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Identification of Enriched Conjugated Linoleic Acid Isomers in Cultures of Ruminal Microorganisms after Dosing with 1-^{13}C-Linoleic Acid

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Most studies of linoleic acid biohydrogenation propose that it converts to stearic acid through the production of *cis*-9 *trans*-11 CLA and *trans*-11 C18:1. However, several other CLA have been identified in ruminal contents, suggesting additional pathways may exist. To explore this possibility, this research investigated the linoleic acid biohydrogenation pathway to identify CLA isomers in cultures of ruminal microorganisms after dosing with a ^{13}C stable isotope. The ^{13}C enrichment was calculated as [(M+1/M)×100] in labeled minus unlabeled cultures. After 48 h incubation, significant ^{13}C enrichment was observed in seven CLA isomers, indicating their formation from linoleic acid. All enriched CLA isomers had double bonds in either the 9,11 or 10,12 position except for *trans*-9 *cis*-11 CLA. The *cis*-9 *trans*-11 CLA exhibited the highest enrichment (30.65%), followed by enrichments from 21.06 to 23.08% for *trans*-10 *cis*-12, *cis*-10 *trans*-12, *trans*-9 *trans*-11, and *trans*-10 *trans*-12 CLA. The remaining two CLA (*cis*-9 *cis*-11 and *cis*-10 *cis*-12 CLA) exhibited enrichments of 18.38 and 19.29%, respectively. The results of this study verified the formation of *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA isomers from linoleic acid biohydrogenation. An additional five CLA isomers also contained carbons originating from linoleic acid, indicating that pathways of linoleic acid biohydrogenation are more complex than previously described.

***Keywords*:** biohydrogenation, linoleic acid, conjugated linoleic acid, enrichment, stable isotope, ruminal microorganisms

Fatty acids with conjugated double bond arrangements have exhibited high biopotency compared to methylene-interrupted isomers. For instance, conjugated linoleic acid (CLA) was shown to modulate a multitude of biological functions affecting human health including lipid metabolism, carcinogenesis, immune function, obesity, diabetes, and atherogenesis (DeLany and West, 2000; Ryder *et al*., 2001; Pariza, 2004; Wahle *et al*., 2004). Particularly, the *cis*-9 *trans*-11 CLA isomer has been reported to function as an antioxidant and anticarcinogenic in animal models (Ip *et al*., 1999; Mosley *et al*., 2002). However, physiological functions appear to be isomer specific.

Conversion of linoleic acid to CLA is carried out naturally by anaerobic microorganisms that inhabit the gut of animals in a process called biohydrogenation. Biohydrogenation accomplishes the conversion of unsaturated fatty acids to stearic acid with accumulation of CLA and *trans* monoenes as intermediates. Biohydrogenation is particularly relevant in cattle and other ruminant species because it accounts for the high concentration of saturated fatty acids in meat and milk despite polyunsaturated fatty acids being the primary fatty acids consumed. Because of the large volume (up to 200 L) of anaerobic fermentation occurring in stomach compartments of cattle, the microbial population in the rumen provides an ideal ecosystem for studying biohydrogenation and the synthesis of CLA.

Biohydrogenation is initiated by the action of an isomerase that maintains the same number of double bonds but produces positional and geometric isomers including CLA. The interaction of the *cis*-9 electrons of linoleic acid substrate with the electronegative enzyme region acting as the hydrogen binding site to produce the *cis*-9 *trans*-11 CLA isomer has been proposed (Harfoot and Hazlewood, 1997). Also, the geometry of fatty acid binding to the isomerase and the involvement of specific amino acid residues in the formation of *trans*-10 *cis*-12 CLA by *Propionibacterium acnes* has been described (Liavonchanka *et al*., 2006). Despite detailed information being available on enzymatic action of the isomerase, little is known about the identity of CLA isomers produced from specific polyunsaturated fatty acid substrates. As many as 14 CLA isomers have been identified in ruminal contents taken from cattle (Jenkins *et al*., 2008). Yet, most published pathways of biohydrogenation account for the synthesis of only one or two CLA isomers. The ability to regulate CLA synthesis in ruminal contents to deliver specific isomers with specific physiological functions to animal tissues is dependent on delineating the exact synthetic pathways for all isomers.

