Metabolism of α-linolenic acid during incubations with strained bovine rumen contents: products and mechanisms

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Abstract

Description of α-linolenic acid (*cis*-9,*cis*-12,*cis*-15-18 : 3, ALA) metabolism in the rumen is incomplete. Ruminal digesta samples were incubated with ALA and buffer containing water or deuterium oxide to investigate the products and mechanisms of ALA biohydrogenation. Geometric Δ9,11,15-18 : 3 isomers were the main intermediates formed from ALA. An increase in the $n+1$ isotopomers of Δ9,11,15-18 : 3 was due to ^{2}H labelling at C-13. Isomers of Δ9,11,13-18 : 3, *cis*-7,*cis*-12,*cis*-15-18 : 3 and *cis*-8,*cis*-12,*cis*-15-18 : 3 were also formed. No increase in $n+1$ isotopomers of Δ7,12,15-18 : 3 or Δ8,12,15-18 : 3 was detected. Enrichment in $n+2$ isotopomers of 18 : 2 products indicated that ALA metabolism continued via the reduction of 18 : 3 intermediates. Isomers of Δ9,11,15-18 : 3 were reduced to Δ11,15-18 : 2 labelled at C-9 and C-13. ALA resulted in the formation of Δ11,13-18 : 2 and Δ12,14-18 : 2 containing multiple ^{2}H labels. Enrichment of the $n+3$ isotopomer of Δ12,15-18 : 2 was also detected. Metabolism of ALA during incubations with rumen contents occurs by one of three distinct pathways. Formation of Δ9,11,15-18 : 3 appears to be initiated by H abstraction on C-13. Octadecatrienoic intermediates containing *cis*-12 and *cis*-15 double bonds are formed without an apparent H exchange with water. Labelling of Δ9,11,13-18 : 3 was inconclusive, suggesting formation by an alternative mechanism. These findings explain the appearance of several bioactive fatty acids in muscle and milk that influence the nutritional value of ruminant-derived foods.

Key words: Biohydrogenation: Conjugated linoleic acid: Conjugated linolenic acid: Linolenic acid: Rumen bacteria

Clinical, biomedical and *in vitro* studies have provided evidence that conjugated 18-C fatty acids (FA) including isomers of conjugated linoleic acid (CLA) exhibit potential anti-inflammatory, immuno-modulatory, anti-obesity and anti-carcinogenic activities as well as improve biomarkers of cardiovascular health[1,2]. Isomers of conjugated linolenic acid (CLN) containing a conjugated diene double-bond system also have similar biological effects[3,4] with potential as therapeutics for regulating blood glucose and body composition in humans[5]. Recent studies in experimental animal models or *in vitro* have also indicated that isomers of CLN containing a conjugated triene system have anti-carcinogenic and anti-lipogenic activity and may influence immune function[6]. A more complete understanding of the mechanisms responsible for the formation of CLN and CLA isomers from α-linolenic acid (*cis*-9,*cis*-12,*cis*-15-18 : 3, ALA) is essential for the synthesis of these compounds in amounts required for the prevention of chronic human diseases or establishing biological activity in a range of mammalian species.

Ruminant fat is relatively abundant in CLA and CLN isomers containing a conjugated diene arrangement of double bonds[7–9], but only trace amounts of CLN isomers containing a conjugated triene double-bond system, which are typically found in certain plant seeds including pomegranate, tung, bitter gourd, catalpa and pot marigold[10]. *In vitro* studies have demonstrated that isomers of CLN trienes, prepared by alkaline treatment, have higher cytotoxic activity during incubations with human tumour cells than their non-conjugated counterparts or CLA isomers[11].

Meat and milk from ruminants are the principal source of CLA in the human diet, with *cis*-9,*trans*-11 as the major isomer[12,13]. Ruminant foods also contain a wide range of CLA isomers with double bonds located from Δ7,9 to Δ12,14 and isomers of Δ9,11,15-CLN[7–9]. Geometric isomers of Δ9,11-CLA and Δ10,12-CLA are formed during the initial isomerisation of linoleic acid (*cis*-9,*cis*-12-18 : 2, LA) in the rumen[14–16]. However, other CLA isomers accumulate in the rumen of cattle fed diets rich in ALA[17–19]. Metabolism of ALA in the rumen is thought to involve

an initial isomerisation to yield *cis*-9,*trans*-11,*cis*-15-CLN, which is sequentially reduced to *trans*-11,*cis*-15-18:2 and *trans*-11-18:1 with 18:0 as an end product[20–22]. Historical studies of ruminal ALA biohydrogenation do not consider isomers of CLA as intermediates. However, alternative pathways of ALA biohydrogenation, involving the formation of *cis*-9,*trans*-11,*trans*-15-CLN[9], *trans*-9,*trans*-11,*cis*-15-CLN[23], *cis*-9,*trans*-13,*cis*-15-CLN[24] and *trans*-10,*cis*-12,*cis*-15-CLN[25], have been proposed but not proven. Incubations of ^{13}C-labelled ALA with bovine rumen contents were reported to result in the accumulation of fourteen uncharacterised 18:3 intermediates[26], highlighting the complexity of ruminal ALA biohydrogenation.

Recent studies have characterised a range of intermediates formed during the incubation of ALA with ovine or bovine rumen contents[23,26,27], but none has provided unambigious evidence on the biochemical pathways responsible. The present study investigated the products formed and possible mechanisms involved in the initial stages of ALA biohydrogenation by rumen microbiota based on an examination of the incorporation of ^{2}H in FA intermediates formed during incubations of ALA with bovine rumen fluid diluted with water or deuterium oxide (D_2O).

Methods

Collection of rumen contents

All experimental procedures involving animals were approved by the National Animal Ethics Committee (Hämeenlinna, Finland), in accordance with the guidelines outlined in the European Community Council Directive 86/609/EEC[28]. Four multiparous Finnish Ayrshire cows of mean 253 (SD 11·3) d in lactation and 601 (SD 60) kg live weight, and fitted with a rumen cannula (i.d. 100 mm; Bar Diamond, Inc.), were used as donors. Each cow received a diet based on a mixture of timothy and meadow fescue grass silage and concentrates (forage: concentrate ratio 60:40, on a DM basis). Concentrates comprised rolled barley (293 g/kg), rolled oats (270 g/kg), molassed sugar beet pulp (130 g/kg), rapeseed expeller (280 g/kg) and a proprietary mineral and vitamin premix (27 g/kg; Onni-Kiven-näinen). Silage and concentrates were offered four times daily at 06.45, 13.00, 16.00 and 19.00 hours for 14 d before starting *in vitro* incubations. Cows were housed in a barn fitted with individual stalls with continuous access to water, and were milked at 06.30 and 16.45 hours. Samples of rumen contents (500 ml) were collected into plastic bottles from each cow immediately before morning feeding, placed in a 39°C water bath and transported to the laboratory.

Incubations with rumen contents

Batch culture incubations were performed in 100 ml glass flasks[16]. Strained rumen contents were diluted 1:2 (v/v) with modified McDougall buffer[16] prepared using de-ionised water or 99% [2]H-enriched water (D_2O; Cambridge Isotope Laboratories, Inc.). A sample of 50 ml of diluted rumen fluid, 400 mg of ground dry hay, 5 mg of ALA (10-1803-30; Larodan Fine Chemicals AB) prepared as a suspension in aqueous Tween 80[16] and 5 mg of 19:0 (10-1900-13; Larodan Fine Chemicals AB) dissolved in ethanol (5 mg/ml) was incubated under carbon dioxide at 39°C for 0, 1·5, 3·0 and 12 h. Control incubations containing hay, Tween 80 and rumen fluid diluted with de-ionised water were also established over the same time course. At each designated time point, flasks were placed immediately in ice-cold water, and the contents were stored at −20°C. Incubations were performed in triplicate with samples of rumen contents from each cow.

Lipid extraction and fatty acid analysis

Flask contents were freeze-dried for 24 h at −90°C under a partial pressure of 103 Pa (Brown Christ Gamma 2; Melsungen AG). In total, 200 mg of freeze-dried incubation contents were mixed with 0·5 ml of de-ionised water and the pH was adjusted to 2·0 with 2 M-hydrochloric acid. Lipids were extracted in 4 ml of heptane–isopropanol (3:2, v/v). Extraction was repeated and both organic phases recovered were combined, washed with de-ionised water, dried over 200 mg of sodium sulphate and evaporated to dryness under a constant stream of N_2 at 30°C. Fatty acid methyl esters (FAME) were prepared from total lipids using a two-step base–acid catalysed procedure[29]. Samples of FAME were also converted to 4,4-dimethyloxazoline (DMOX) derivatives by incubation overnight with 2-amino-2-methyl-1-propanol under an N_2 atmosphere at 170°C[29].

