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Efficiency of monolaurin in mitigating ruminal methanogenesis and modifying C-isotope fractionation when incubating diets composed of either C_3 or C_4 plants in a rumen simulation technique (Rusitec) system

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Mitigation of methanogenesis in ruminants has been an important goal for several decades. Free lauric acid, known to suppress ruminal methanogenesis, has a low palatability; therefore, in the present study the aim was to evaluate the mitigation efficacy of its esterified form (monolaurin). Further, ^{13}C-isotope abundance (δ^{13}C) and ^{13}C–^{12}C fractionation during methanogenesis and fermentation were determined to evaluate possible microbial C-isotope preferences. Using the rumen simulation technique, four basal diets, characterised either by the C_3 plants grass (hay) and wheat (straw and grain), or the C_4 plant (^{13}C excess compared with C_3 plants) maize (straw and grain), and a mixture of the latter two, were incubated with and without monolaurin (50 g/kg dietary DM). Added to hay, monolaurin did not significantly affect methanogenesis. When added to the other diets ($P<0{\cdot}05$ for the wheat-based diet) methane formation was lowered. Monolaurin decreased fibre disappearance (least effect with the hay diet), acetate:propionate ratio, and protozoal counts. Feed residues and SCFA showed the same δ^{13}C as the diets. Methane was depleted in ^{13}C while CO_2 was enriched in ^{13}C compared with the diets. Monolaurin addition resulted in ^{13}C depletion of CO_2 and enrichment in CH_4 (the latter only in the hay diet). In conclusion, monolaurin proved to effectively decrease methanogenesis in the straw–grain diets although this effect might partly be explained by the concomitantly reduced fibre disappearance. The influence on ^{13}C-isotope abundance and fractionation supports the hypothesis that ruminal microbes seem to differentiate to some extent between C-isotopes during methanogenesis and fermentation.

Methane: Fatty acids: Carbon isotopes: Rusitec

Enteric methane (CH_4) emissions from livestock, and thereby mainly ruminants, are estimated to be the second largest source of global agricultural non-CO_2 greenhouse gases[1]. In 2000, global enteric CH_4 emissions were estimated to amount to 85·6 Gg equivalent to 1·8 Mt CO_2 equivalents and are projected to increase by 32 % until 2020 relative to 1990[1]. The search for ideal CH_4-mitigating strategies revealed that diet supplementation seems to be especially promising[2]. There is an increasing body of literature indicating that supplementing diets with lipids that are not protected from ruminal digestion can diminish enteric CH_4 emissions[2]. Saturated medium-chain fatty acids (MCFA), including caprylic acid, capric acid[3], lauric acid[4,5] and myristic acid[6,7], as well as combinations of the latter two[8], are among the most promising lipids for that purpose. Also coconut oil, a lipid especially rich in lauric and myristic acid, has proved to be very effective in mitigating CH_4 formation in the gut of the ruminant (for example, Machmüller *et al.*[9], Jordan *et al.*[10] and Yabuuchi *et al.*[11]). So far, only a few studies have investigated the efficiency of potentially CH_4-abating supplementation strategies in different diet types[12,13]. Added to a concentrate-based diet, myristic acid showed a larger effect in suppressing CH_4 formation in sheep than when supplemented to a forage-based diet[6]. Machmüller[12] concluded that, in the case of diets rich in structural carbohydrates, non-esterified rather than esterified MCFA should be fed, as the efficiency of esterified fatty acids strongly depends on the rate of ruminal lipolysis. Besides their anti-methanogenic effects, lipids are also helpful in increasing the energy density of the diet, which may improve animal performance in some situations[10]. However, feeding higher amounts of lipids to ruminants may have adverse side-effects. These include the possibility of a depressed DM intake[2] and, sometimes, a reduced ruminal fibre degradation[8,14]. Furthermore, the most efficient anti-methanogenic MCFA, lauric acid, is known to be of low palatability, particularly due to its soapy taste, which may result in substantial feed refusals[15]. This should be different with esterified lauric acid. At least, according to a large series of reports, refusals were never nearly as high when feeding coconut oil, consisting of

Abbreviations: δ^{13}C, ^{13}C-isotope abundance; IRMS, isotope ratio mass spectrometer; MCFA, medium-chain fatty acids; Rusitec, rumen simulation technique.

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about half of lauric acid in esterified form[14], than those reported for pure lauric acid[15]. Finally, the immediacy of the potentially adverse effects in the rumen should be reduced with monolaurin, since monolaurin, an ester of lauric acid, is known to be less corrosive and, therefore, might be less irritating for the ruminal environment than free lauric acid.

So far, analyses of stable C-isotopes in ruminant science have mostly been used to trace the feeding regimen in terms of dietary proportions of C_3 and C_4 plants[16,17] that cattle have been subjected to by concluding from isotope ratios analysed in body tissue (meat), milk, urine or faeces. Few studies have analysed the stable C-isotopes of CH_4 released from ruminants (for example, Rust[18], Schulze *et al.*[19], Bilek *et al.*[20] and Levin *et al.*[21]) and from ruminal fluid *in vitro*[22]. Diets tested were either mixtures of C_3 and C_4 plants in varying proportions or pure C_3 and pure C_4 plant diets. However, none of these studies investigated all versions analysed in the present study; neither did they determine the ^{13}C isotope abundance ($\delta^{13}C$) and isotope fractionation in single SCFA. According to the authors' best knowledge, monolaurin was tested in the present study for the first time for its effectiveness in ruminal CH_4 mitigation. Generally monolaurin is well known for its antimicrobial efficiency against gram-positive bacteria[23], but it has been shown that also specific gram-negative bacterial species can be affected[24].