Because linoleic acid is believed to be the parent compound for most of the CLA isomers found in digestive contents of cattle, and that only one or two CLA isomers are usually identified in pathways of linoleic acid biohydrogenation, this study was conducted to establish the number and identity of CLA isomers that originate from linoleic acid biohydrogenation. To accomplish this objective, the carboxy carbon of linoleic acid was tagged with a ^{13}C stable isotope to trace its appearance in other C18:2 intermediates produced in cultures of mixed ruminal microorganisms. Once the ^{13}C label was identified in a C18:2 compound, the double bond position and

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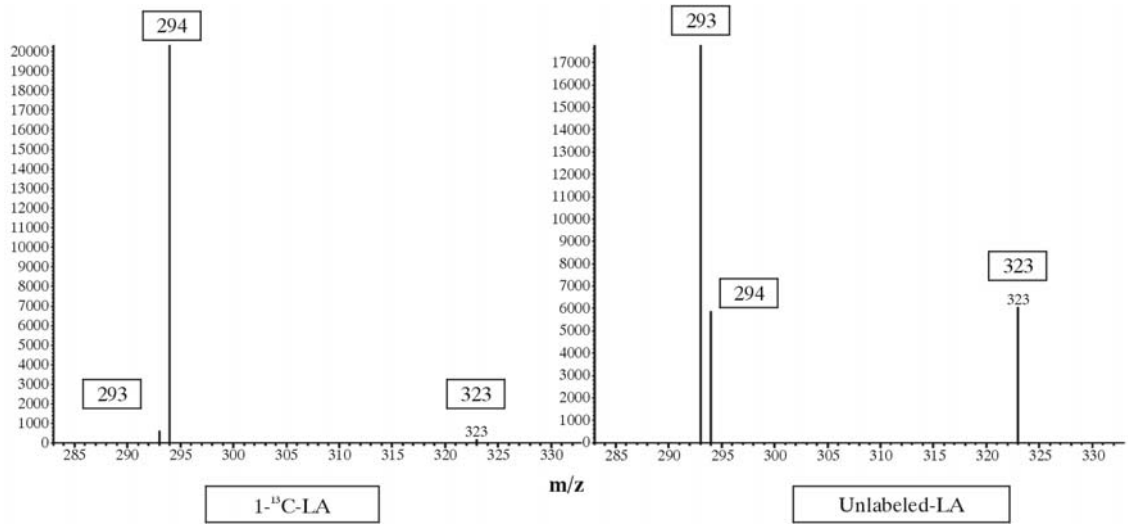

Fig. 1. The mass spectrometry spectrum of 1-^{13}C-linoleic acid and unlabeled linoleic acid with methyl ester.

cis-trans geometry was determined by tandem MS/MS. This approach allowed verification that CLA were produced from linoleic acid including its exact structural identity.

Material and Methods

Materials

TMR (Total mixed rations) were obtained from Clemson University Dairy Farm (including 54.00% corn silage, 2.90% grass hay, 4.90% alfalfa hay, 28.40% grain mix, and 9.80% moisture) and Emersol 315 (including 2.50% palmitic, 1.00% stearic, 27.20% oleic, 57.50% linoleic, 5.10% linolenic, and 6.70% other fatty acid) were purchased from Continental Industrial Chemicals (USA). Unlabeled linoleic acids (*cis*-9 *cis*-12 C18:2; >99.00% chemical purity) were purchased from Sigma-Aldrich (USA) and 1-^{13}C-linoleic acids (>99.00% chemical purity; 99.00% isotopic purity) were purchased from Larodan AB, Inc. (Sweden).

In vitro microbial cultures

Whole digesta contents were collected from the rumen of a fistulated Holstein cow two hours after the AM feeding. The contents were filtered through cheesecloth to remove large particles, and the filtrate containing mixed ruminal microorganisms was then transferred directly to the laboratory in a sealed container and used within 20 min. Six cultures in 125 ml Erlenmeyer flasks containing 0.5 g of diet (TMR+5% Emersol 315), 10 ml of filtered rumen fluid, 40 ml of *in vitro* media, and 2 ml of reducing solution (Goering and Van Soest, 1970) were divided into two groups of three cultures each of mixed rumen microbial population. Cultures containing either 50 mg unlabeled linoleic acid or 35 mg unlabeled linoleic acid + 15 mg 1-^{13}C-linoleic acid in 1 ml of ethanol were run at 39°C water bath under anaerobic conditions. Two 5 ml samples were taken from each culture flask at 0, 24, and 48 h while being stirred with a magnetic bar under CO_2, and then transported immediately in an ice bath, and then stored freezer at -5°C.

Analysis of Fatty Acid Methyl Esters (FAME)

Culture samples were freeze-dried and transesterified to methyl esters in sodium methoxide followed by methanolic HCl procedure (Kramer *et al*., 1997). FAME contents were then quantified by gas chromatography (GC) using internal standard (C17:0, heptadecanoic acid). A Hewlett-Packard 5890 gas chromatograph was equipped with a P-2380, fused silica capillary column (100 m×0.25 mm) with 0.2 μm film thickness (Supelco Inc., USA). The conditions used were initially 140°C for 3 min with a ramp of 3.7°C/min up to 220°C holding for 20 min. Helium was used as the carrier gas at 20 cm/sec.