FAME were quantified using a GC (model 6890; Hewlett-Packard) equipped with a flame-ionisation detector and a 100-m fused silica capillary column (i.d. 0·25 mm) coated with a 0·2-μm film of cyanopropyl polysiloxane (CP-SIL 88; Agilent Technologies Inc.). The total FAME profile in a 2-μl sample at a split ratio of 1:50 was determined using a temperature gradient programme[30], and H_2 as a carrier gas operated at 206·8 kPa for 50 min, which was increased at a rate of 34·5 kPa/min to a final pressure of 310·3 kPa, that was maintained for a further 7 min. The nominal initial flow rate was 2·1 ml/min. FAME and DMOX derivatives were analysed by GC-MS using a GC equipped with a quadrupole selective mass detector (model 5973N; Agilent Technologies Inc.), operated at 230°C in the positive electron ionisation mode using an ionisation voltage of 70 eV. Chromatography was performed using the same temperature gradient and column type used for GC analysis of FAME and helium as the carrier gas[29]. The analysis was repeated to separate methyl esters of *trans*-10,*cis*-15-18:2 and *trans*-11,*cis*-15-18:2 using an alternative 100-m column coated with a highly polar ionic liquid SLB-IL111 column (100 m × 0·25 mm i.d., 0·2-μm film thickness; Sigma-Aldrich)[31,32]. Helium was used as the carrier gas, operated at a nominal initial flow rate of 1·0 ml/min at a constant pressure of 264·8 kPa with a temperature programme as follows: initial oven temperature was maintained at 168°C for 30 min, increased at 1°C/min to 200°C and maintained at 200°C temperature for 10 min. The distribution of CLA and CLN isomers was determined by HPLC (model 1090; Hewlett-Packard) using four silver-impregnated silica columns (ChromSpher 5 Lipids, 250 × 4·6 mm; 5-μm particle size; Agilent Technologies Inc.) coupled in series. Methyl esters of CLA or CLN were separated under isocratic conditions at 22°C using

0·1 % or 0·2 % (v/v) acetonitrile in heptane, respectively, at a flow rate of 1 ml/min and monitoring column effluent at 233 and 268 nm[30]. Isomers were identified based on retention time comparisons with methyl ester standards containing a mixture of CLA isomers (Sigma-Aldrich), or geometric isomers of 8,10,12-CLN and 9,11,13-CLN (Larodan Fine Chemicals AB).

FA were identified based on GC-MS analysis of FAME and DMOX derivatives and interpretation of mass spectra according to published guidelines[33,34]. For most products, the deduced FA structure was verified by comparison with an online reference spectra library[35]. Double-bond geometry was deduced based on relative retention times and known elution order for a mixture of geometric Δ9,12-18:2 and Δ9,12,15-18:3 methyl esters (Sigma-Aldrich) during GC analysis[36]. The double-bond geometry of 9,11,15-18:3 isomers was inferred based on the elution order reported in the literature[8,9,23].

Enrichment of m/z $n+1$, $n+2$ and $n+3$ isotopomers (molecular ion +1, +2 and +3, respectively) was determined by GC-MS analysis of FAME. Enrichment in water was determined by gas isotope ratio MS using a VG SIRA 10 (VG Isotech) gas isotope ratio MS fitted with a split flight tube and H/^{2}H collector[15]. Corrections for H^{3+} were made using dedicated software at the time of measurement. The position of ^{2}H labelling of incubation products was determined by GC-MS analysis of DMOX derivatives.

Data analysis

Amounts of FA in incubation flasks were analysed by ANOVA for repeated measures with a statistical model that included the fixed effect of incubation time, treatments (control and test incubations containing added ALA with de-ionised water or D_2O) and their interaction and random effect of replicate nested within cow assuming a compound symmetry covariance structure using the MIXED procedure of SAS (version 9.2; SAS Institute, Inc.). Denominator df were calculated using the Kenward-Rogers method. The same statistical model was used to compare the abundance of $n+1$, $n+2$ and $n+3$ isotopomers of products formed during incubations of ALA with D_2O or de-ionised water (natural enrichment). Enrichment of $n+1$, $n+2$ and $n+3$ isotopomers was calculated from the m/z ratios at n, $n+1$, $n+2$, $n+3$ and $n+4$ by deconvolution[15,37]. Changes in the enrichment of $n+1$, $n+2$ and $n+3$ isotopomers of products formed during 0, 1·5, 3·0 and 12 h of incubation of ALA with D_2O were analysed by ANOVA for repeated measures with a statistical model that included the fixed effect of incubation time and random effect of replicate nested within cow assuming a compound symmetry covariance structure and calculation of denominator df by the Kenward-Rogers method. Fixed effects were considered significant at $P < 0.05$. Least square means with pooled standard errors are reported.

Results

Metabolism of α-linolenic acid during incubations with strained rumen contents

Strained ruminal digesta samples collected from four lactating cows were diluted with buffer containing de-ionised water or

D_2O and incubated with ALA under anaerobic conditions for up to 12 h. Concentrations of added ALA declined rapidly (Fig. 1), resulting in the appearance ($P < 0.05$) of 18:3 and 18:2 intermediates relative to control incubations (Fig. 2). The relative disappearance of ALA (Fig. 1) and formation of intermediates were similar (Fig. 2), but not identical during incubations of ALA with rumen fluid diluted with water or D_2O.

Formation of octadecatrienoic acids during incubation of α-linolenic acid with strained rumen contents

Addition of ALA increased ($P < 0.05$) the formation of CLN isomers compared with hay and rumen fluid alone (Table 1). Cis-9,trans-11,cis-15-18:3 was the most abundant CLN isomer in all incubations, but smaller amounts of trans-9,trans-11,cis-15-CLN were also detected (Table 1). Cis-9,trans-11,Cis-15-CLN was formed in the greatest quantity immediately after the addition of ALA to strained rumen contents, but disappeared from flask contents over the course of 12 h incubations. Formation of trans-9,trans-11,cis-15-CLN from ALA was greatest during the first 1·5–3 h of incubation, but the amounts declined thereafter. Small amounts of trans-9,trans-11,cis-13-CLN were also formed (Table 1). Addition of ALA with rumen contents increased ($P < 0.05$) the appearance of cis-7,cis-12,cis-15-18:3, cis-8,cis-12, cis-15-18:3 and trans-8,cis-12,cis-15-18:3 in flask contents, with the highest amounts detected after 1·5 and 3 h of incubation.

All incubation flasks contained Δ10,12,15-CLN. Addition of ALA increased ($P < 0.05$) Δ10,12,15-CLN formation during 1·5 h of incubation with rumen contents diluted in water (Table 1). However, formation of 10,12,15-CLN between 0 and 3 h did not differ ($P > 0.05$) between incubations of ALA with D_2O and the control. After 12 h, the amounts of Δ10,12,15-CLN were lower ($P < 0.05$) for incubations containing D_2O (Table 1).

Mass spectra of methyl esters prepared from flask contents over the course of 0 to 12 h incubations of ALA with D_2O
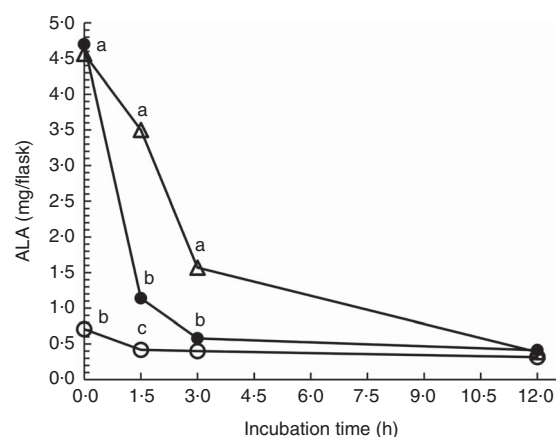

