0·011	0·65
Osmolality (mOsm/kg)	252	265	273	242	260	268	247^{B}	263^{AB}	271^{A}	7·2	0·27	0·013	0·95

D, diet type; S, supplementation; D × S, interaction (diet type × supplementation); VFA, volatile fatty acids; A:P, acetate:propionate.
^{a,b,c}Mean values within a row with unlike superscript lower case letters were significantly different ($P<0·05$).
^{A,B}Mean values within a row with unlike superscript capital letters were significantly different ($P<0·05$).
*For details of diets and procedures, see Table 1 and p. 531.

Table 3. Effect of myristic acid on microbial counts in rumen fluid*
(Mean values and standard errors of the means)

	Concentrate-based diet type			Forage-based diet type			Supplementation						
Myristic acid (g/kg DM)…	0	50	50	0	50	50	0	50	50		*P* values		
Ca (g/kg DM)…	4·2	4·2	9·0	4·2	4·2	9·0	4·2	4·2	9·0				
n…	6	6	6	6	6	6	12	12	12	SEM	D	S	D × S
Bacteria (×10^9/ml)	12·3	11·6	11·6	11·1	10·0	8·5	11·7	10·8	10·1	1·80	0·20	0·65	0·87
Ciliate protozoa (×10^5/ml)													
Entodiniomorphids	3·51	4·94	1·32	6·93	3·63	2·29	5·22	4·29	1·81	1·71	0·47	0·15	0·40
Holotrichs	2·36	0·09	0·00	0·17	0·00	0·04	1·27	0·04	0·02	0·94	0·34	0·33	0·43
Total ciliate protozoa	5·87	5·03	1·32	7·10	3·63	2·33	6·49^{A}	4·33^{AB}	1·83^{B}	1·84	0·85	0·061	0·73
Methanogens (×10^8/ml)													
Total archaea	10·55	9·20	10·00	11·67	8·92	9·68	11·11^{A}	9·06^{B}	9·84^{AB}	0·686	0·76	0·023	0·50
Methanobacteriales	2·93	2·22	2·26	3·19	2·51	2·35	3·06	2·37	2·30	0·412	0·54	0·15	0·97
Methanococcales	3·12	3·48	3·55	4·20	3·97	3·51	3·66	3·73	3·53	0·456	0·19	0·91	0·48
Methanomicrobiales	1·68	1·73	1·65	2·18	1·84	1·63	1·93	1·78	1·64	0·342	0·50	0·70	0·74
Methanosarcinales	2·23	1·44	1·47	2·11	1·52	1·10	2·17	1·48	1·29	0·464	0·72	0·16	0·89
Proportion of the different orders (%)													
Methanobacteriales	28·7	25·1	25·8	27·9	25·8	27·7	28·3	25·4	26·7	1·75	0·66	0·28	0·75
Methanococcales	31·8	38·8	38·9	39·2	40·3	40·0	35·5	39·5	39·5	2·88	0·18	0·30	0·49
Methanomicrobiales	17·1	19·9	18·8	17·4	18·2	19·3	17·2	19·0	19·0	1·72	0·82	0·49	0·79
Methanosarcinales	22·4	16·2	16·5	15·5	15·8	13·0	18·9	16·0	14·7	2·46	0·087	0·24	0·44

D, diet type; S, supplementation; D × S, interaction (diet type × supplementation).
^{A,B} Mean values within a row with unlike superscript capital letters were significantly different ($P < 0·05$).
* For details of diets and procedures, see Table 1 and p. 531.

generally had little effect on VFA concentrations and profiles. The average rumen fluid osmolality was 247 mOsm/kg when feeding the basal diets and increased ($P<0·05$, *post hoc* test) to 271 mOsm/kg with 14 : 0 and Ca addition, parallel to VFA concentration.