The ^{13}C enrichment in individual FAME was analyzed by GC-MS (Agilent 6890N gas chromatograph with a model 5973 quadrapole mass selective detector). The GC-MS was equipped with a 100 m ×0.25 mm chrompack CP-Sil 88 column with 0.20 μm film thickness. The carrier gas was helium at 20 cm/sec with splitless injection. Column temperature was programmed initially 140°C for 5 min with a ramp of 4°C per min up to 220°C for 20 min. The ions chosen for fatty acid analysis in selected ion mode was the quasimolecular ion [M], a [M+1] ion to indicate the labeled compound, and [(M+1)+29] ion that represented the [M+$C_2H_5^+$] ion (Fig. 1).

FAME double bond positions and geometry were verified by gas chromatography (GC), followed by covalent adduct chemical ionization tandem mass spectrometry (CACI-MS/MS) according to methods outlined in detail previously (VanPelt and Brenna, 1999; Michaud *et al*., 2002, 2003; Lawrence and Brenna, 2006). Briefly, FAME were separated with a CP-Sil 88 capillary column (100 m×0.32 mm×0.25 μm), and temperature programmed from 80°C to 120°C at a rate of 10°C/min and then increased to 220°C at a rate 2.5°C/min. Total run time was 60 min. FAME eluting into the MS undergo an ion-molecule reaction to form an adduct with molecular weight 54 mass units above that of the parent FAME. Collisional dissociation of this adduct yields diagnostic ions that are characteristic of double bond position. In the case of CLA, relative diagnostic ion intensity is indicative of double bond geometry (Michaud *et al*., 2003), and can otherwise be discerned by GC relative retention time.

Calculations and statistical analysis

Fatty acid contents and ^{13}C enrichments were analyzed by analysis of variance (ANOVA) using the GLM (general linear model) procedure of SAS (2003). The fatty acid contents in the samples were

Table 1. Concentration (mg/5 ml culture) of stearic acid (C18:0), C18:1 isomers, C18:2 isomers and other isomers detected at 0, 24, and 48 h

Fatty acid	Fatty acid content (mg)			SEM
	0 h	24 h	48 h	
C18:0	1.74[a]	2.34[ab]	2.71[b]	0.23
C18:1 isomers				
trans-11 C18:1	0.13[a]	2.40[b]	3.32[c]	0.15
trans-12 C18:1	ND	<0.01	<0.01	<0.01
cis-9 C18:1	1.46[a]	1.40[a]	1.26[b]	0.03
cis-11 C18:1	ND	0.17	0.18	0.01
cis-12 C18:1	0.02[a]	0.81[b]	0.82[b]	0.03
unidentified C18:1	0.02[a]	0.04[ab]	0.05[b]	0.01
C18:2 isomers				
unidentified C18:2 (I)	0.03[a]	0.07[b]	0.07[b]	<0.01
C18:2n-8	0.07[a]	0.16[b]	0.13[c]	0.01
C18:2n-7	ND	0.27	0.25	0.03
cis-9 *cis*-12 C18:2	6.43[a]	2.11[b]	1.01[c]	0.09
unidentified C18:2 (II)	<0.01[a]	0.01[b]	0.01[b]	<0.01
cis-9 *trans*-11 CLA	0.01[a]	0.01[a]	0.02[b]	<0.01
cis-10 *trans*-12 CLA	0.05[a]	0.18[b]	0.09[a]	0.01
trans-10 *cis*-12 CLA	<0.01[a]	0.02[b]	0.02[b]	<0.01
cis-9 *cis*-11 CLA	<0.01[a]	0.05[b]	0.03[c]	0.01
cis-10 *cis*-12 CLA	<0.01[a]	0.01[b]	0.02[c]	<0.01
trans-9 *trans*-11 and *trans*-10 *trans*-12 CLA	0.02[a]	0.15[b]	0.13[b]	0.01
Other isomers				
C20:0	0.05[a]	0.04[b]	0.04[b]	<0.01
C18:3	0.02[a]	0.02[a]	0.01[b]	<0.01
C18:3n-3+C20:1n-9	0.17[a]	0.03[b]	0.02[b]	0.01

Values in a row are the least square means of fatty acid content for each treatment time followed by the pooled SEM (n=6).
[a, b, c] Least squares means within a row with different superscript letters indicate significant difference ($P<0.05$).
ND, not detected.