In the present study the hypothesis tested was that an effective lipid source acts differently on methanogenesis when the carbohydrate types available for fermentation differ, which might be expressed in a different C-isotope fractionation. An *in vitro* approach using the rumen simulation technique (Rusitec) was chosen for the present study as this system allows the following of all processes taking place in the rumen quantitatively, which would be much more difficult to control in an *in vivo* approach including all extra-ruminal processes. In order to determine whether differences in CH_4 formation and C-isotope fractionation exist, four basal diets characterised by either C_3 plants (two diets; grass hay, wheat), C_4 plants (one diet; maize) or C_3 and C_4 plants (one diet; maize and wheat mixture) were compared, either at a similar (C_3 straw plus grain diet *v.* C_4 straw plus grain diet) or at a differing carbohydrate profile (C_3 grass hay diet *v.* C_4 straw plus grain diet, i.e. easily degradable fibre *v.* starch).

Materials and methods

In vitro *system and experimental diets*

The *in vitro* experiment was conducted using an eight-fermenter Rusitec system as described in detail by Soliva & Hess[25]. With this *in vitro* system four different basal diets were tested at 15 g DM/d both with and without monolaurin (chemically: $C_{15}H_{30}O_4$, glycerol monolaurate) supplemented at 50 g/kg (on a DM basis) in a completely randomised design in six replicates per treatment. In the present study, Lauricidin® (purity >95 %; Med-Chem Laboratories, Galena, IL, USA) was used where, according to the producer's statement, lauric acid is esterified at the external position with glycerol and is shaped into mini-pellets without fill material. The basal diets consisted either of meadow-grass hay rich in ryegrass (second cut, beginning of shooting; forage-only), maize or wheat (always straw and grain mixed in the proportions being equivalent in estimated net energy content to that of the hay) (Table 1). A fourth basal diet consisted of a 1:1 mixture of the maize and the wheat diet. All diets were balanced in their calculated net energy for lactation content according to the Swiss Federal Research Station for Animal Production (RAP)[26]. To increase the limiting dietary contents of ruminally degradable protein in the straw–concentrate-based diets, urea, being low in C content to minimise the addition of non-C_3 or non-C_4 plant carbon, was used as an N source.

Experimental procedures and sampling

In six experimental runs, each time including all dietary treatments and lasting for 10 d, the daily portions of experimental feeds were put into nylon bags (70 × 140 mm) with a pore size of 100 μm[27]. Before that, hay and straw were ground to pass

Table 1. Composition of the experimental diets

Monolaurin (g/kg DM)...	0				50			
Basal diet type...	Maize	Maize and wheat*	Wheat	Hay	Maize	Maize and wheat*	Wheat	Hay
Basal diet (mg/g DM)†								
Hay	–	–	–	1000	–	–	–	1000
Maize grain	250	125	–	–	250	125	–	–
Maize straw	750	375	–	–	750	375	–	–
Wheat grain	–	225	450	–	–	225	450	–
Wheat straw	–	275	550	–	–	275	550	–
Supplement (mg/g basal diet DM)								
Urea	43	43	42	5	44	43	42	5
Glycerol monolaurate	–	–	–	–	50	50	50	50
Analysed nutrient composition (g/kg DM)								
Organic matter	933	956	978	904	936	957	979	908
Crude protein	196	195	193	162	187	186	184	154
Neutral-detergent fibre	521	490	460	544	497	468	439	518
Starch	169	223	277	<2	161	213	264	<2
Total sugars	25	17	13	77	24	16	13	73
Net energy for lactation (MJ/kg DM)	5·4	5·5	5·6	5·6	5·4	5·5	5·6	5·6

* 1:1 Mixture of the maize and wheat diets.
† Supplemented at an amount of 15 g DM/d.

a 5 mm sieve whereas the grains were ground to a size of 3 mm. Ruminal fluid was obtained from a lactating rumen-fistulated Brown Swiss cow which was fed hay *ad libitum* and concentrate (1 kg/d administered in two portions). The cow was kept according to the Swiss guidelines for animal welfare. Before inoculation, ruminal fluid was strained through four layers of medicinal gauze with a pore size of about 1 mm. At the beginning of each experimental run the fermenters were filled with 100 ml pre-warmed buffer[25] and 900 ml strained ruminal fluid. Thereafter, two nylon bags were administered whereby the first one was filled with solid ruminal content (about 40 g fresh matter) and the second one with the respective experimental diet. On the second experimental day the bag containing the solid ruminal content was exchanged with another bag containing the experimental diet. Each feed bag was incubated for 48 h. To maintain anaerobic conditions the system was flushed with gaseous N_2 for 3 min after exchanging the feed bags. The incubation temperature was kept constant at 39·5°C. Buffer flow to the fermenters was continuous and averaged 397 (SD 69) ml/d, resulting in a dilution rate of about 40 % per d. The resulting incubation fluid outflow was collected in bottles chilled at −20°C.