Neither basal diet type nor supplementation significantly affected counts of bacteria, entodiniomorphid or holotrich ciliate protozoa (Table 3). On average, concentrations were $10·9 \times 10^9$/ml, $3·8 \times 10^5$/ml and $0·4 \times 10^5$/ml for bacteria, entodiniomorphid and holotrich ciliate protozoa, respectively. For total ciliate protozoa concentration, the mean value of the groups supplemented with 14 : 0 and extra dietary Ca was lower ($P<0·05$, *post hoc* test) than that of the unsupplemented groups. Myristic acid supplementation decreased ($P<0·05$) archaea concentration in rumen fluid but only in combination with 4·2 g Ca/kg DM. The counts of the four individual methanogenic orders were not significantly affected by 14 : 0 supplementation. The basal diet type neither altered counts nor proportion of rumen methanogen orders. On average, the methanogenic population was composed of 27 % Methanobacteriales, 38 % Methanococcales, 18 % Methanomicrobiales and 17 % Methanosarcinales. The sum of the counts of the four different order-specific probes was $9·6 \times 10^7$ (SD 3·3) $\times 10^7$/ml equivalent to 97 (SD 28) % of the average counts using the *Archaea* domain-specific probe, illustrating that the order-specific 16S rRNA oligonucleotide probes used were appropriate to characterise the rumen methanogenic population. Counts of Methanococcales, Methanomicrobiales and Methanosarcinales were positively correlated ($P<0·05$) with counts of entodiniomorphid ciliate protozoa (Table 4). The counts of total rumen archaea were positively correlated with counts of Methanobacteriales ($P<0·05$), Methanomicrobiales ($P<0·05$) and Methanosarcinales ($P<0·01$) but not with counts of Methanococcales. Accordingly, a correlation ($P<0·001$) was found between counts of Methanobacteriales, Methanomicrobiales and Methanosarcinales. Counts of Methanococcales only were positively correlated ($P<0·01$) with counts of Methanomicrobiales. No significant correlation occurred between total counts of bacteria and counts of the other rumen microbes.

Depending on the basal diet, the average DM intake differed among treatment groups (Table 5). Feed refusals occurred in the case of two animals (2 and 24 % of DM allowance) when fed the concentrate-based diet type supplied with 14 : 0 at 4·2 g Ca/kg DM. The animals ingested 0·09 to 0·10 kg water/d with the feed. The intake of tap water was decreased ($P<0·01$) when 14 : 0 was supplied, especially in combination with the concentrate-based diet type (interaction, $P<0·05$). The effects on water excretion via urine corresponded with the intake of tap water. The daily excreted amount of faecal water was not affected but that of faecal OM was. With the forage-based diet type, more ($P<0·001$) OM was excreted with faeces than with the concentrate-based diet type. Independent of dietary Ca level, 14 : 0 supplementation increased ($P<0·01$) OM excretion via faeces. The total tract OM digestibility remained unchanged by the treatments and averaged at 0·69. On average, the digestibilities of non-NDF carbohydrates, NDF and ADF were 0·93, 0·49 and 0·27 for the concentrate-based diet and 0·87, 0·59 and 0·52 for the forage-based diet. The 14 : 0 supplementation did not significantly affect digestibility of NDF and ADF, but hemicellulose digestibility was reduced ($P<0·01$). The effect on hemicellulose degradation was smaller with the extra Ca supply. The combined supplementation of 14 : 0 and Ca decreased ($P<0·05$, *post hoc* test) the digestibility of non-NDF carbohydrates, but only in the concentrate-based diet type (interaction, $P<0·05$).

The two unsupplemented basal diets resulted in a similar CH_4 release of about 25 litres/d. Adding 50 g 14 : 0/kg feed DM to the concentrate-based diet type resulted in a significant reduction of daily CH_4 release by up to 58 %, and this was not significantly changed by additional dietary Ca. When the same dietary percentage of 14 : 0 was added to the forage-based diet type, the reduction in CH_4 release was only 22 % and the difference compared with the unsupplemented diet became insignificant with the increased dietary Ca level. The diurnal pattern of CH_4 release (Fig. 1) showed that CH_4 production was always similar throughout the day with the two unsupplemented basal diets. The supplementation of 14 : 0 decreased both the highest and lowest values measured throughout the day. Concerning the diurnal pattern, extra dietary Ca had similar effects in the concentrate-based and in the forage-based diet type. Relating the daily CH_4 release to gross energy intake and OM digested (Table 5) further

Table 4. Relationships between rumen microbial counts (n 36) (Pearson's correlation coefficients)