calculated by comparing the fatty acid peak areas on the chromatograms with the internal standard. Data are shown to Means±SEM as mg of fatty acid contents per 5 ml microbial *in vitro* cultures. Abundances of selected ions in samples were exhibited as [(M+1/M]. Enrichment was identified as $[(M+1/M]_{labeled}$-$[(M+1/M]_{unlabeled}$ cultures to adjust for natural occurrence of the ^{13}C isotope. Enrichment % Means±SEM taken from ANOVA were shown for each fatty acid. Least significant difference was conducted to identify the main effect and differences of fatty acid contents and enrichments of labeled fatty acids by treatment within incubation time. The fatty acid contents and enrichments data were subsequently tested for their differences from zero using Student's t-test ($P<0.05$).

Results and Discussion

The fatty acid content (Table 1) in cultures investigated here supports the previous research in this area (Kellens *et al*., 1986; Griinari and Bauman, 1999; Nam and Garnsworthy, 2007a, 2007b). Linoleic acid decreased 84.29% (5.42 mg) over 48 h in a 5 ml microbial *in vitro* culture, accompanied by an increase in *trans*-11 C18:1 (3.19 mg) and stearic acid (0.97 mg) of 58.86% and 17.90%, respectively. A previous *in vitro* study (Van Nevel and Demeryer, 1996) found that more than 90% of the linoleic acid was hydrogenated at 6 h of incubation, while the *in vivo* experiment (Wu *et al*., 1991) determined that approximately 80% of the linoleic acid disappeared. In addition, this study found that the concentration of *cis*-12 C18:1 also increased (0.80 mg) over time, as the linoleic acid disappeared. Several *cis*-C18:1 isomers may originate from linoleic and linolenic acid (Kemp *et al*., 1975). In total approximately 95% of the linoleic acid lost was accounted for by the increase in the sum of these three fatty acids: stearic, *trans*-11 and *cis*-12 C18.

The C18:2 isomers, including the CLA, were present in very small amounts or were not detected at 0 h. After 24 h, the *cis*-10 *trans*-12 CLA content was highest at 0.18 mg followed by the *trans*-9 *trans*-11 and *trans*-10 *trans*-12 CLA at 0.15 mg. Other CLA accounted for less than 0.05 mg at the end of incubation. These results do not agree with previous research which demonstrated that *cis*-9 *trans*-11 CLA is the predominant CLA isomer under similar conditions (Kellens *et al*., 1986; Griinari and Bauman, 1999; Griinari *et al*., 2000; Nam and Garnsworthy, 2007a). For example, *cis*-9 *trans*-11 CLA comprised 15% of the fatty acid content after 10 min of incubation in the presence of mixed ruminal bacteria (Nam and Garnsworhty, 2007a). The pattern of CLA production in the study reported here may be related to the rapid biohydrogenation rate of *cis*-9 *trans*-11 CLA to *trans*-11 C18:1, thereby quickly reducing the concentration of the former and increasing the latter.

Previous research (Kellens *et al*., 1986; Proell *et al*., 2002) found a disappearance of linoleic acid simultaneous with in-

Table 2. Enrichment of stearic acid (C18:0), linolenic acid (C18:3n-3) and other isomers

Fatty acid	Enrichment (%)			SEM
	0 h	24 h	48 h	
C18:0	-0.020[a]	1.930[b]	2.690[b]	0.96
C18:3n-3+C20:1n-9	-0.110[a]	-0.810[b]	-0.010[c]	0.44
C18:3	0.890[a]	-1.770[b]	-3.300[c]	2.40
C20:0	6.980[a]	-3.590[b]	-3.340[c]	8.43

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=3).
[a, b, c] Least squares means within a row with different superscript letters indicate significant difference (P<0.05).

creasing *trans* isomers and stearic acid concentration during 50 h of incubation with mixed ruminal microbes. More recently, the linoleic acid content decreased significantly within 60 min of incubation with mixed ruminal bacteria and within 12 h of incubation with mixed ruminal fungi (Nam and Garnsworthy, 2007a, 2007b). In our research, biohydrogenation proceeded more slowly than in previous studies (Kellens *et al*., 1986; Nam and Garnsworthy, 2007a, 2007b). This slower rate may be related to the injection of highly concentrated linoleic acid that may inhibit the biohydrogenation process. In this study, linoleic acid was inoculated 0.96 mg/ml, a dose five times higher than the 0.20 mg/ml of the other study (Nam and Garnsworthy, 2007a, 2007b). Biohydrogenation and the growth of ruminal bacteria can be inhibited by inoculation of as little as 50 μg linoleic acid/ml (Maia *et al*., 2006).