Incubation fluid samples, collected directly from the fermenters via a three-way valve using a syringe equipped with a plastic tube 3 h before exchanging the feed bags, were analysed daily for redox potential and pH using the respective electrodes connected to a pH meter (model 634; Methrom AG, Herisau, Switzerland). Part of the incubation fluid samples taken were centrifuged for 5 min at 4000 rpm (Varifuge® K; Heraeus, Osterode, Germany) and the supernatant fraction was stored at −20°C before being analysed for SCFA concentrations and the $\delta^{13}C$ values of the SCFA. The first were determined by using high pressure liquid chromatography (System Hitachi Lachrom; Merck, Tokyo, Japan) according to the method of Ehrlich *et al.* [28]. For the determination of $\delta^{13}C$, 4 M-sodium chloride was added to the samples and pH was adjusted to 2·5 using 5 M-HCl. The SCFA were then extracted with solid-phase micro extraction adapted with a specific fibre (Carbowax/Divinylbenzene, Yellow-Green no. 57 337-U; Supelco Inc., Bellefonte, PA, USA). The isotope composition of the individual SCFA was analysed via online coupling to a GC-combustion isotope ratio mass spectrometer (IRMS) (Thermo Delta plus XL with Combustion Interface III and Thermo Trace GC; Thermo Electron Corp., Waltham, MA, USA). Measurements followed modified procedures as described by Dias & Freeman[29] as well as Berg *et al.* [30]. The temperature programme of the GC was as follows: 60°C for 1 min, heating-up to 110°C at a rate of 20°C/min, heating-up to 135°C at a rate of 0·5°C/min, heating-up to 220°C at a rate of 60°C/min, 3 min at 220°C. Oxidation and reduction reactors in the combustion interface were maintained at 940°C and 640°C, respectively. The NiO, CuCO and Pt wires in the combustion unit were oxidised with O_2 for 12 h at 940°C before being used. The solid-phase micro extraction/GC-IRMS method had an accuracy of ±0·5 ‰ of $\delta^{13}C$.

After 48 h of incubation, dietary residues were washed with cold water in a washing machine and frozen at −20°C until nutrient analyses were performed. Later the lyophilised and ground residues were analysed for DM and organic matter, via total ash (automatically by TGA-500; Leco Corporation, St Joseph, MI, USA), N (C/N analyser, Leco-Analysator Typ FP-2000; Leco Instrumente GmBH, Kircheim, Germany; crude protein = 6·25 × N) and neutral-detergent fibre. Analyses of neutral-detergent fibre were carried out with the Fibretec System M (Tecator, 1020 Hot Extraction, Höganäs, Sweden) with the addition of α-amylase but without sodium sulfite as suggested by Van Soest *et al.* [31]. Starch content was determined polarimetrically[32] (model 343; Perkin Elmer, Boston, MA, USA). Samples were extracted with hot ethanol (80 %) for the determination of total sugar content. After being filtered the samples were analysed with a colorimetric method using an orcin/sulfuric acid reagent in an auto-analyser (Cartridge Gesamtzucker (total sugar cartridge), Autoanalyzer II; Bran-Luebbe GmbH, Norderstedt, Germany).

The fermentation gases produced during 24 h were collected in gas-tight aluminium bags (TECOBAG 8 litres, PETP/AL/PE – 12/12/75 quality; Tesserau Container GmbH, Bürstadt, Germany). Gas was analysed daily for concentrations of CH_4, CO_2 and H_2 with a GC (model 5890 Series II; Hewlett Packard, Avondale, PA, USA) equipped with a flame ionisation detector (to determine CH_4), a thermal conductivity detector (to determine CO_2 and H_2) and a 2·34 m × 2·3 mm column, 80/100 mesh (Porapak Q; Fluka Chemie AG, Buchs, Switzerland). The total amount of gas produced was quantified by water displacement[25]. This was accomplished by pressing the fermentation gas out of the gas-tight aluminium bags using plates of 2 kg weight. Fermentation gas was flushed into an Erlenmeyer flask and the water displaced from this flask then was collected in a second, graduated, flask.

Subsamples of the fermentation gases were analysed with a trace gas analyser (ANCA-TG II; SerCon Ltd, Crewe, Cheshire, UK) for the $\delta^{13}C$ of CO_2 and CH_4. CO_2 was separated cryogenically from CH_4, N_2 and O_2, and subsequently measured in continuous-flow with a Sercon Ltd GEO 20/20 mass spectrometer. Methane was separated cryogenically from the other gases and combusted at 1000°C in a furnace containing CuO, Ni and Pt wires. Calibration and linearity corrections were accomplished by measuring variable amounts of an internal standard gas (1 % H_2, 1 % O_2, 7·99 % CH_4, 40·18 % CO_2, rest is N_2; PanGas, Dagmersellen, Switzerland). The $\delta^{13}C$ of the feeds and the fermentation residues was determined using an elemental analyser (model NCS 2500; Carlo-Erba, Rodano, Italy) coupled in continuous flow with an IRMS (Optima, Micromass, Crewe, Cheshire, UK). Sample material was combusted in the presence of O_2 in an oxidation column at 1030°C. Combustion gases then passed a reduction column (650°C), and the N_2 and CO_2 produced were separated chromatographically and transferred into the IRMS via an open split for on-line isotope measurements. This method had an accuracy of ±0·5 ‰ of $\delta^{13}C$ for CH_4, and of ±0·2 ‰ for CO_2.