Fig. 1. Disappearance of α-linolenic acid (ALA) during 0 to 12 h incubations of ground hay with strained rumen fluid diluted in de-ionised water ($\bigcirc$), rumen fluid diluted in de-ionised water and 5 mg of added ALA ($\bullet$) or rumen fluid diluted in 56·6 (SEM 1·33) % moles per cent excess (MPE) deuterium oxide and 5 mg of added ALA ($\triangle$). Rumen contents were collected from four cows and incubated at 39°C under carbon dioxide. Each point represents the least square mean of n 12 measurements (pooled SEM 0·083 mg/flask). [a,b,c] Mean values for each incubation time with unlike letters were significantly different ($P < 0.05$).
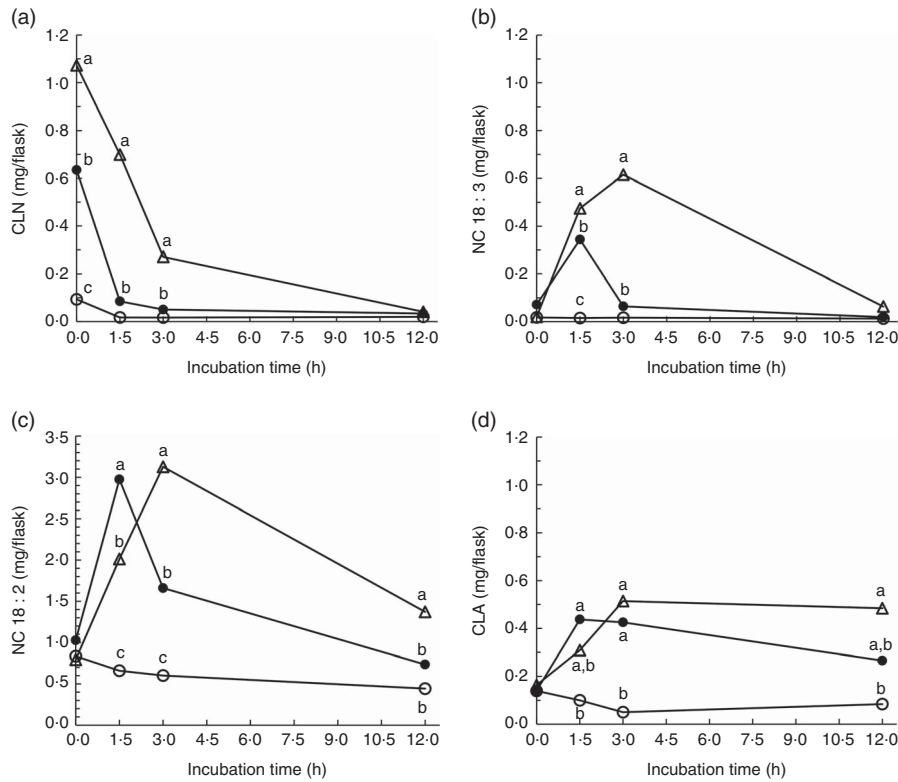
Fig. 2. Formation of (a) total conjugated linolenic acid (CLN), (b) non-conjugated 18 : 3 (NC 18 : 3), (c) non-conjugated 18 : 2 (NC 18 : 2) and (d) conjugated linoleic acid (CLA) during 0 to 12 h incubations of ground hay with strained rumen fluid diluted in de-ionised water (○), rumen fluid diluted in de-ionised water and 5 mg of added *α*-linolenic acid (ALA) (●) or rumen fluid diluted in 56·6 ± 1·33 % moles per cent excess deuterium oxide and 5 mg of added ALA (△). Rumen contents were collected from four cows and incubated at 39°C under carbon dioxide. Each point represents the mean of *n* 12 measurements (SEM 0·026, 0·020, 0·098 and 0·043 mg/flask for total CLN, NC 18 : 3, NC 18 : 2 and CLA, respectively). [a,b,c] Mean values for each incubation time with unlike letters were significantly different ($P < 0.05$).

indicated that geometric isomers of Δ9,11,15-CLN contained a single ^{2}H label. Independent analysis of the enrichment in water allowed for the comparison of the ratio of moles per cent excess (MPE) in FAME with the MPE of water. All samples of water had a similar enrichment of 56·6 (SE 1·33) %. Calculated labelling (MPE intermediate/MPE water) averaged 0·79 and 0·83 for *cis*-9,*trans*-11,*cis*-15-18 : 3 and *trans*-9,*trans*-11,*cis*-15-CLN, respectively (Table 2). The mass spectrum of the DMOX derivative of *cis*-9,*trans*-11,*cis*-15-CLN formed during incubations of ALA with D$_2$O containing buffer indicated enrichment of ion fragments from the molecular ion to m/z 262 (Fig. 3). The occurrence of ion fragment isotopomers with $m/z < 262$ was comparable with the natural abundance of 22·2 %. Analysis of ion fragments located the incorporation of ^{2}H on C-13 of the FA moiety. The mass spectrum of *trans*-9,*trans*-11,*cis*-15-CLN indicated a similar pattern of enrichment with labelling on C-13 (data not presented).

Labelling (MPE sample/MPE water) of $n+1$ isotopomers of *trans*-9,*trans*-11,*cis*-13-CLN increased ($P < 0.001$) during 12 h incubations with ALA (Table 2). Abundance of $n+1$ isotopomers of *trans*-9,*trans*-11,*cis*-13-CLN formed during 1·5, 3 and 12 h of incubation of ALA with D$_2$O was higher ($P < 0.001$) compared with natural abundance (average 35·7 and 21·8 %, respectively). Enrichment of $n+1$ isotopomers of *cis*-7,*cis*-12, *cis*-15-18 : 3, *cis*-8,*cis*-12,*cis*-15-18 : 3 and *trans*-8,*cis*-12,*cis*-15-18 : 3 formed after 1·5 and 3 h of incubation with ALA averaged

0·01, −0·19 and 0·01, respectively (Table 2). Relative abundance of $n+1$ isotopomers for Δ7,12,15-18 : 3 and Δ8,12,15-18 : 3 formed in the presence of D$_2$O did not differ ($P > 0.05$) from natural enrichment. Furthermore, the mass spectra of the DMOX derivatives of *cis*-7,*cis*-12,*cis*-15-18 : 3 (online Supplementary Fig. S1) and *cis*-8,*cis*-12,*cis*-15-18 : 3 (online Supplementary Fig. S2) provided no evidence of ^{2}H labelling.

The Δ10,12,15-CLN isomer eluted immediately after *cis*-9, *trans*-11,*cis*-15-CLN during GC-MS analysis. It was not possible to obtain reliable mass spectra for the methyl ester or DMOX derivative of Δ10,12,15-CLN formed during 0 to 12 h incubations of ALA with D$_2$O. Calculated labelling at $n+1$ of Δ10,12,15-CLN formed after 1·5 and 3 h incubations averaged 0·28 (Table 2). Relative abundance of $n+1$ isotopomers of Δ10,12,15-CLN was higher compared with natural enrichment ($P < 0.05$), but the position of the ^{2}H label could not be located. Concentrations of 10,12,15-CLN were too low to allow the MPE at $n+2$ to be estimated accurately.

Formation of octadecadienoic acids during incubation of α-linolenic acid with strained rumen contents

Cis-12,*cis*-15-18 : 2 and *trans*-11,*cis*-15-18 : 2 represented the major non-conjugated 18 : 2 products formed during incubations with ALA, with smaller amounts of *trans*-10,*cis*-15-18 : 2

Table 1. Amounts of 18:3 intermediates formed during 0 to 12 h incubations of α-linolenic acid with rumen contents diluted in buffer prepared using de-ionised H_2O or deuterium oxide (D_2O)*
(Mean values with their pooled standard errors)

Treatments…	0 h CON	0 h H$_2$O	0 h D$_2$O	1·5 h CON	1·5 h H$_2$O	1·5 h D$_2$O	3 h CON	3 h H$_2$O	3 h D$_2$O	12 h CON	12 h H$_2$O	12 h D$_2$O	SEM‡	P† Time	P† Treatment	P† Time × treatment
Amount (μg/flask)																
cis-9,trans-11,cis-15	83·1[c]	612[b]	1040[a]	4·21[b]	32·8[b]	648[a]	4·29[c]	17·5[a,b]	188[a]	4·48	7·66	15·2	25·67	<0·001	<0·001	<0·001
trans-9,trans-11,cis-15	1·31	7·38	6·33	1·23[b]	15·5[a]	24·0[a]	0·85[b]	4·47[b]	36·3[a]	2·62	1·69	2·66	2·697	0·003	0·002	<0·001
Δ10,12,15	3·20[b]	6·32[a]	5·51[a,b]	4·47[b]	9·04[a]	7·04[a,b]	5·89	8·02	7·81	8·73[a]	9·18[a]	4·19[b]	0·661	0·006	0·006	<0·001
trans-9,trans-11,cis-13	8·22[b]	15·7[a,b]	23·3[a]	7·96[b]	23·9[a]	27·2[a]	6·56[c]	19·6[b]	37·4[a]	5·26[b]	14·7[a,b]	21·6[a]	2·220	0·010	<0·001	0·010
cis-7,cis-12,cis-15	1·76	24·1	2·02	3·03[c]	99·5[b]	155[a]	5·05[b]	19·2[b]	185[a]	3·43	3·44	6·08	6·770	<0·001	<0·001	<0·001
cis-8,cis-12,cis-15	5·68[a]	28·4[a]	4·68[a]	3·95[b]	174[a]	193[a]	3·32[b]	25·8[b]	257[a]	1·75	5·76	42·1	14·56	<0·001	<0·001	<0·001
trans-8,cis-12,cis-15	10·5[a]	19·3[a]	11·6[a]	8·36[b]	70·5[a]	60·4[a]	8·26[b]	19·2[b]	78·8[a]	7·62	10·0	9·86	2·891	<0·001	<0·001	<0·001

[a,b,c] Mean values within a row for each incubation time with unlike superscript letters were significantly different ($P < 0.05$).