The enrichment analysis found that linoleic acid was isomerized and hydrogenated to several intermediates at the end of 48 h after the injection of the 1-^{13}C-linoleic acid (Tables 2, 3, 4). A total of 21 fatty acid peaks were identified including 6 C18:1 isomers and 11 C18:2 isomers of which 7 were CLA. This finding is inconsistent with currently accepted pathways (Griinari and Bauman, 1999) for the biohydrogenation of linoleic acid. Many researchers believe that ruminal microbes produce only *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA, which are converted to *trans*-11 C18:1 and *trans*-10 C18:1, respectively, before the formation of stearic acid (C18:0). However, since 7 CLA were identified in this study, including *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA, biohydrogenation appears to be more complicated than previously thought.

Enrichment of linoleic acid remained statistically constant from 0 to 24 h (22.20% to 28.15%, respectively), then increased

Table 3. Enrichment of C18:1 isomers

Fatty acid	Enrichment (%)			SEM
	0 h	24 h	48 h	
trans-11 C18:1	NS	12.50[a]	14.51[b]	1.09
trans-12 C18:1	ND	14.55[a]	2.90[b]	2.67
cis-9 C18:1	0.03[a]	5.60[b]	14.07[c]	1.17
cis-11 C18:1	ND	16.99[a]	18.66[b]	1.88
cis-12 C18:1	NS	14.14	13.57	0.66
Unidentified C18:1	NS	9.92	11.47	1.31

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=3).
[a, b, c] Least squares means within a row with different superscript letters indicate significant difference (P<0.05).
NS, not significant from zero
ND, not detected.

Table 4. Enrichment of C18:2 isomers

Fatty acid	Enrichment (%)			SEM
	0 h	24 h	48 h	
unidentified C18:2 (I)	5.88[a]	20.05[b]	22.09[c]	0.70
C18:2n-8	20.19[a]	24.80[b]	32.54[c]	2.27
C18:2n-7	ND	19.14[a]	23.71[b]	2.57
cis-9 *cis*-12 C18:2	22.20[a]	28.15[a]	37.21[b]	3.49
unidentified C18:2 (II)	11.24[a]	1.710[b]	3.600[b]	0.88
cis-9 *trans*-11 CLA	12.80[a]	30.90[b]	30.65[b]	3.82
cis-10 *trans*-12 CLA	13.14[a]	16.72[b]	23.08[c]	1.73
trans-10 *cis*-12 CLA	15.04[a]	18.99[b]	22.60[c]	1.02
cis-9 *cis*-11 CLA	15.40	16.74	18.38	1.24
cis-10 *cis*-12 CLA	9.510[a]	14.66[b]	19.29[c]	1.07
trans-9 *trans*-11 and *trans*-10 *trans*-12 CLA	14.25[a]	17.39[b]	21.06[c]	0.91

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=3).
[a, b, c] Least squares means within a row with different superscript letters indicate significant difference (P<0.05).
ND, not detected.

from 24 to 48 h (Table 4). However, constant enrichment of linoleic acid during incubation was expected because the microbial system in the rumen cannot produce the linoleic acid, which originated from other fatty acid sources such as oleic and linolenic acid. Without the additional linoleic acid produced in the culture, it was expected that the ruminal microbes would utilize both isotope-labeled and unlabeled linoleic acid in the biohydrogenation process with equal efficiency. The increased enrichment of isotope-labeled linoleic acid from 24 to 48 h, however, may be due to the method of sample collecting. In this study, 5 ml sample was collected at 0, 24, and 48 h from the same *in vitro* cultures. However, when samples were collected by individual cultures, i.e. without subsampling, the enrichment of the linolenic acid was almost constant - 0 h (33.47%), 3 h (33.26%), 24 h (33.14%), and 48 h (35.49%). This result suggests a fatty acid by sub-sampling might be distributed evenly throughout in the cultures, causing an increase in the enrichment after 24 h during the biohydrogenation process.