Calculations and statistical evaluation

The abundance of ^{13}C relative to ^{12}C was determined in comparison with a generally accepted reference standard ($\delta^{13}C$) (‰) = $((R_{sample} - R_{reference})/R_{reference}) \times 1000$, where R_{sample} is the isotope ratio (^{13}C:^{12}C) of the sample, and $R_{reference}$ represents the isotope ratio of the conventional δ-notation for

carbon with respect to the Vienna Pee Dee Belemnite (VPDB) standard. The factor α, describing the fractionation between the respective two fermentation gases, was calculated as $\alpha_{CO2/CH4} = (\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)$[33]. Because α is usually close to 1·0 the fractionation will be expressed as the enrichment factor $\varepsilon_{(CO2-CH4)} = (\alpha - 1) \times 1000$. The equations used for calculating the C-isotope fractionation between diet and individual SCFA were $\varepsilon_{(diet-SCFA)} = (\alpha - 1) \times 1000$, with $\alpha_{diet/SCFA} = (\delta^{13}diet + 1000)/(\delta^{13}SCFA + 1000)$. In order to avoid influences of the fermenters, diets were arranged completely randomised. For all data, the mean values of the last 5 d of each experimental run were subjected to ANOVA using the general linear model (GLM) procedure of SAS (version 9.1; SAS Institute Inc., Cary, NC, USA) with diet and lipid supplementation as fixed effects while experimental run was assumed to be random. Multiple comparisons among means were performed with Tukey's method and differences were declared significant at $P<0·05$.

Results

Independently of the presence of monolaurin, there was a clear basal diet effect ($P<0·001$) on incubation fluid pH, with the lowest pH found with the hay diet ($P<0·001$), while monolaurin supplementation resulted in an increase of pH in all treatments ($P<0·001$). Monolaurin addition led to a decrease of total SCFA concentration ($P<0·001$), and the molar proportion of propionate was increased ($P<0·001$) at the cost of butyrate and acetate. In molar proportions of propionate and *n*-butyrate, an interaction ($P=0·003$ and $P=0·011$ respectively) between diet type and monolaurin supplementation was present due to the lack of response to monolaurin with the hay diet. The acetate:propionate ratio was affected ($P<0·001$) by basal diet type and monolaurin supplementation. There was also an interaction ($P<0·001$) between the two factors, as monolaurin had a larger depressive effect in the wheat and the maize–wheat mixed basal diet compared with the other diets. The addition of monolaurin reduced ($P<0·001$) ciliate protozoal counts (Table 2).

Monolaurin supplementation decreased ($P<0·001$) nutrient disappearances. The difference in crude protein disappearance between the hay diet and the other diets was higher in the presence of monolaurin (interaction, $P<0·001$). Concerning the daily amount of CH_4 produced during fermentation, the effect of the basal diet type was significant ($P=0·001$), although this was apparent only for the lipid-supplemented diets when considered separately (Table 3). The addition of monolaurin resulted in a decline in CH_4 formation (monolaurin effect: $P<0·001$), except for the hay diet. The most pronounced decrease in daily CH_4 formation due to monolaurin was found in the wheat diet (−63 %) and, less substantially, in the mixed and the maize diet (−38 and −37 %, respectively). In multiple comparisons among means, the monolaurin supplementation to the wheat diet significantly decreased CH_4 related to total SCFA formation. Monolaurin addition decreased CO_2 ($P=0·048$), except for the hay diet, but did not affect the daily amount of H_2.

Diets differed in their carbon isotope values, with the maize diets being most enriched in ^{13}C, showing $\delta^{13}C$ of about −15 ‰ in the unsupplemented and −17 ‰ in the monolaurin-supplemented treatments (Table 4). The hay diet, both supplemented and unsupplemented with monolaurin, was the one most depleted in ^{13}C with a $\delta^{13}C$ of −30 ‰. After 48 h of incubation the feed residues still showed a similar $\delta^{13}C$ profile as the original diets. There was no effect of monolaurin supplementation.

The $\delta^{13}C$ ratios of the individual SCFA primarily reflected diet differences ($P<0·001$) in $\delta^{13}C$; however, some changes were also observed. The $\delta^{13}C$ of acetate was more positive than that of the respective diets, meaning richer in the ^{13}C isotope, with the largest enrichment of about 6 ‰ occurring with the wheat diet, followed by the mixed and the hay diet. Monolaurin supplementation had a significant effect on the $\delta^{13}C$ values of several SCFA. Accordingly, the wheat diet resulted in about 1·6 ‰ more ^{13}C-depleted propionate with monolaurin than the corresponding diet, which had not been the case without monolaurin (interaction, $P=0·018$). With respect to the C-isotope fractionation from diet to SCFA, there was a clear basal diet effect ($P<0·001$) on the enrichment factors ε(diet–acetate), ε(diet–propionate) and ε(diet–*n*-butyrate; $P=0·027$). A monolaurin effect ($P<0·001$) on the enrichment was observed with ε(diet–*iso*-butyrate), where ε decreased by about 2 ‰ with the maize and the wheat diet, and by about 5 ‰ with the mixed diet. However, no influence of monolaurin was found with the hay diet, resulting in a monolaurin × diet interaction ($P=0·042$). Monolaurin also influenced ($P=0·016$) the enrichment factor ε(diet–*n*-valerate), with increases in the wheat (+2·2 ‰) and the hay diet (+1·7 ‰) and a slight decrease in the maize-containing diets. Interactions ($P<0·001$) between diet type and monolaurin supplementation were found in ε(diet–propionate) and ε(diet–*n*-valerate), as well as ε(diet–acetate; $P=0·019$).