* Incubations established in 100-ml flasks containing 400 ml ground dried hay and 50 ml of diluted rumen fluid maintained at 39°C under carbon dioxide containing no additional α-linolenic acid (CON) or 5 mg of added α-linolenic acid and rumen fluid diluted with de-ionised H_2O or D_2O.
† Significance due to incubation time, incubation treatment and their interaction.
‡ Pooled SEM for n 36 measurements.

Table 2. Enrichment of $n+1$, $n+2$ and $n+3$ isotopomers of 18:3 intermediates formed during incubations of α-linolenic acid (ALA) with strained rumen contents diluted in ^2H-containing buffer*
(Mean values with their pooled standard errors)

Incubation time (h)…	n+1 0	n+1 1·5	n+1 3	n+1 12	n+1 SEM‡	n+2 0	n+2 1·5	n+2 3	n+2 12	n+2 SEM‡	P† n+1	P† n+2
Fatty acid												
cis-9,trans-11,cis-15-18:3	0·92[a]	0·82[b]	0·79[b]	0·62[c]	0·010	−0·05[b]	−0·03[b]	0·01[a]	0·02[a]	0·019	<0·001	<0·001
trans-9,trans-11,cis-15-18:3	0·92[a]	0·81[b]	0·82[b]	0·76[b]	0·035	−0·06	0·05	0·13	0·04	0·054	0·009	0·051
Δ10,12,15-18:3	ND	0·32[a]	0·23[b]	ND	0·014	ND	ND	ND	ND	ND	0·003	–
trans-9,trans-11,cis-13-18:3	0·10[d]	0·14[c]	0·20[b]	0·34[a]	0·009	−0·09[b]	−0·09[b]	−0·08[b]	−0·05[a]	0·007	<0·001	<0·001
cis-7,cis-12,cis-15-18:3	ND	−0·02[b]	0·03	ND	0·023	ND	−0·04	−0·07	ND	0·035	0·180	0·235
cis-8,cis-12,cis-15-18:3	ND	−0·18	−0·19	ND	0·022	ND	0·06	0·06	ND	0·014	0·450	0·645
trans-8,cis-12,cis-15-18:3	0·03	−0·02[b]	0·03	0·14[a]	0·030	0·03	−0·02	−0·04	0·02	0·023	0·009	0·099

[a,b,c] Mean values within a row for each incubation time with unlike superscript letters were significantly different ($P < 0.05$).

* Enrichment calculated from the ratio of moles per cent excess (MPE) in the incubation product/MPE in water. Mean 56·6 (SE 1·33) % MPE enrichment in deuterated water. Natural abundance of $n+1$ and $n+2$ isotopomers was determined for the same products formed during incubations of ALA with strained rumen contents diluted in de-ionised water.
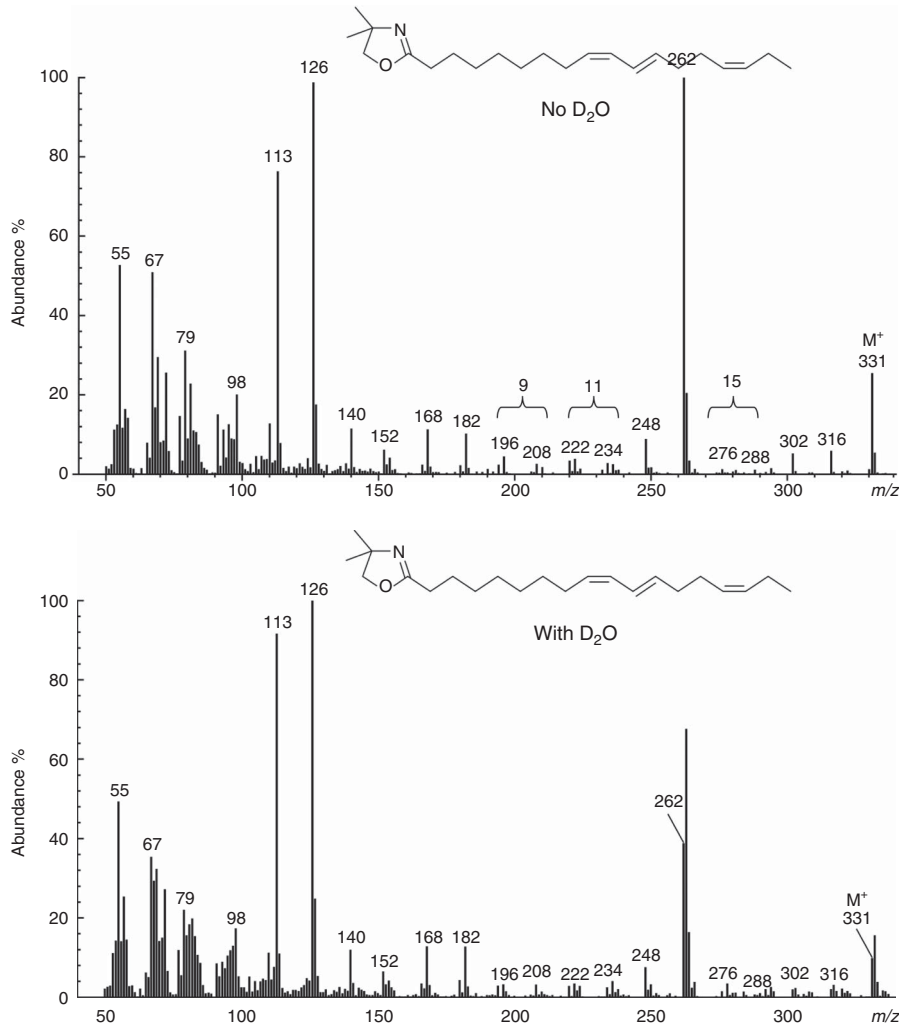† Significance due to incubation time.
‡ Pooled SEM for n 12 measurements.

Fig. 3. Mass spectrum of the 4,4-dimethyloxazoline derivative of *cis*-9,*trans*-11,*cis*-15-18 : 3 formed from α-linolenic acid during incubations with strained rumen contents diluted with buffer prepared using de-ionised water or deuterium oxide. Gaps of 12 atomic mass units between *m/z* 196 and 208, 222 and 234 and 276 and 288 located double bonds at Δ9, 11 and 15, respectively. Enrichment of ion fragments at *m/z* 262 and 263 indicate labelling on C-13 of the fatty acid moiety during incubations with [2]H-containing buffer.

also detected (Table 3). Incubation of ALA with rumen contents diluted with water increased ($P < 0.05$) the amount of *cis*-12, *trans*-14-CLA, *trans*-11,*cis*-13-CLA, *trans*-11,*trans*-13-CLA, *trans*-12,*cis*-14-CLA, *trans*-12,*trans*-14-CLA and *trans*-13,*trans*-15-CLA in flask contents (Table 3). However, the amounts of CLA products formed differed during incubations of ALA with D_2O or water. *Trans*-10,*cis*-12-CLA and *trans*-10,*trans*-12-CLA were detected in all flask contents but the amount of *trans*-10, *trans*-12-CLA did not change ($P = 0.47$) over the course of all incubations (Table 3). The amount of *trans*-10,*cis*-12-CLA in samples containing added ALA with water or D_2O decreased ($P < 0.05$) during incubation from 3 to 12 h.