		Ciliate protozoa		Methanogens			
	Bacteria	ENT	HOL	ARC	MEB	MEC	MEM
ENT	−0·21						
HOL	−0·23	0·01					
ARC	0·26	0·18	−0·12				
MEB	0·10	0·32	0·13	0·34*			
MEC	−0·12	0·37*	−0·15	0·15	0·22		
MEM	−0·05	0·36*	0·08	0·35*	0·65***	0·50**	
MES	0·21	0·36*	0·06	0·46**	0·74***	0·25	0·75***

ENT, entodiniomorphid ciliates; HOL, holotrich ciliates; ARC, Total archaea; MEB, Methanobacteriales; MEC, Methanococcales, MEM, Methanomicrobiales; MES, Methanosarcinales.
* $P<0·05$; ** $P<0·01$; *** $P<0·001$.

Table 5. Effect of myristic acid on intake, excretion, total tract nutrient digestibility and methane emission*
(Mean values and standard errors of the means)

	Concentrate-based diet type			Forage-based diet type			Supplementation						
Myristic acid (g/kg DM)…	0	50	50	0	50	50	0	50	50		*P* values		
Ca (g/kg DM)…	4·2	4·2	9·0	4·2	4·2	9·0	4·2	4·2	9·0				
n…	6	6	6	6	6	6	12	12	12	SEM	D	S	D × S
Feed DM (kg/d)													
Allowance	0·707	0·743	0·752	0·828	0·869	0·880	0·768	0·806	0·816				
Intake	0·707^{b}	0·711^{b}	0·752^{b}	0·828^{a}	0·869^{a}	0·880^{a}	0·768^{B}	0·790^{AB}	0·816^{A}	0·0117	0·0001	0·0024	0·26
Water intake (kg/d)													
Tap water	7·78^{a}	5·80^{ab}	5·09^{b}	7·22^{ab}	5·57^{ab}	7·16^{ab}	7·50^{A}	5·68^{B}	6·12^{B}	0·539	0·34	0·0081	0·049
Feed	0·09	0·09	0·09	0·10	0·10	0·10	0·10	0·09	0·10	0·002	0·0001	0·36	0·34
Excretion (kg/d)													
Urine water	6·56^{a}	4·24^{b}	3·40^{b}	5·62^{ab}	3·84^{b}	5·47^{ab}	6·09^{A}	4·04^{B}	4·43^{B}	0·518	0·57	0·0018	0·020
Faecal water	0·38	0·40	0·41	0·39	0·46	0·43	0·38	0·43	0·42	0·035	0·33	0·33	0·75
Faecal OM	0·19^{c}	0·21^{bc}	0·22^{bc}	0·24^{ab}	0·27^{a}	0·27^{a}	0·21^{B}	0·24^{A}	0·24^{A}	0·007	0·0001	0·0018	0·77
Total tract nutrient digestibility													
OM	0·716	0·690	0·694	0·696	0·675	0·675	0·706	0·683	0·685	0·0107	0·056	0·075	0·97
Cell-wall fractions													
NDF	0·492^{b}	0·467^{b}	0·508^{ab}	0·593^{a}	0·525^{ab}	0·530^{ab}	0·543	0·496	0·519	0·0194	0·0011	0·079	0·15
ADF	0·272^{c}	0·284^{c}	0·328^{bc}	0·520^{a}	0·432^{ab}	0·433^{ab}	0·396	0·358	0·381	0·0295	0·0001	0·46	0·066
Hemicellulose	0·697^{a}	0·639^{ab}	0·676^{ab}	0·678^{ab}	0·629^{b}	0·640^{ab}	0·688^{A}	0·634^{B}	0·658^{AB}	0·0146	0·081	0·0055	0·68
Non-NDF CH	0·929^{a}	0·894^{ab}	0·880^{b}	0·871^{b}	0·877^{b}	0·867^{b}	0·900^{A}	0·886^{AB}	0·874^{B}	0·0089	0·0006	0·026	0·037
CH_4 emission													
Litres/d	25·1^{a}	10·5^{c}	13·2^{c}	25·5^{a}	19·8^{b}	21·8^{ab}	25·3^{A}	15·2^{C}	17·5^{B}	0·86	0·0001	0·0001	0·0001
kJ/MJ GE intake	74·0^{a}	29·4^{c}	35·1^{c}	64·5^{a}	45·6^{b}	49·4^{b}	69·2^{A}	37·5^{B}	42·2^{B}	2·21	0·0009	0·0001	0·0001
g/kg OM digested	37·8^{a}	16·1^{d}	19·4^{d}	33·7^{ab}	25·8^{c}	28·2^{bc}	35·7^{A}	21·0^{B}	23·8^{B}	1·38	0·0004	0·0001	0·0001