Compared with the diets, CH_4 (−65 to −73 ‰) was markedly depleted in ^{13}C while, on the other hand, CO_2 (−11 to −17 ‰) was enriched in ^{13}C. For both gases, CH_4 and CO_2, a clear ($P<0·001$) basal diet effect was obvious. Additionally, a diet type × monolaurin interaction ($P<0·001$) in $\delta^{13}CH_4$ occurred, with an increase of 3·8 ‰ found when supplementing monolaurin to the wheat diet, while there was a decrease in all other diets. Monolaurin addition resulted in a ^{13}C-depleted CO_2 ($P<0·001$). The diet type × monolaurin interaction found in $\delta^{13}CO_2$ ($P=0·014$) was less clear in that respect. The enrichment factor $\varepsilon(CO_2-CH_4)$ was very high (56 to 61 ‰) and was affected by diet type ($P<0·007$) and monolaurin supplementation ($P<0·001$; mostly decreased by monolaurin). There was an interaction ($P<0·001$) based on that $\varepsilon(CO_2-CH_4)$ was highest with the wheat diet compared with the other treatments only without, but not with, monolaurin.

Discussion

Lipids are among the most promising nutritional strategies for abating enteric methane formation. Their effectiveness depends on several factors including level of supplementation, fatty acid chain length, and the diet type fed to the ruminant. If lipid supplementation does not exceed 50 g/kg dietary DM, effects on feed intake and digestibility are likely to be low[34]. Comparing *in vivo* conditions with a water:food ratio in the rumen of about 4·5[35] and *in vitro* conditions with water:food ratios of at least ten times higher might have different effects, as the dosage of supplements normally is related to feed and

Table 2. Effects of diet type and fatty acid addition on fermenter fluid traits and degree of ruminal nutrient disappearance (averages of days 6–10) (*n* 6)

(Mean values with pooled standard errors)

Monolaurin (g/kg DM)...	0				50					*P*		
Basal diet type...	Maize	Maize and wheat*	Wheat	Hay	Maize	Maize and wheat*	Wheat	Hay	SEM	Diet	Lipid	Diet × lipid
Fermenter fluid traits												
Redox potential (mV) (*n* 4)	−296^{d}	−263^{b,c,d}	−239^{b}	−291^{c,d}	−294^{d}	−249^{b,c}	−188^{a}	−302^{d}	7·5	<0·001	0·044	0·019
pH	7·07^{a,b}	7·05^{b,c}	7·03^{b,c}	6·82^{d}	7·19^{a}	7·14^{a,b}	7·10^{a,b}	6·93^{c,d}	0·034	<0·001	<0·001	0·852
SCFA (mmol/d)	46·2^{a,b}	45·6^{a,b}	41·5^{a,b,c}	48·9^{a}	34·8^{c}	37·2^{b,c}	35·1^{c}	46·6^{a,b}	2·19	<0·001	<0·001	0·100
Molar porportions (%)												
Acetate	56·6^{a,b}	51·0^{b,c}	51·4^{b,c}	63·7^{a}	52·2^{b}	44·2^{c,d}	40·2^{d}	60·4^{a}	1·704	<0·001	<0·001	0·118
Propionate	18·3^{b,c}	16·1^{c}	15·0^{c}	18·8^{b,c}	25·4^{a,b}	27·6^{a}	30·3^{a}	17·8^{b,c}	1·725	0·073	<0·001	0·003
n-Butyrate	18·2^{b}	24·2^{a}	24·8^{a}	14·0^{b}	15·3^{b}	17·9^{b}	19·0^{a,b}	15·9^{b}	1·284	<0·001	0·001	0·011
iso-Butyrate	0·20	0·34	0·38	0·42	0·30	0·49	0·43	0·38	0·081	0·161	0·280	0·691
n-Valerate	6·09^{b}	7·96^{a,b}	8·01^{a,b}	2·74^{c}	6·07^{b}	9·59^{a}	9·20^{a}	5·07^{b,c}	0·657	<0·001	0·009	0·351
iso-Valerate	0·58	0·44	0·49	0·24	0·68	0·28	0·84	0·40	0·155	0·066	0·340	0·444
Acetate:propionate ratio	2·98^{a,b}	3·34^{a}	3·28^{a}	3·28^{a}	2·43^{b,c}	1·98^{c}	1·82^{c}	3·21^{a}	0·148	<0·001	<0·001	<0·001
Protozoa (× 10^{3}/ml)	4·39^{b,c}	4·52^{b,c}	7·51^{a,b}	9·61^{a}	0·26^{c}	0·19^{c}	0·13^{c}	1·46^{c}	1·101	0·039	<0·001	0·253
Nutrient disappearance (g/g supply)												
Organic matter	0·535^{b}	0·530^{b}	0·542^{b}	0·645^{a}	0·404^{c}	0·433^{c}	0·461^{c}	0·548^{b}	0·0138	<0·001	<0·001	0·334
Crude protein	0·855^{b,c}	0·866^{a,b}	0·894^{a}	0·756^{d}	0·825^{c}	0·831^{c}	0·855^{b,c}	0·655^{e}	0·0075	<0·001	<0·001	<0·001
Neutral-detergent fibre	0·245^{c}	0·212^{c}	0·198^{c}	0·457^{a}	0·105^{d}	0·088^{d}	0·094^{d}	0·341^{b}	0·0153	<0·001	<0·001	0·899

$^{a-e}$ Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* 1:1 Mixture of the maize and wheat diets.