The enrichment of stearic acid was increased at all incubation times from 0.00% at 0 h to a final enrichment of 2.69% at 48 h (Table 2). These results suggest that the stearic acid originated during the biohydrogenation of linoleic acid at that time. Stearic acid accounted for only 17.90% of the C18:2 lost by 48 h; these results do not support the findings (Kellens *et al*., 1986), who determined that stearic acid concentration increased significantly at 50 h incubation with ruminal microbes while the linoleic acid disappeared. The study found that stearic acid comprised 100.0% of the identified fatty acids for linoleic acid biohydrogenation until 90 min of incubation in the presence of mixed ruminal bacteria (Nam and Garnsworthy, 2007a). It is possible, as mentioned previously, that the high linoleic acid concentrations in the cultures inhibited the biohydrogenation process (Beam *et al*., 2000), especially from intermediates C18:1 isomer to stearic acid. Lower enrichment might also be attributed to the isotope introduction method. In addition, other isomers such as C18:3, C20:0, and C20:1n-9 were identified but not enriched. Because ruminal

anaerobes do not have the ability to desaturate fatty acids, especially C18:2, with the addition of a double bond, no enrichment of C18:3 was found during the incubation time. Since ruminal anaerobes cannot add acetyl units to the fatty acid structure by reacting with elongases, C20:0 cannot be formed, accounting for the elongation of labeled C18:0. For that reason, C18:3, C20:0, and C20:1n-9 intermediates were not derived from the labeled linoleic acid by the rumen microorganisms. However, other possible sources of unlabeled unsaturated fatty acids were present in the diet substrate *in vitro*. It is likely that the isomerization and biohydrogenation products in the mixed rumen microorganism cultures affected the formation of all intermediates of linoleic acid.

The enrichment of all C18:1 isomers was either negligible or not detected at 0 h incubation, indicating that none originated from linoleic acid biohydrogenation (Table 3). The *trans*-11, *trans*-12, *cis*-9, *cis*-11, *cis*-12, and unidentified positional C18:1 isomers were identified as intermediates of linoleic acid biohydrogenation. From 0 to 24 h, enrichment of *trans*-11 C18:1 increased to 14.51% but did not change from 24 to 48 h. *Trans*-11 C18:1 generally is considered the major intermediate of linoleic acid biohydrogenation in the rumen (Griinari *et al*., 2000). In addition, our results suggest those intermediates of *cis*-9, *cis*-11, *cis*-12, and unidentified C18:1 were enriched by 48 h. 1-^{13}C-elaidic acid (*trans*-9 C18:1) was hydrogenated, becoming stearic acid and *trans*-C18:1 isomers by ruminal microorganisms (Proell *et al*., 2002). This study also found that 17.60% of the oleic acid (*cis*-9 C18:1) originated from elaidic acid (*trans*-9 C18:1). Similarly the *cis*-12 bond of linoleic acid is converted to *trans*-11 bond by isomerase (Harfoot and Hazlewood, 1997). As all of these studies suggest, the formation of *cis*/*trans* C18:1 positional isomers is possible during the biohydrogenation of linoleic acid. Although *trans*-10 C18:1 has been suggested as an intermediate of the biohydrogenation of *trans*-10 *cis*-12 CLA (Griinari and Bauman, 1999), the amount, if present in this research, was too small to detect.

The enrichment of C18:2 isomers, with the exception of C18:2n-7, was detectable at 0 h incubation, even though it was theoretically impossible for them to be formed at this point (Table 4). One possible explanation for this apparent contradiction is that C18:2n-7 might be converted to form CLA or other C18:2 isomers within a few seconds and, thus, have such a short half-life that it is undetectable using this assay. Similarly, the ruminal bacteria *Megasphaera elsdenii* YJ-4 produced *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA very quickly, reaching maximal levels after only 2 min of incubation (Kim *et al*., 2002).

The enrichment of *cis*-9 *trans*-11 CLA reached a maximum at 24 h (30.90%) and did not increase significantly from 24 to 48 h (30.65%), while the accumulation of other CLA continued to increase throughout incubation. These results indicate the ^{13}C label is transferred to other intermediates during biohydrogenation. Likewise, *cis*-9 *trans*-11 CLA may be a potential precursor of other CLA and/or C18:1 isomers. In addition, *trans*-10 *cis*-12, considered the second major isomer, and *trans*-9 *trans*-11 and *trans*-10 *trans*-12 CLA increased slightly over all incubation times, the final proportions of these CLA reaching 22.60% and 21.06% by 48 h. Three CLA isomers (*cis*-9 *trans*-11, *trans*-10 *cis*-12, and *trans*-9 *trans*-11 CLA) are produced by several species of bacteria in the rumen (Griinari and Bauman, 1999). *Butyrivibrio fibrisolvens* are important ruminal bacteria, normally used as models for producing *cis*-9 *trans*-11 CLA, but they do not produce *trans*-10 *cis*-12 CLA, which are produced by *Propionibacterium acnes* and *P. freudenreichii* (Jiang *et al*., 1998; Kim *et al*., 2000). One important finding of the study was that linoleic acid was hydrogenated to produce *cis*-9 *trans*-11 CLA and smaller amounts of *trans*-9 *trans*-11 CLA. Subsequently, *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA were converted to *trans*-9 *trans*-11 CLA via the *Bifidobacterium* species. Instead of direct formation of these CLA, Ogawa *et al*. (Ogawa *et al*., 2001; Coakley *et al*., 2006) suggested another route of biohydrogenation by which 10-hydroxy-*cis*12-C18:1 and 10-hydroxy-*trans*12-C18:1 became intermediates of linoleic acid before the formation of *cis*-9 *trans*-11 CLA and *trans*-9 *trans*-11 CLA by *Lactobacillus acidophilus* AKU 1137 under microaerobic conditions.