D, diet type; S, supplementation; D × S, interaction (diet type × supplementation); OM, organic matter; NDF, neutral-detergent fibre; ADF, acid-detergent fibre; CH, carbohydrates; GE, gross energy.
^{a,b,c,d} Mean values within a row with unlike superscript lower case letters were significantly different ($P<0·05$).
^{A,B,C} Mean values within a row with unlike superscript capital letters were significantly different ($P<0·05$).
* For details of diets and procedures, see Table 1 and p. 530.

documented that the high efficacy of the 14 : 0 supplementation depended on the basal diet type (interaction, $P<0{\cdot}001$). The mean values for the unsupplemented basal diets were not significantly different.

Discussion

Effects of the basal diet type

Feeding strategies for ruminants, which achieve a significant CH_4 suppression, are limited (Moss *et al.* 2000). Methanogenesis is an important terminal step in the anaerobic fermentation of OM within the rumen. Carbohydrates are the main energy source for the rumen microbes and the production of CH_4 is closely related to their fermentation (Czerkawski, 1969). Compared with structural carbohydrates (for example, cellulose), it was observed that the fermentation of non-structural carbohydrates (starch, sugars) results in less CH_4 per unit of substrate fermented (Hungate, 1966). Thus, increasing the dietary proportion of concentrate, i.e. the proportion of easily fermentable carbohydrates, appears to be an effective feeding strategy in decreasing rumen methanogenesis (for example, Van Nevel & Demeyer, 1996). However, when studying the relationship between diet composition, intake level and CH_4 production, Moe & Tyrrell (1980) revealed that the nature of the carbohydrates digested (i.e. cellulose, hemicellulose or soluble residues) is less important at feed intake levels below 1·5 times maintenance. This is also supported by data of Blaxter & Clapperton, (1965), which showed that at maintenance level, independent of the type of diet, i.e. forage or mixed diets, the percentage of gross energy converted to CH_4 increases with increasing digestibility of the dietary energy. This relationship will be negative only at an intake level exceeding twice the maintenance requirements. In the present study, the feed was provided at 1·3 times maintenance requirements (Swiss Federal Research Station of Animal Production, 1999). This explains why almost equal levels of CH_4 were produced per unit of apparently digested OM with both unsupplemented basal diets although the composition of the OM digested was different.

The effect of feeding level on the expression of diet type differences in CH_4 release may be the result of associated changes in passage rate. Okine *et al.* (1989) demonstrated that the overall relationship between CH_4 production and passage rate of ruminal particulate matter is negative and that ruminal passage rate explained at least 28 % of the variation in CH_4 production of steers. Additionally, at a low feeding level only weak effects of concentrate proportion on rumen fluid pH and VFA profile are expected (Zhao *et al.* 1993), which was confirmed by the present study. Rumen methanogenesis was shown to be a pH-dependent process (Van Kessel & Russell, 1996). Overall, this illustrates that the CH_4-suppressing effect of an increased concentrate proportion cannot be regarded as a general phenomenon.

Effects of myristic acid supplementation

In the present experiment, the 14 : 0 supplementation was 50 g/kg DM, resulting in daily supplies of either 35·2 or 40·9 g, depending on the basal diet type. This decreased daily CH_4 emission of the animals by 40 % on average. At the same time 14 : 0 had only small effects on total tract digestibility of OM and fibre. Accordingly, the greatest difference between the treatments with and without 14 : 0 supplementation still remained (−41 %) when CH_4 release was related to the apparently digested feed OM. These effects of 14 : 0 are in line with previous results using 14 : 0 *in vitro* (Dohme *et al.* 2001*a*) or coconut oil *in vivo* (Machmüller & Kreuzer, 1999). Nevertheless, with 14 : 0 supplementation there might have been a shift of fibre fermentation from the rumen to the hindgut as was found by Sutton *et al.* (1983) in sheep fed coconut oil. Since hindgut fermentation differs from rumen fermentation in producing substantially lower amounts of CH_4 (Immig, 1996), this would have only marginally counteracted the suppressing effect of 14 : 0 on rumen methanogenesis.