Mass spectra of methyl esters were used to calculate the MPE ratios of $n+1$, $n+2$ and $n+3$ isotopomers of 18 : 2 products formed during incubations with added ALA (Table 4). Enrichment of $n+1$ isotopomers of *cis*-12,*cis*-15-18 : 2, *trans*-10,*cis*-15-18 : 2, *trans*-11,*cis*-15-18 : 2, *trans*-11,*cis*-13-CLA, *trans*-11,*trans*-13-CLA and *cis*-9,*trans*-11-CLA from 1·5 to 12 h incubations averaged 1·13, 1·03, 1·01, 0·89, 0·83 and 0·18,

respectively (Table 4). Labelling at $n+2$ for the same intermediates averaged 0·99, 0·75, 0·74, 0·63, 0·46 and −0·01, respectively. Average enrichment of $n+3$ isotopomers was 0·43, 0·17, 0·13, 0·05, 0·16 and <0·01, respectively. For 3 to 12 h incubations, enrichment of $n+1$, $n+2$ and $n+3$ isotopomers of *trans*-12,*trans*-14-CLA averaged 1·0, 0·91 and 0·52, respectively.

The molecular ion of the DMOX derivative of *trans*-11,*cis*-15-18 : 2 formed during incubations of ALA with D_2O at *m/z* 335 ($n+2$) confirmed the incorporation of two ^{2}H labels in the FA moiety (Fig. 4). Relative abundances of ion fragments at *m/z* 210 and 211 and at *m/z* 264, 265 and 266 located the incorporation of a single ^{2}H on C-9 and another on C-13. The mass spectrum of the DMOX derivative of *cis*-12,*cis*-15-18 : 2 formed in the presence of D_2O indicated a molecular ion at *m/z* 336 ($n+3$), confirming an octadecadienoic acid structure and incorporation of three ^{2}H atoms, but the position of each label could not be determined accurately (online Supplementary Fig. S3). Furthermore, the amounts of *trans*-10,*cis*-15-18 : 2 in

Table 3. Amounts of 18:2 intermediates formed during 0 to 12 h incubations of α-linolenic acid with rumen contents diluted in buffer prepared using de-ionised H_2O or deuterium oxide (D_2O)*
(Mean values with their standard errors)

| Treatments... | Incubation time (h) | | | | | | | | | | | | SEM‡ | P† | | |
| | 0 h | | | 1·5 h | | | 3 h | | | 12 h | | | | Time | Treatment | Time × treatment |
	CON	H_2O	D_2O	CON	H_2O	D_2O	CON	H_2O	D_2O	CON	H_2O	D_2O				
Amount (µg/flask)																
cis-12,cis-15	9·42	21·9	3·50	18·5^b	1300^a	233^b	10·6^b	507^a	706^a	7·96	145	244	45·60	<0·001	<0·001	<0·001
trans-10,cis-15	0·00	0·00	0·00	0·00^a	383^b	0·00^a	0·00^b	174^a	21·6^b	0·00	51·9	8·13	18·93	<0·001	0·064	0·005
trans-11,cis-15	97·4	192	65·2	75·5^b	835^a	951^a	66·8^c	412^b	1450^a	53·3^c	150^b	527^a	33·67	<0·001	<0·001	<0·001
cis-9,trans-11	78·3^a	54·5^b	66·1^{a,b}	19·5^b	19·4^b	54·9^a	16·9^b	15·9^b	37·3^a	12·7	11·0	10·2	3·261	<0·001	0·005	<0·001
cis-11,trans-13	1·37	1·16	1·72	0·50^b	1·61^b	58·8^a	0·00^b	1·07^b	81·2^a	0·00^b	0·38^{a,b}	7·21^a	1·170	<0·001	<0·001	<0·001
cis-12,trans-14	0·40	0·55	0·31	1·68^b	26·6^a	8·98^b	0·56^b	9·52^b	34·5^a	1·23	2·99	8·80	2·805	0·002	0·008	<0·001
trans-10,cis-12	24·5	33·2	36·7	16·3^b	22·1^{a,b}	40·9^a	6·02^b	14·9^{a,b}	32·6^a	11·9	6·75	12·2	4·507	0·003	0·011	0·072
trans-11,cis-13	12·1	13·9	5·53	14·6^c	37·5^b	61·1^a	6·49^c	24·0^b	85·6^a	14·3	13·7	12·7	3·409	<0·001	<0·001	<0·001
trans-12,cis-14	1·24	1·18	0·62	3·88	47·4	1·54	1·30^b	118^a	11·1^b	2·35^b	51·8^{a,b}	96·8^a	16·46	0·035	0·029	0·009
trans-10,trans-12	4·23	5·50	6·10	5·62	7·19	8·14	2·93	5·82	9·96	5·83	5·39	6·51	1·368	0·468	0·064	0·333
trans-11,trans-13	7·40	19·1	6·35	19·1^b	223^a	55·9^b	7·68^b	132^{a,b}	209^a	16·3^b	90·3^b	244^a	24·37	0·005	0·004	<0·001
trans-12,trans-14	2·47	3·77	1·70	10·3	42·6	4·00	3·49^b	89·3^a	20·9^b	5·51^b	68·2^a	101^a	7·765	<0·001	0·004	<0·001
trans-13,trans-15	1·46	3·76	0·78	2·42	3·05	0·86	1·90^b	8·15^a	1·05^b	8·87^{a,b}	12·4^a	3·54^b	1·056	0·001	0·006	0·052

^{a,b,c} Mean values within a row for each incubation time with unlike superscript letters were significantly different (P<0·05).
* Incubations established in 100-ml flasks containing 400 ml ground dried hay and 50 ml of diluted rumen fluid maintained at 39°C under carbon dioxide containing no additional α-linolenic acid (CON) or 5 mg of added α-linolenic acid and rumen fluid with de-ionised H_2O or D_2O.
† Significance due to incubation time, incubation treatment and their interaction.
‡ SEM for n 36 measurements.

Table 4. Enrichment of $n+1$, $n+2$ and $n+3$ isotopomers of 18:2 intermediates formed during incubations of α-linolenic acid (ALA) with strained rumen contents and ^2H-containing buffer*
(Mean values with their pooled standard errors)

| Incubation time (h)... | Isotopomers | | | | | | | | | | | | | | | P† | | |
| | $n+1$ | | | | | $n+2$ | | | | | $n+3$ | | | | | | | |
	0	1·5	3	12	SEM‡	0	1·5	3	12	SEM‡	0	1·5	3	12	SEM‡	$n+1$	$n+2$	$n+3$
Fatty acid																		
cis-12,cis-15-18:2	−0·05^b	1·12^a	1·13^a	1·14^a	0·024	0·05^b	0·96^a	1·01^a	1·00^a	0·012	0·05^b	0·38^a	0·39^a	0·53^a	0·066	<0·001	<0·001	<0·001
trans-10,cis-15-18:2	ND	ND	1·00	1·06	0·057	ND	ND	0·70^b	0·80^a	0·045	ND	ND	0·15	0·19	0·070	0·234	0·042	0·474
trans-11,cis-15-18:2	0·49^b	1·04^a	1·02^a	0·97^a	0·022	0·32^c	0·78^a	0·75^a	0·68^b	0·017	0·05	0·12	0·12	0·15	0·038	<0·001	<0·001	0·165
cis-9,trans-11-18:2	0·33^a	0·20^b	0·26^b	0·07^c	0·021	−0·01	−0·01	−0·00	−0·01	0·007	−0·01	−0·01	−0·01	−0·01	0·002	<0·001	0·327	0·818
trans-10,cis-12-18:2	−0·02^c	0·04^b	0·13^a	0·05^b	0·018	0·01^a	−0·05^b	−0·08^b	−0·06^b	0·011	ND	ND	ND	ND	ND	<0·001	<0·001	–
trans-11,cis-13-18:2	0·04^c	0·99^a	1·00^a	0·69^b	0·020	0·01^c	0·72^a	0·72^a	0·47^b	0·024	0·01	−0·02	0·06	0·10	0·056	<0·001	<0·001	0·259
trans-11,trans-13-18:2	0·26^c	0·70^b	0·86^a	0·93^a	0·035	0·05^d	0·33^c	0·45^b	0·62^a	0·023	−0·00^c	0·08^b	0·09^b	0·30^a	0·022	<0·001	<0·001	<0·001
trans-12,trans-14-18:2	0·27^c	0·55^b	0·92^a	1·08^a	0·057	0·00^d	0·16^c	0·79^b	1·03^a	0·030	0·01^c	0·03^c	0·42^b	0·61^a	0·016	<0·001	<0·001	<0·001