Cis-10 *trans*-12 CLA, and *cis*-10 *cis*-12 CLA were detected at all incubation times. The enrichment of these CLA increased from 0 to 24 h and from 24 to 48 h to final values of 23.08%, and 19.29% respectively. The enrichment of *cis*-9 *cis*-11 CLA was similar at 0 h (15.40%) but did not increase significantly from 0 to 48 h incubation. According to the calculations, all of these CLA originate from labeled linoleic acid derived by bacterial isomerases; however, which biochemical pathways are involved is still unclear.

As the CLA isomers that have been identified, at least 14 can be divided into the four double bond geometric configurations of *cis*/*cis, trans*/*trans, cis*/*trans,* and *trans*/*cis* (Duckett *et al*., 2002; Piperova *et al*., 2002; Shingfield *et al*., 2003). Furthermore, each of these CLA was identified in the intestinal contents of ruminants. However, only seven CLA originated from linoleic acid biohydrogenation in this study. The remaining CLA isomers (>7) might be formed by biohydrogenation of other polyunsaturated fatty acids. All identified CLA carried a double bond at carbon 9 and 12, and all possible double bond geometries (*cis*/*cis*, *trans*/*trans*, *cis*/*trans*, and *trans*/*cis*) were identified, except for *trans*-9 *cis*-11 CLA, by linoleic acid biohydrogenation. The isomerization of linoleic acid is initiated at carbon number 11 by hydrogen abstraction which then produces several CLA isomers of carbons 9 and 12 with differences in isomerization capacity by different ruminal bacteria species (Liavonchanka *et al*., 2006).

Although the results of the linoleic acid biohydrogenation pathway using stable isotope utilization by mixed ruminal microbes had not been evaluated previously (Griinari and Bauman, 1999), the current study verified the formation of *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA isomers from linoleic acid via biohydrogenation. An additional five CLA isomers contained carbons that originated from linoleic acid, indicating that the pathways of linoleic acid biohydrogenation are more complex than previously thought. Additional studies are necessary to identify the enteric biological pathway of lipid metabolism. The results of such an investigation may lead to benefits in the area of human health such as carcinogenesis, immune function, obesity, diabetes, and atherogenesis.

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References

Beam, T.M., T.C. Jenkins, P.J. Moate, R.A. Kohn, and D.L. Palmquist. 2000. Effects of amount and source of fat on the rates of lipolysis and biohydrogenation of fatty acids in ruminal contents. *J. Dairy Sci*. 83, 2564-2573.

Coakley, M., M.C. Johnson, E. McGrath, S. Rahman, R.P. Ross, G.F. Fitzgerald, R. Devery, and C. Stanton. 2006. Intestinal *Bifidobacteria* that produce *trans*-9, *trans*-11 conjugated linoleic acid: a fatty acid with antiproliferative activity against human colon SW480 and HT-29 cancer cells. *Nutr. Cancer* 56, 95-102.

DeLany, J.P. and D.B. West. 2000. Changes in body composition with conjugated linoleic acid. *J. Am. Coo. Nutr*. 19, 487S-493S.

Duckett, S.K., J.G. Andrae, and F.N. Owens. 2002. Effect of high-oil corn or added corn oil on ruminal biohydrogenation of fatty acids and conjugated linoleic acid formation of beef steers fed finishing diets. *J. Anim. Sci*. 80, 3353-3360.

Goering, H.K. and P.J. Van Soest. 1970. Forage fiber analysis. Agric. Handbook No. 379. ARS, USDA, Washington, DC, USA.

Griinari, J.M. and D.E. Bauman. 1999. Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants, Vol. 1., pp. 180-200 in Advances in Conjugated Linoleic Acid Research, *In* M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza, and G.J. Nelson (ed.). AOCS Press, Champaign, IL, USA.

Griinari, J.M., B.A. Corl, S.H. Lacy, P.Y. Chouinard, K.V. Nurmela, and D.E. Bauman. 2000. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ(9)-desaturase, *J. Nutr*. 130, 2285-2291.