It has been shown that the growth of rumen microbial species of all three domains of life, *Archaea*, *Eucarya* and *Bacteria*, could be directly affected by non-esterified MCFA. In a pure culture study, the growth of *Methanobrevibacter ruminantium* was found to be inhibited by adding 10 : 0, 12 : 0 or 14 : 0 at concentrations of 0·05, 0·25 or 0·50 g/l medium (Henderson, 1973). Matsumoto *et al.* (1991) showed that rumen ciliates are suppressed by MCFA, particularly by 10 : 0 and 12 : 0. In an *in vitro* screening test some species of Gram-positive bacteria were also inhibited by MCFA, whereas Gram-negative bacteria seemed to be less sensitive (Galbraith *et al.* 1971). The latter indicates that probably the cell-wall structure of the microbes will determine their sensitivity against the MCFA. Kabara (1978) assumed that fatty acids inhibit microbes by affecting their lipid membranes, thus changing the permeability of the cell membrane. Among the four rumen methanogen orders there are also differences in the Gram staining. Methanobacteriales are Gram positive, Methanosarcinales are Gram variable, and Methanococcales and Methanomicrobiales are Gram negative (Boone *et al.* 1993).

In the present study, 14 : 0 supplementation significantly decreased the rumen archaeal population but showed relatively weak effects on other microbial counts. However, when 14 : 0 was fed, the animals significantly decreased tap water intake by 24 % on average compared with the unsupplemented diet. With decreased water consumption, the expansion of rumen volume following drinking will be small (Warner & Stacy, 1968) and the rumen fluid dilution rate will decrease (Rogers & Davis, 1982). Therefore, in the present study, all concentrations measured in the rumen fluid have to be interpreted carefully since they may not reflect the effects on total production (Rogers & Davis, 1982). This is not only valid for rumen microbial counts but also for the related traits concerning microbial metabolism, i.e. concentration of NH_3 and VFA. Assuming a lower rumen fluid volume and a lower rumen fluid dilution rate with the 14 : 0 supplementation would mean that: (i) the inhibiting effect of 14 : 0 on archaea and ciliate protozoa was more pronounced; (ii) the decrease in total NH_3 quantity was even larger; (iii) the increase in VFA production (particularly acetate and propionate) was smaller than apparent.

In contrast, alterations in the correlation coefficients between the rumen microbial counts and in the molar proportions of VFA will not be affected by variations in water intake and rumen fluid volume. In the present study, there was no obvious relationship between counts of holotrich ciliates and methanogens, whereas counts of entodiniomorphid ciliates were positively correlated with counts of Methanococcales, Methanomicrobiales and Methanosarcinales. This presumably reflects the symbiotic relationship between rumen methanogens and ciliate protozoa (Williams & Coleman, 1997). With the exception of the Methanococcales, all other counts of methanogen orders were significantly correlated to total archaeal counts and intercorrelated. The population of the Methanococcales might therefore actually be less sensitive to 14 : 0 compared with the other methanogen orders. The Methanomicrobiales seem to take an intermediate position as they were the only order showing a correlation with Methanococcales counts. Concerning the molar proportions of VFA, a lower acetate:propionate value was found with 14 : 0 supplementation. This suggests a decreased capacity of the archaea to produce CH_4 as it reflects a shift from the hydrogen-consuming process of methanogenesis towards propionate production (Whitelaw *et al.* 1984).

As presumed additional consequences of the decreased water intake with the 14 : 0 supply, the average rumen pH was lower and the actual 14 : 0 concentration in the rumen fluid was higher than would have been the case without suppressed water consumption. These two aspects contributed to the high efficiency of the 14 : 0 supplementation against methanogenesis since they are known to increase the antimicrobial activity of 14 : 0 (Galbraith & Miller, 1973; Henderson, 1973). The reduced pH was also accompanied by an increasing rumen osmolality (respectively osmotic pressure). The physiological mechanism that decreased water intake of the animals when consuming 14 : 0 is as yet unknown. To our knowledge it is the first time that such a relationship between lipid intake and *ad libitum* tap water intake has been described.