^{a,b,c} Mean values within a row for each incubation time with unlike superscript letters were significantly different (P<0·05).
* Enrichment calculated from the ratio of moles per cent excess (MPE) in the incubation product/MPE in water. Mean 56·6 (SE 1·33) % MPE enrichment in deuterated water. Natural abundance of $n+1$, $n+2$ and $n+3$ isotopomers was determined for the same products formed during incubations of ALA with strained rumen contents diluted in de-ionised water.
† Significance due to incubation time.
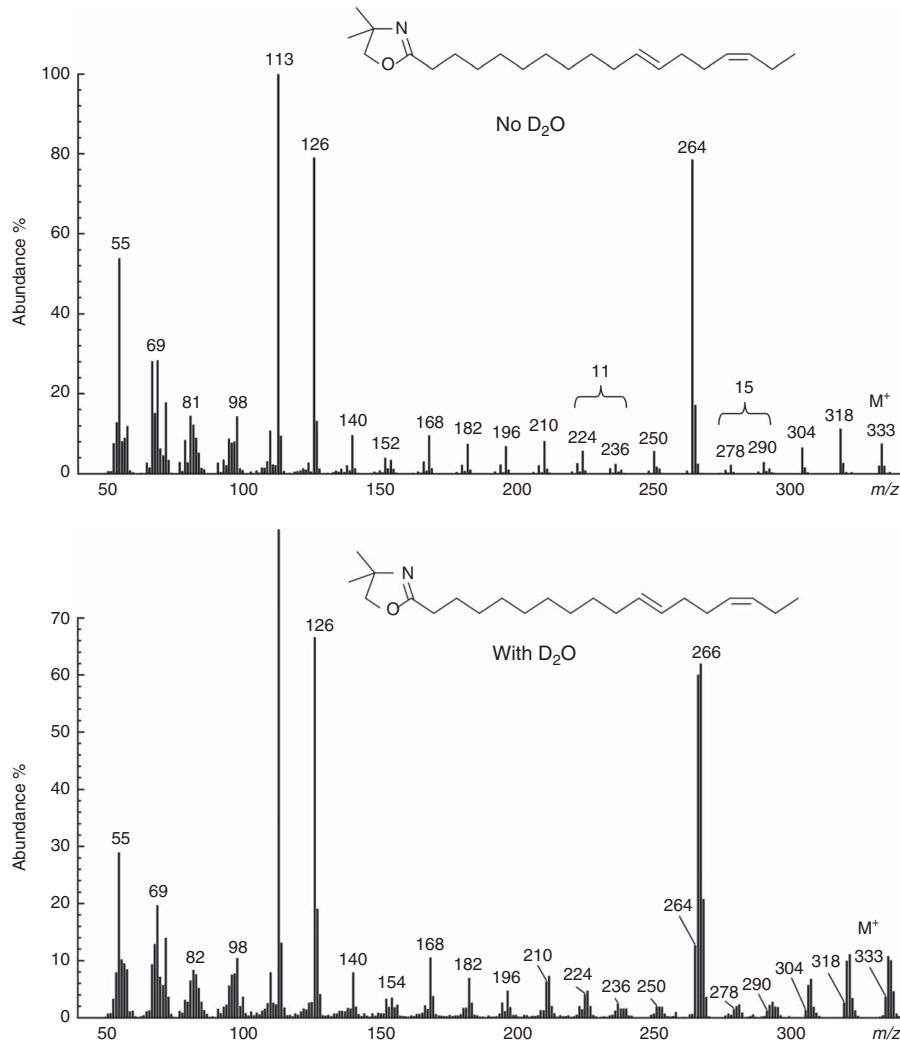‡ Pooled SEM for n 12 measurements.

Fig. 4. Mass spectrum of the 4,4-dimethyloxazoline derivative of *trans*-11,*cis*-15-18 : 2 formed from α-linolenic acid during incubations with strained rumen contents diluted with buffer prepared using de-ionised water or deuterium oxide. An abundant ion at m/z 264 along with gaps of 12 atomic mass units between m/z 224 and 236 and 278 and 290 confirmed a Δ11,15 double-bond arrangement. Enrichment of ion fragments at m/z 210 and 211 ($n+1$) and 264 and 266 ($n+2$) indicated labelling on C-9 and C-13 of the fatty acid moiety during incubations with ^{2}H-containing buffer.

flask contents were too low to obtain reliable MS spectra. Mass spectra of the methyl esters of *trans*-11,*cis*-13-CLA and *trans*-11, *trans*-13-CLA revealed a molecular ion at m/z 296 ($n+2$), indicating that both intermediates formed from ALA during incubations with D_2O contained two ^{2}H labels. The mass spectrum of the DMOX derivative of *trans*-11,*trans*-13-CLA (Fig. 5) indicated enrichment in ion fragments at m/z 334 and 335 ($n+1$ and $n+2$, respectively), confirming the incorporation of two or more ^{2}H atoms. An increase in ion fragment isotopes at m/z 211 ($n+1$) and m/z 306 ($n+2$) suggested labelling at C-9 and C-16, respectively. However, during the course of incubations with D_2O, the abundance of the ion fragment at m/z 336 ($n+3$) increased, which along with enrichment of $n+2$ isotopomers with $m/z < 306$ suggested that another ^{2}H label was incorporated between C-9 and C-16. The mass spectrum of the DMOX derivative of *trans*-12,*trans*-14-CLA revealed a molecular ion at m/z 336 (data not presented), indicating incorporation of three ^{2}H atoms, but the locations could not be established.

Formation of octadecenoic acids, oxygenated fatty acids and stearic acid during incubation of α-linolenic acid with strained rumen contents

A mixture of *trans*-4 to *trans*-16-18 : 1 and *cis*-9 to *cis*-16-18 : 1 accumulated during incubations with ALA. *Trans*-11,-13,-14,-15 and -16-18 : 1 and *cis*-15-18 : 1 represented the most abundant octadecenoic intermediates. Owing to extensive labelling during incubations with D_2O, none of the 18 : 1 isomers could be resolved during GC analysis, preventing the amounts synthesised and enrichment patterns to be accurately determined. Nevertheless, the mass spectrum of the DMOX derivative of *trans*-11-18 : 1 indicated the incorporation of ^{2}H atoms on C-9, C-13 and C-15 of the FA moiety (data not presented).

Incubations with ALA also resulted in the formation of 9-O-18 : 0, 10-O-18 : 0 and 13-O-18 : 0, none of which was labelled after 12 h (data not presented). For all incubations, 18 : 0 was the end product containing multiple ^{2}H labels.
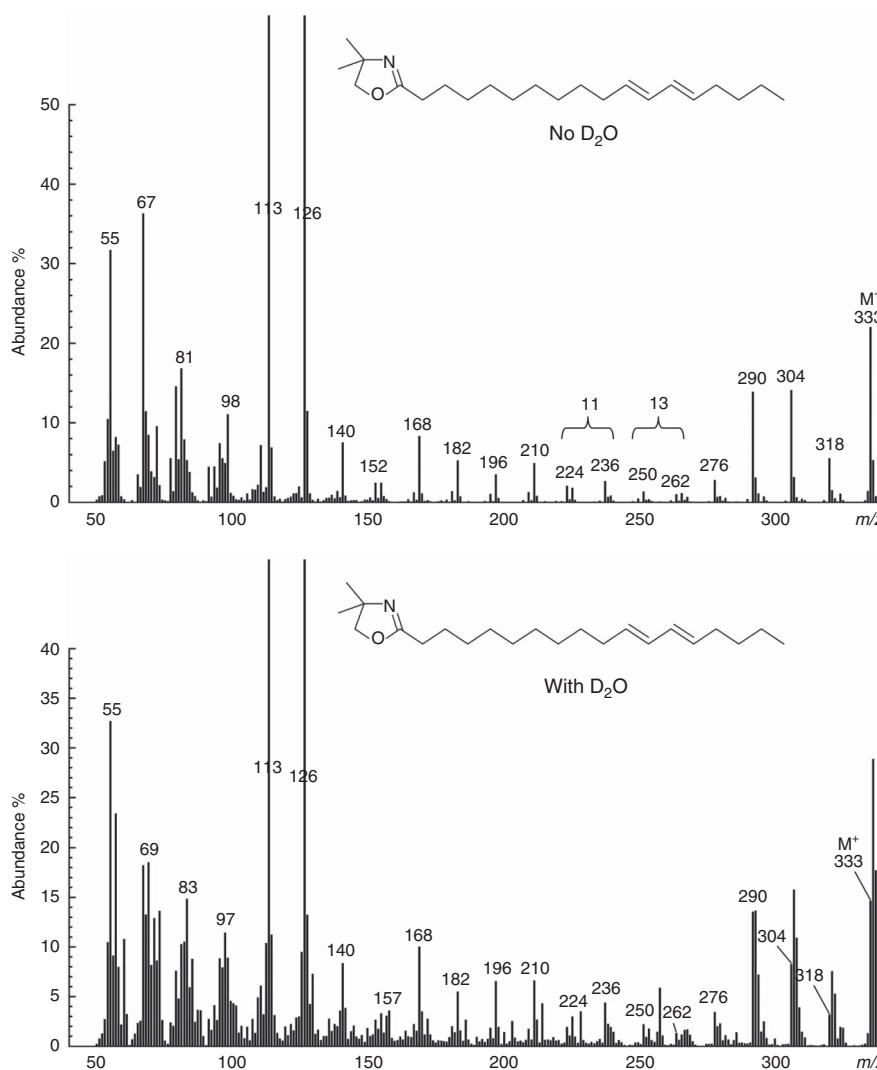
Fig. 5. Mass spectrum of the 4,4-dimethyloxazoline derivative of *trans*-11,*trans*-13-18 : 2 from *α*-linolenic acid during incubations with strained rumen contents diluted with buffer prepared using de-ionised water or deuterium oxide. Gaps of 12 atomic mass units between m/z 224 and 236 and 250 and 262 located double bonds at Δ11 and 13, respectively. Enrichment of ion fragments at m/z 210 and 211 ($n+1$) and 304 and 306 ($n+2$) provide tentative evidence of labelling on C-9 and C-16 of the fatty acid moiety during incubations with ^{2}H-containing buffer.