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Table 3. Effects of diet type and fatty acid addition on the formation of fermentation gases (averages of days 6–10) (*n* 6)
(Mean values with pooled standard errors)

Monolaurin (g/kg DM)…	0				50				SEM	*P*		
Basal diet type…	Maize	Maize and wheat*	Wheat	Hay	Maize	Maize and wheat*	Wheat	Hay		Diet	Lipid	Diet × lipid
Gaseous emissions (mmol/d)												
CH_4	5·15^{a,b}	5·51^{a,b}	6·37^{a}	6·13^{a}	3·52^{b,c}	3·30^{b,c}	2·99^{c}	6·99^{a}	0·476	0·001	<0·001	0·011
CH_4 (mmol/mol SCFA)	114^{a,b}	128^{a,b}	155^{a}	128^{a,b}	114^{a,b}	92·7^{b}	83·9^{b}	147^{a}	9·82	0·145	<0·001	0·002
CO_2	45·3^{a,b}	42·8^{a,b}	46·8^{a}	45·6^{a,b}	32·7^{b}	33·7^{a,b}	33·7^{a,b}	48·1^{a}	5·10	0·030	0·048	0·142
H_2	0·066^{b}	0·178^{a,b}	0·173^{a,b}	0·148^{a,b}	0·107^{a,b}	0·138^{a,b}	0·426^{a}	0·070^{a,b}	0·0644	0·027	0·433	0·150

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
*1:1 Mixture of the maize and wheat diets.

not to ruminal fluid or incubation liquid, respectively. Therefore, in the present *in vitro* experiment supplementation of lipids was chosen to amount to 50 g related to feed DM. In detail, four basal diets isoenergetic in terms of net energy for lactation but differing in their carbohydrate profile were supplemented with monolaurin, an esterified form of lauric acid. This, and using diets containing either C_3 or C_4 plants or a mixture of both, was intended to facilitate the expression of differentiation in the effects on methanogenesis and C-isotope fractionation during ruminal fermentation.

Effects on ruminal methanogenesis, nutrient disappearance and formation of short-chain fatty acids

Some of the differences in the effects on fermentation and methanogenesis found among the four basal diets were as expected. These include an increase in the molar proportion of acetate with the hay diet, as it contained more and better degradable fibre. It was somewhat unexpected that this mainly happened in association with lower butyrate and, only less so, propionate proportions. Among the straw–grain-based diets, differences in SCFA were mostly small. Unexpectedly, in the absence of monolaurin, all four diets did not differ very clearly regarding CH_4 formation. Hindrichsen *et al.* [36] demonstrated that the differences in the methanogenic potential of forage-only diets and diets with a forage:concentrate ratio of 1:1 were smaller than expected from shifts taking place at very high concentrate proportions. Still, the lack of any difference between the hay diet and the wheat diet in the present study was astonishing and may have resulted from the long incubation time of 48 h for all feeds, including concentrate, and the high pH level due to buffering. By contrast, the difference to the maize diet and the maize–wheat diet (with hay being by some 20 % higher) was in the range expected. Starch fermentation favours propionate formation which is inversely related to CH_4[37]. As in the absence of monolaurin the propionate proportion of total SCFA was lower in the wheat than in the hay diet; the lack of a CH_4 effect seems reasonable despite the concomitant decrease in fibre fermentation.

Monolaurin proved to be effective in suppressing ruminal methanogenesis in some of the basal diets. The mitigation of CH_4 formation found with the wheat diet exceeded 50 %, a level similar to that found previously with Rusitec using about the same dietary proportion of non-esterified lauric acid in a mixed forage–concentrate diet[38]. Kabara[23] described monolaurin to have an even higher antimicrobial potential than non-esterified lauric acid when being tested in direct contact with different microbes. However, in the rumen it is more likely that a rapid lipolysis occurs, making monolaurin approximately equally efficient as non-esterified lauric acid. The present experiment was not primarily designed to identify the factors responsible for the anti-methanogenic activity of monolaurin. This leaves open the extent to which a direct suppression of methanogens[4,5], an indirect suppression via anti-protozoal effects (a considerable proportion of methanogens is associated with protozoa[39]; the assessment of effects on protozoa in Rusitec is, however, very limited) and a concomitant decline in nutrient disappearance would explain this effect. The latter had also been found in previous studies with the addition of MCFA[8,12] and in the

Table 4. ^{13}C-isotope abundance (δ^{13}C) values of the diets, residues, SCFA and fermentation gases, and treatment effects on the enrichment factors $\varepsilon(CO_2-CH_4)$ and ε(diet−SCFA) (averages of days 6–10) (*n* 6)

(Mean values with pooled standard errors)