Interactions between myristic acid supplementation and diet composition

As mentioned earlier, the effects of 14 : 0 supplementation on rumen microbes will depend on the concentration of non-esterified fatty acids in the medium, as was shown by Henderson (1973) for the inhibition of methanogens. Galbraith *et al.* (1971) concluded that, to exert antimicrobial activity, non-esterified fatty acids have to be in solution and have to remain sufficiently lipophilic to permit adsorption on to the cell surface of the microbes. Therefore, dietary components that influence the concentration of free 14 : 0 in rumen fluid will be decisive for the extent of its CH_4-suppressing effect. In this context, *in vitro* investigations with MCFA revealed two important interactions: (i) with the fibre content of the diet (Machmüller *et al.* 2001); (ii) with the dietary Ca content (Machmüller *et al.* 2002).

Harfoot *et al.* (1974) demonstrated that fatty acids might attach either to rumen microbes or to feed particles. Thus, for the present study two basal diets were prepared differing in their forage:concentrate value, which is associated with differences in feed particle structure and inertness. Highly significant interactions between these basal diets and supplementation were found in the CH_4 emissions of the animals, which is in line with previous *in vitro* findings (Dong *et al.* 1997; Machmüller *et al.* 2001). In the present study, with the forage-based diet the decrease in CH_4 emissions by 14 : 0 was less than half the decrease achieved with the concentrate-based diet. Since the two basal diets did not affect average rumen pH, it can be assumed that this was mainly a result of the different amount and structure of the dietary particulate matter. With the forage-based diet, probably more 14 : 0 was attached to the feed particles and less to the methanogens than with the concentrate-based diet. The use of the forage-based diet also decreased the effects of 14 : 0 on rumen fluid NH_3, water intake and water excretion, whereas other variables, such as pH, VFA and archaeal counts, seemed to be unaffected by the combinations of basal diet type and 14 : 0.

In the rumen, non-esterified fatty acids could also chelate with cations, especially with the alkaline earth metal Ca, resulting in insoluble and inactive complexes (El Hag & Miller, 1972). The formation of Ca soaps is known to alter the effects of fatty acids on rumen fermentation (for example, Jenkins & Palmquist, 1982). Concerning methanogenesis, significant interactions between supplementation of 12 : 0 and Ca was recorded *in vitro* (Machmüller *et al.* 2002). Accordingly, in the present study, increasing the dietary Ca content from 4·2 and 9·0 g/kg DM significantly decreased the effect of the 14 : 0 supplementation on CH_4 release from the animals and even reversed the effect of 14 : 0 on other traits such as rumen archaea, acetate:propionate and hemicellulose digestion. However, although the Ca:14 : 0 molar value was increased from 1 : 2 to 1 : 1, the reversal of the CH_4-suppressing effect of 14 : 0 was incomplete. There are three reasons why Ca probably had this limited effect. First, the process of saponification is directly related to rumen pH (Palmquist *et al.* 1986) with the highest rates found at a pH of 7. Therefore, the decrease in pH, which accompanied 14 : 0 supplementation, could have limited soap formation. Second, calcium carbonate, a common Ca source but known to have a low solubility in rumen fluid (Keyser *et al.* 1985), was used to increase the Ca content of the diets. Third, nothing is known about the Ca availability from the other dietary components (i.e. hay, soyabean meal and barley). However, the effect of Ca on methanogenesis did not differ noticeably between the two basal diets, suggesting that the Ca ions will interact with free 14 : 0 even when a proportion of 14 : 0 is inactivated by attachment to the feed particles.

Conclusions

The present results demonstrate that 14 : 0 is a promising feed additive to suppress CH_4 emissions from ruminants, with decreases of up to 58 %. However, 14 : 0 seems to be less effective in diets with a high forage proportion, a common diet type in many agricultural systems worldwide. Concerning dietary Ca level, a high efficacy of 14 : 0 is guaranteed only when the Ca supply does not exceed the actual requirements of the animals. A favourable side effect of supplementing diets with 14 : 0 is a decreased

water consumption, which is particularly interesting in regions with water scarcity. Generally, the present results show that, because of the impact of the animal, *in vivo* verifications of CH_4 mitigation strategies are indispensable and that *in vivo* the concentrations of rumen microbes involved in methanogenesis, i.e. methanogens or ciliate protozoa, are not reliable as indicators for the success of a strategy.

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