The molecular ion of the DMOX derivative of 18 : 0 at m/z 342 (337 + 5) indicated that up to five ^{2}H atoms were incorporated during the reduction of ALA to 18 : 0, but the locations could not be determined.

Discussion

Most reports based on incubations with ruminal digesta[23] or pure cultures of rumen bacteria[38] indicate that biohydrogenation of ALA is initiated by isomerisation of the *cis*-12 double bond to yield a *cis*-9,*trans*-11,*cis*-15-CLN intermediate catalysed by a 12-*cis*,11-*trans* isomerase[26]. Alternative pathways of ALA biohydrogenation have been proposed[9,23–25], but the mechanisms and products formed are not known. In the present investigation, incubations of ALA with ruminal contents were made with or without D$_2$O to better understand the fate of ALA in the rumen. Control incubations

were also performed, allowing identification of products formed from ALA during incubations with strained rumen contents. The non-ionic surfactant Tween 80 was used to disperse ALA that has been shown to result in more extensive biohydrogenation during incubations with rumen contents compared with sonication or dissolving substrates in ethanol[39]. Incubations with rumen contents diluted with water or D$_2$O resulted in the appearance of similar products, but the rate of added ALA disappearance and reaction kinetics were slower in the presence of the ^{2}H isotope.

Cis-9,*trans*-11,*cis*-15-CLN was the major product synthesised from ALA, but *trans*-9,*trans*-11,*cis*-15-CLN was also detected. Both Δ9,11,15-CLN products formed during incubations of ALA with D$_2$O were labelled on C-13. Enrichment of the $n+1$ isotopomer of *cis*-9,*trans*-11,*cis*-15-CLN was greatest immediately after the addition of ALA. Incorporation of the ^{2}H label in *trans*-9,*trans*-11,*cis*-15-CLN was consistent across all incubation times. Labelling of geometric 9,11,15-CLN isomers is analogous

to the incorporation of ^{2}H in 9,11-CLA formed during incubations of LA with D$_2$O and pure cultures of *Butyrivibrio fibrisolvens*[15,38,40], *Bifidobacterium breve* and *Propionibacterium freudenreichii* subsp. *shermanii* DSM 4902T[41], ovine rumen contents[15] or human mixed faecal bacteria[41]. The appearance of a single label on C-13 of 9,11-CLA isomers was explained by H abstraction on C-11, which for reasons of thermodynamic stability was followed by re-arrangement of the double bond and the assimilation of a proton from water[15,38,41]. Abstraction of a single H at C-11 and formation of a radical intermediate would also explain an increase in $n+1$ isotopomers and the labelling pattern of 9,11,15-CLN isomers formed from ALA. The *cis*-9,*trans*-11,*cis*-15-CLN isomer was formed in much higher amounts than *trans*-9,*trans*-11,*cis*-15-CLN, which may be related to possible differences in the thermodynamics of these reactions.

A common mechanism for the synthesis of Δ9,11-CLA from LA and Δ9,11,15-CLN from ALA during incubations with mixed rumen microbiota is not unexpected, given that the same 12-*cis*,11-*trans* isomerase isolated from *B. fibrisolvens* is capable of both reactions[40]. Functional studies of the Δ^{12}-*cis*-Δ^{11}-*trans*-isomerase isolated from *B. fibrisolvens*[38] suggest that the reaction is initiated by π electrons of C-9 double bond interacting with the hydrophobic binding site of the enzyme[40]. Other bacteria are capable of converting ALA to CLN isomers *in vitro*. *Lactobacillus plantarum* AKU 1009a catalyses the synthesis of *cis*-9,*trans*-11,*cis*-15-CLN and *trans*-9,*trans*-11,*cis*-15-CLN from ALA[42,43]. Geometric isomers of 9,11,15-CLN are also formed during incubations of ALA with strains of *Bifidobacterium*[44–46] and *Propionibacterium*[46]. It remains unclear as to whether the mechanisms of 9,11,15-CLN synthesis from ALA are common to both food-producing bacterial strains and ruminal bacteria.

Incubations of ALA with rumen contents resulted in the formation of *trans*-9,*trans*-11,*cis*-13-CLN. Enrichment of the $n+1$ isotopomer of *trans*-9,*trans*-11,*cis*-13-CLN increased over the course of 12-h incubations, but was consistently lower compared with incorporation of ^{2}H in Δ9,11,15-CLN products (average 0·20 and 0·81, respectively). However, the labelling pattern of *trans*-9,*trans*-11,*cis*-13-CLN was inconclusive. Nevertheless, an increase in the abundance of the $n+1$ isotopomer above natural enrichment suggests that the conversion of *trans*-9,*trans*-11,*cis*-13-CLN from ALA involves an exchange of H with water, by a mechanism that apparently differs from Δ9,11,15-CLN formation. The isomerase isolated from *Propionibacterium acnes* is capable of converting ALA to CLN isomers with *trans*-11,*trans*-13,*cis*-15-CLN as the main product with trace amounts of *trans*-10,*cis*-12,*cis*-15-CLN also being formed[47]. Incubation of ALA with rumen contents resulted in the formation of Δ10,12,15-CLN but in amounts too low to conclude about the possible mechanisms responsible.

Incubations of ALA with rumen contents also resulted in the formation of *cis*-7,*cis*-12,*cis*-15-18 : 3 and Δ8,12,15-18 : 3 isomers. Assignment of double-bond geometry of these products based on GC retention times would require further validation based on NMR or reductive ozonolysis. In earlier investigations, complementary Ag$^+$-TLC and GC-MS analysis identified *cis*-5,*cis*-12-18 : 2, *cis*-6,*cis*-12-18 : 2, *cis*-7,*cis*-12-18 : 2,

cis-8,*cis*-12-18 : 2 and *trans*-8,*cis*-12-18 : 2 as intermediates of LA metabolism during incubations with ruminal digesta[16]. The appearance of the 18 : 3 intermediates containing a *cis*-12 and *cis*-15 double-bond arrangement provides the first evidence that transformation of ALA may also involve migration of the *cis*-9 double bond. Enrichment in $n+1$ isotopomers of Δ7,12,15-18 : 3 and Δ8,12,15-18 : 3 during incubations of ALA with D$_2$O did not differ from natural enrichment, indicating that formation of these products does not involve H exchange with water. *Cis-trans* isomerisation of doubly deuterated *cis*-9-18 : 1 to *trans*-9-18 : 1 does not result in the loss of ^{2}H[48]. Thus, an absence of labelling confirms that formation of Δ7,12,15-18 : 3 and Δ8,12,15-18 : 3 from ALA does not involve prior formation of Δ9,11,13-CLN or Δ9,11,15-CLN. It is not possible to conclude on whether double-bond migration is catalysed by a *cis-trans* isomerase or via an alternative series of reactions, however. Hydrogenation of LA with iridium, palladium and ruthenium catalysts is known to generate *trans*-8,*cis*-12-18 : 2 and *cis*-9,*trans*-13-18 : 2 with *cis*-8,*cis*-12-18 : 2 and *cis*-9,*cis*-13-18 : 2 predicted as minor products[49]. Under these circumstances, double-bond migration has been explained by the release of the H atom from the adjacent methylene group at Δ8 or Δ14 of the semi-hydrogenated C–C bond and subsequent rotation of the C–C bond during abstraction of the H atom[49]. Further investigations involving incubations of ALA with living and irradiated rumen contents would be required to establish whether a *cis-trans* isomerase or non-enzymatic reaction catalyses re-arrangement of the *cis*-9 double bond.