Monolaurin (g/kg DM)...	0				50					*P*		
Basal diet type...	Maize	Maize and wheat*	Wheat	Hay	Maize	Maize and wheat*	Wheat	Hay	SEM	Diet	Lipid	Diet × lipid
δ^{13}C ratios†												
Diets	−15·2	−21·2	−28·7	−30·4	−16·9	−23·3	−28·6	−30·3				
Diet residues‡	−14·5^{a}	−21·7^{c}	−27·9^{d}	−29·6^{e}	−14·6^{a}	−20·8^{b}	−28·0^{d}	−29·6^{e}	0·12	<0·001	0·094	0·001
Acetate	−12·9^{a}	−17·3^{b}	−22·9^{c}	−25·7^{d}	−13·7^{a}	−17·1^{b}	−22·9^{c}	−26·0^{d}	0·47	<0·001	0·501	0·731
Propionate	−14·8^{a}	−20·2^{b}	−28·6^{c}	−29·3^{c}	−15·3^{a}	−21·2^{b}	−30·1^{c}	−28·5^{c}	0·37	<0·001	0·040	0·018
n-Butyrate	−18·5^{a}	−23·5^{b}	−29·3^{c}	−34·6^{d}	−18·5^{a}	−24·6^{b}	−29·8^{c}	−34·3^{d}	0·51	<0·001	0·268	0·536
iso-Butyrate	−23·4^{a}	−30·8^{b,c}	−35·0^{d,e}	−36·8^{e}	−23·2^{a}	−28·3^{b}	−33·1^{c,d}	−36·2^{d,e}	0·73	<0·001	0·018	0·347
n-Valerate	−21·2^{a}	−26·3^{b}	−34·6^{d}	−34·4^{e}	−22·2^{a}	−28·1^{c}	−34·6^{e}	−35·9^{e}	0·38	<0·001	<0·001	0·590
iso-Valerate	−21·5^{a}	−26·5^{b}	−33·7^{c}	−34·8^{c}	−22·4^{a}	−28·3^{b}	−35·4^{c}	−34·6^{c}	0·09	<0·001	0·108	0·646
CH_4	−65·5^{a}	−68·3^{b,c}	−73·4^{e}	−66·4^{a,b}	−67·4^{a,b}	−69·1^{c}	−69·6^{d}	−67·1^{a,b}	0·51	<0·001	0·755	<0·001
CO_2	−10·9^{a}	−13·9^{b}	−16·5^{c}	−13·4^{b}	−13·2^{b}	−15·4^{c}	−17·7^{d}	−14·0^{b}	0·25	<0·001	<0·001	0·014
C-isotope enrichment factor ε§												
Diet−acetate	−2·31^{a}	−3·88^{a,b,c}	−6·02^{c,d}	−4·83^{b,c,d}	−3·18^{a,b}	−6·34^{d}	−5·83^{c,d}	−4·42^{a,b,c,d}	0·48	<0·001	0·051	0·019
Diet−propionate	−0·39^{b}	−0·96^{b,c}	−0·17^{b}	−1·12^{b,c}	−1·57^{b,c}	−2·18^{c}	1·61^{a}	−1·83^{b,c}	0·38	<0·001	0·218	<0·001
Diet−*n*-butyrate	3·43^{a}	2·45^{a,b}	0·59^{b}	2·31^{a,b}	1·69^{a,b}	1·34^{a,b}	1·26^{a,b}	2·11^{a,b}	0·52	0·027	0·239	0·224
Diet−*iso*-butyrate	8·38^{a,b}	9·96^{a}	6·51^{b,c}	6·61^{a,b,c}	6·50^{b,c}	5·11^{b,c}	4·71^{c}	6·18^{b,c}	0·76	0·047	<0·001	0·042
Diet−*n*-valerate	6·17^{a}	5·34^{a,b,c}	3·99^{c}	4·12^{b,c}	5·50^{a,b,c}	4·92^{a,b,c}	6·21^{a}	5·80^{a,b}	0·39	0·128	0·016	<0·001
Diet−*iso*-valerate	6·44	5·47	5·18	4·60	5·68	5·07	7·05	4·48	0·91	0·274	0·824	0·479
CO_2-CH_4	58·4^{b}	58·3^{b}	61·4^{a}	56·9^{b,c}	58·1^{b,c}	57·7^{b,c}	55·9^{c}	57·1^{b,c}	0·65	0·007	<0·001	<0·001

$^{a-e}$ Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* 1:1 Mixture of the maize and wheat diets.

† δ^{13}C calculated as δ (‰) = $((R_{sample} - R_{reference})/R_{reference}) \times 1000$, with R_{sample} being the isotope ratio of the sample ($^{13}C:^{12}C$) and $R_{reference}$ representing the isotope ratio of the standard for carbon (Vienna Pee Dee Belemnite; VPDB).

‡ Statistical analyses of the δ^{13}C ratios of the residues were done for the first four experimental runs (*n* 4).

§ $\varepsilon_{(CO2-CH4)}$, ε (‰) = $(\alpha - 1) \times 1000$, with $\alpha_{CO2/CH4} = (\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)$[38]. $\varepsilon_{(diet-SCFA)}$, ε (‰) = $(\alpha - 1) \times 1000$, with $\alpha_{diet/SCFA} = (\delta^{13}\text{diet} + 1000)/(\delta^{13}\text{SCFA} + 1000)$[38].

present study where monolaurin addition resulted in a reduction in nutrient disappearance. Relating CH_4 to the amount of SCFA produced, a measure for nutrient fermentation, did not reveal a monolaurin-induced effect, except for the wheat diet. The shifts towards propionate at the cost of acetate and butyrate, caused by monolaurin, are consistent with the findings on neutral-detergent fibre disappearance. Out of seven cultured rumen bacterial species, those contributing to propionate production were found to be less susceptible to MCFA than the others[40]. The present study again confirmed the different levels of efficiency in suppressing methanogenesis in different diet types. Accordingly, as was also shown earlier *in vitro*[13] and *in vivo*[6], the effects of MCFA are much more pronounced in concentrate-based diets than in forage-based or forage-only diets. In the present case, monolaurin even proved to be completely ineffective in the forage-only diet type. Potential reasons for this diet-dependent efficiency of MCFA include binding of the fatty acids to fibre particles[6].