Trans-11,*cis*-15-18 : 2 was the major 18 : 2 product formed from ALA, consistent with the established pathway of ALA biohydrogenation[22–25,27]. The $n+2$ isotopomer of *trans*-11,*cis*-15-18 : 2 during incubations of ALA with D$_2$O were highly enriched after 1·5 h of incubation with single ^{2}H labels incorporated on C-9 and C-13. The labelling pattern is consistent with Δ11,15-18 : 2 intermediates being formed from Δ9,11,15-CLN labelled on C-13, with reduction of the *cis*-9 double bond involving H abstraction on C-10 and incorporation of a single H from water on C-9. Previous experiments have shown that the reduction of *cis*-9,*trans*-11-18 : 2 to *trans*-11-18 : 1 results in labelling on C-9 during incubations of LA and D$_2$O with ruminal digesta[15] or human intestinal bacteria[41].

Under routine GC analysis with a polar 100-m capillary column, *trans*-10,*cis*-15-18 : 2 and *trans*-11,*cis*-15-18 : 2 elute as a single peak that can only be separated using a 100-m GC column with an ionic liquid stationary phase[32] or Ag$^+$ solid-phase extraction and semi-preparative HPLC[50]. Re-analysis of FAME in the present study using the SLB-IL111 column provided confirmation that *trans*-10,*cis*-15-18 : 2 is formed from ALA. An increase in the $n+2$ isotopomer indicates that transformation of ALA to *trans*-10,*cis*-15-18 : 2 by rumen microbiota involves exchange of two H ions from water. It has been suggested that *trans*-10,*cis*-15-18 : 2 is a product formed from the reduction of *trans*-10,*cis*-12,*cis*-15-CLN in the rumen[25,32]. Even though enrichment of $n+1$ isotopomers of Δ10,12,15-CLN was detected during incubations of ALA with D$_2$O, the labelling pattern of the *trans*-10,*cis*-15-18 : 2 product was inconclusive. *L. plantarum* AKU 1009a is capable of

converting ALA to *trans*-10,*cis*-15-18:2 via the formation of *cis*-9,*trans*-11,*cis*-15-18:3 and *trans*-9,*trans*-11,*cis*-15-18:3 as intermediates[43]. It is not clear whether the same transformation also occurs in the rumen.

Incubations of ALA with rumen contents also resulted in the formation of *cis*-12,*cis*-15-18:2. The $n+1$, $n+2$ and $n+3$ isotopomers of *cis*-12,*cis*-15-18:2 were progressively enriched during incubations of ALA with D_2O, but the position of ^2H labels could not be located. It is possible that *cis*-12,*cis*-15-18:2 originates from the reduction of *cis*-7,*cis*-12,*cis*-15-18:3 or Δ8,12,15-18:3 or from the direct reduction of ALA. However, the increase in the $n+3$ isotopomer of *cis*-12,*cis*-15-18:2 suggests that an alternative mechanism may be responsible. All incubations contained LA, but the amounts did not increase following ALA addition. No enrichment in $n+1$, $n+2$ or $n+3$ isotopomers was detected over the course of incubations with D_2O, indicating that ALA is not converted to LA by rumen microbiota.

The major pathways of ALA biohydrogenation do not consider isomers of CLA as intermediates. In the present investigation, small amounts of *trans*-11,*cis*-13-CLA, *trans*-11,*trans*-13-CLA and *trans*-12,*trans*-14-CLA were formed from ALA. Enrichment of $n+2$ isotopomers indicates that formation of Δ11,13-CLA involves an exchange of H with water. The mass spectrum of the DMOX derivative was difficult to interpret but provided some indications of labelling on C-9 and C-16. Over the course of 12-h incubations, an increase in the enrichment of $n+3$ isotopomers was detected, indicating that the formation of *trans*-11,*trans*-13-CLA involved the incorporation of another ^2H label, but the location could not be established with a high degree of certainty. Earlier studies have shown that formation of *cis*-9,*trans*-11-CLA from LA results in labelling on C-13[15]. Further reduction of *cis*-9,*trans*-11-CLA to *trans*-11-18:1 results in the incorporation of ^2H on C-9[41]. Assuming that the same mechanisms are also involved in transforming CLN products to CLA, then labelling on C-9 would be expected if Δ11,13-CLA is formed by the reduction of Δ9,11,13-CLN. Given the uncertainties in the labelling pattern, it was not possible to confirm Δ9,11,15-CLN as a precursor for Δ11,13-CLA formation or recent reports that strains of *B. fibrisolvens* convert *trans*-11,*cis*-15-18:2 to *trans*-11,*cis*-13-CLA[51]. Much earlier investigations reported that *cis*-9,*trans*-11,*cis*-13-CLN is hydrogenated to *trans*-11-18:1, which in the presence of D_2O resulted in labelling at C-9, C-10, C-13 and C-14, whereas *cis*-9,*trans*-11,*trans*-13-CLN was not hydrogenated[52].

Mechanisms explaining the transformation of ALA to *trans*-12,*trans*-14-CLA are not resolved from the present investigation. An increase in the $n+3$ isotopomer of *trans*-12,*trans*-14-CLA was detected after 12 h of incubation with ALA, but the labelling pattern was not informative. A possible explanation is that *trans*-12,*trans*-14-CLA is formed from the isomerisation of *trans*-11,*cis*-15-18:2 or geometric isomers of 11,13-CLA by a mechanism that involves an exchange of H with water. Earlier investigations with ^{13}C-labelled ALA reported significant ^{13}C enrichment in Δ8,10-CLA, Δ9,11-CLA, Δ10,12-CLA and Δ11,13-CLA intermediates, suggesting that multiple CLA isomers are formed from ALA during incubations with rumen contents[26]. Earlier studies have reported formation of *trans*-11,*trans*-13-CLA and *cis*-11,*trans*-13-CLA from ALA[27]. In the present study, addition of ALA did not increase Δ8,10-CLA, Δ9,11-CLA or Δ10,12-CLA formation and or enrich *cis*-9,*trans*-11-18:2 at $n+2$. This would exclude ALA being transformed to CLA products other than geometric isomers of Δ11,13 and Δ12,14-CLA in the rumen.

Multiple 18:1 isomers accumulated during incubations of ALA with rumen contents. It was not possible to resolve all 18:1 isomers during GC and GC-MS analysis because of the broad peak shapes of isomers containing multiple ^2H labels. However, *cis*-12-18:1, *cis*-15-18:1, *trans*-11-18:1, *trans*-12-18:1, *trans*-13-18:1, *trans*-14-18:1, *trans*-15-18:1 and *trans*-16-18:1 were detected in flask contents during incubations of ALA with and without D_2O. The mass spectrum of the DMOX derivative of *trans*-11-18:1 indicated the incorporation of ^2H atoms on C-9, C-13 and C-15, consistent with this isomer originating from the sequential reduction of *cis*-9,*trans*-11,*cis*-15-CLN (labelled on C-13) and *trans*-11,*cis*-15-18:2 (labelled on C-9 and C-13), with the reduction of the *cis*-15 double bond being associated with the assimilation of ^2H on C-15. In all incubations, 18:0 was detected as the major end product of ALA biohydrogenation, which was found to contain up to five ^2H labels.

Overall, incubations of physiological amounts of ALA with strained rumen contents offer an explanation for the appearance of *cis*-9,*trans*-11,*trans*-15-CLN, Δ9,11,13-CLN, *trans*-11,*trans*-13-18:2, *trans*-12,*trans*-14-18:2, *trans*-10,*cis*-15-18:2, *trans*-11,*cis*-15-18:2 and *cis*-12,*cis*-15-18:2 in bovine muscle[7,53], adipose[50,53] and milk fat[8,54]. Data from this and earlier investigations indicate that the abundance of specific FA containing one or more *trans* double bonds can be expected to be higher in meat or milk from ruminants fed diets rich in ALA, but the implications on the health of human consumers are, however, uncertain.

Conclusions

Incubations of ALA with rumen contents with or without D_2O indicated that biohydrogenation proceeds via several distinct mechanisms leading to the formation of a diverse range of intermediates, many of which have not been characterised previously. Products formed by alternative metabolic pathways were quantitatively less important than the established intermediates of ALA biohydrogenation in the rumen. The complexity of the ruminal microbiota may account for a large part of the diversity of such reactions. Other than for the main routes of ALA metabolism, we have little knowledge of which microbial species might catalyse the different reactions.

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Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/doi:10.1017/S0007114516001446

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