Effects on ruminal carbon isotope fractionation

The differences in $\delta^{13}C$ between the diets consisting either of C_3 or C_4 plants were about 13·5 ‰. A difference of about the same magnitude as seen between C_3 and C_4 plants might be expected in the CH_4 produced by cows fed those diets[21]. In the present study, the C-isotope signature was obvious in feed residues and SCFA, but only to a small degree in the fermentation gases. The $\delta^{13}C$ values of CH_4 resulting from incubating maize (a C_4 plant) did not differ from those originating from the grass hay (a C_3 plant). Further CH_4 from the maize diet was only 8·5 ‰ heavier than the CH_4 produced from the wheat diet (another C_3 plant). The reason for these findings lies in the experimental design. In the present Rusitec study we used the McDougall buffer solution, which included a considerable amount of chemically pure $NaHCO_3$ with a $\delta^{13}C$ of −4·8 ‰ in order to maintain a favourable pH of about 7. According to the carbonate equilibria, the bicarbonate of the buffer exchanges C-isotopes with the CO_2 produced by the fermentation and thus influences the isotope composition of the CO_2 used by the methanogens for CH_4 formation. Therefore, the differences in $\delta^{13}C$ regarding the CO_2 of the different treatments were reduced compared with the differences between the diets themselves. However, this has no influence on the C-isotope fractionation and the relative enrichment between CO_2 − CH_4 as the reduction of CO_2 is energetically the most favourable pathway to generate CH_4[41]. The large $^{13}C-^{12}C$ fractionation between CO_2 and CH_4 has also been observed in other studies[19,22]. The effect of monolaurin on $\delta^{13}CO_2$ may be related to the amount of CO_2 that is converted to CH_4. Regarding the CO_2:CH_4 ratio, it seems that relatively more CO_2 is converted into CH_4 in the unsupplemented treatments. Therefore the remaining CO_2 becomes more enriched in the heavy isotope because ^{12}C is preferentially converted to CH_4.

The buffer has no influence on the isotope fractionation of diet residues and SCFA, and the different fractionation from diet to SCFA. Concerning influences on the $\delta^{13}C$ ratios of individual SCFA, only very little information is available in the literature. Metges *et al.*[22] analysed the SCFA as a whole in the incubation medium of an *in vitro* experiment. They found ratios in SCFA similar to those of the respective C_3 diets. In the present experiment, the smallest enrichment between diet and SCFA was found for propionate, although fractionation differed even among treatments in this SCFA. In almost all treatments acetate was enriched in ^{13}C relative to the diet. This is probably due to the fact that part of the acetate is further transformed to other metabolites, for example, butyrate and amino acids[42], and accordingly the remaining acetate could be enriched in ^{13}C. Comparable situations might explain the high enrichment factors found for the other SCFA. For instance, *iso*-butyrate and *iso*-valerate are derived from the degradation of branched-chain amino acids, and they are used by various bacteria species for the resynthesis of branched-chain amino acids and the *de novo* synthesis of branched long-chain fatty acids[43]. The protein fraction in general is somewhat richer in ^{12}C than the carbohydrate fraction[44]. This would explain the fractionation towards ^{12}C observed in the *iso*-branched-chain SCFA compared with the diet, which consists largely of carbohydrates.

Important effects on C-isotope fractionation included those of monolaurin addition. This supplementation not only strongly influenced ruminal fermentation but also had significant effects on the $\delta^{13}C$ of several SCFA and fermentation gases. The most surprising treatment effect with respect to C-isotope fractionation was the interaction of basal diet and monolaurin found in the fermentation gases. The enrichment $\varepsilon(CO_2-CH_4)$ was highest with the wheat diet when being unsupplemented and was decreased by 5 ‰ with monolaurin addition. The relatively higher fractionation found with the unsupplemented wheat diet might have resulted from the different carbohydrate composition compared with the other diets. With 277 g/kg DM, the wheat diet contained about 40 % more starch than the maize diet. Methanogens need an anaerobic environment with a redox potential being lower than −200 mV[45]. Maybe the redox potential of −188 mV found with the monolaurin-supplemented wheat diet was a major reason for the low amount of CH_4 produced in this treatment. Similar effects have been reported before, with an associated increase of the lactate proportion in ruminal fluid[46], which would contribute to an environment not suitable for many of the fibre-degrading ruminal microbes. Usually lactate is rapidly metabolised to propionate by protozoa to prevent acidosis[47], but monolaurin also seemed to act against the protozoa. In turn, the reduction in protozoal counts could explain the high redox potential, as protozoa significantly contribute to a low redox potential[48].

In the present study the largest enrichment of ^{13}C isotopes (2 ‰) in propionate relative to the diet was found in the monolaurin-supplemented mixed diet. With the hay diets the enrichment factor ε(diet−propionate) was found to range between −1·12 and −1·83 ‰, whereas a depletion in ^{13}C of propionate occurred only in the wheat plus monolaurin treatment ($\varepsilon_{(diet-propionate)} = +1{\cdot}61$ ‰). There are two mechanisms for propionate formation known to exist in the rumen[49]: the randomising (succinate-including) pathway and the non-randomising (acrylate) pathway. The present results suggest that different pathways were used in the different treatments and that C-isotopes were differently discriminated in these two pathways. There is evidence that the contribution of the non-randomising type to propionate formation in hay-only diets is negligible[13]. For those treatments having enrichment factors ε(diet−propionate) in the same range, the same mode of propionate formation might be expected.

Conclusion

The present results provide evidence that monolaurin is an effective methane-mitigating supplement *in vitro*, but only when being added to mixed forage–concentrate diets and not to a forage-only diet. One important mediator of the methane-suppressing effect of monolaurin seems to be an adverse effect on ruminal nutrient disappearance. In order to be successfully applied as a methane-abatement strategy in ruminant nutrition, this needs to be largely compensated for by hindgut digestion. The results obtained with the C-isotope fractionation illustrate that during fermentation ruminal microbes perform fractionation to a certain extent. Determining C-isotope fractionation therefore might evolve into a valuable tool to investigate whether changes in ruminal metabolic pathways during fermentation are occurring. Further investigations are required to demonstrate the usefulness of this approach *in vivo* and to relate the changes to target microbial species.

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The authors state that there is no conflict of interest.

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