Harfoot, C.G. and G.P. Hazlewood. 1997. Lipid metabolism in the rumen. *In* P.N. Hobson and C.S. Stewart (eds.), The rumen microbial ecosystem, pp. 382-426. Chapman and Hall, London, UK.

Ip, C., S. Banni, E. Angioni, G. Carta, J. McGinley, H.J. Thompson, D. Barbano, and D.E. Bauman. 1999. Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. *J. Nutr*. 129, 2135-2142.

Jenkins, T.C., R.J. Wallace, P.J. Moate, and E.E. Mosley. 2008. Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *J. Anim. Sci.* 86, 397-412.

Jiang, J., L. Bjorck, and R. Fonden. 1998. Production of conjugated linoleic acid by dairy starter cultures. *J. Appl. Microbiol*. 85, 95-102.

Kellens, M.J., H.L. Goderis, and P.P. Tobback. 1986. Biohydrogenation of unsaturated fatty acids by a mixed culture of rumen microorganisms. *Biotechnol. Bioeng*. 28, 1268-1276.

Kemp, P., R.W. White, and D.L. Lander. 1975. The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. *J. Gen. Microbiol*. 90, 100-114.

Kim, Y.J., R.H. Liu, D.R. Bond, and J.B. Russell. 2000. Effect of linoleic acid concentration on conjugated linoleic acid production by *Butyrivibrio fibrisolvens* A38. *Appl. Environ. Microbiol*. 66, 5226-5230.

Kim, Y.J., R.H. Liu, K.L. Rychlik, and J.B. Russell. 2002. The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the trans-10, cis-12 isomer of conjugated linoleic acid. *J. Appl. Microbiol*. 92, 976-982.

Kramer, J.K.G., V. Fellner, M.E.R. Dugan, F.D. Sauer, M.M. Mossoba, and M.P. Yurawecz. 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total *trans* fatty acids. *Lipids* 32, 1219-1228.

Lawrence, P. and J.T. Brenna. 2006. Acetonitrile covalent adduct chemical ionization mass spectrometry for double bond localization in non-methylene-interrupted polyene fatty acid methyl esters. *Anal. Chem*. 78, 1312-1317.

Liavonchanka, A., E. Hornung, I. Feussner, and M.G. Rudolph. 2006. Structure and mechanism of the *Propionibacterium acnes* polyunsaturated fatty acid isomerase. *Proc. Natl. Acad. Sci. USA* 103, 2576-2581.

Maia, M.R.G., L.C. Chaudhary, L. Figueres, and R.J. Wallace. 2006. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Antonie van Leeuwenhoek* 91, 303-314.

Michaud, A.L., G.Y. Diau, R. Abril, and J.T. Brenna. 2002. Double bond localization in minor homoallylic fatty acid methyl esters using acetonitrile chemical ionization tandem mass spectrometry. *Anal. Biochem*. 307, 348-360.

Michaud, A.L., M.P. Yurawecz, P. Delmonte, B.A. Corl, D.E. Bauman, and J.T. Brenna. 2003. Identification and characterization of conjugated fatty acid methyl esters of mixed double bond geometry by acetonitril chemical ionization tandem mass spectrometry. *Anal. Chem*. 75, 4925-4930.

Mosley, E.E., G.L. Powell, M.B. Riley, and T.C. Jenkins. 2002. Microbial biohydrogenation of oleic acid to *trans* isomers *in vitro*. *J. Lipid Res*. 43, 290-296.

Nam, I.S. and P.C. Garnsworthy. 2007a. Biohydrogenation of linoleic acid by rumen fungi compared with rumen bacteria. *J. Appl. Microbiol*. 103, 551-556.

Nam, I.S. and P.C. Garnsworthy. 2007b. Factors influencing biohydrogenation and conjugated linoleic acid production by mixed rumen fungi. *J. Microbiol*. 45, 199-204.

Ogawa, J., J. Matsumura, S. Kishino, Y. Omura, and S. Shimizu. 2001. Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol*. 67, 1246-1252.

Pariza, M.W. 2004. Perspective on the safety and effectiveness of conjugated linoleic acid. *Am. J. Clin. Nutr*. 79, 1132-1136.

Piperova, L.S., J. Sampugna, B.B. Teter, K.F. Kalscheur, M.P. Yurawecz, Y. Ku, K.M. Morehouse, and R.A. Erdman. 2002. Duodenal and milk trans octadecenoic acid and conjuaged linoleic acid (CLA) isomers indicate that postabsorptive synthesis is the predominant source of *cis*-9-containing CLA in lactating dairy cows. *J. Nutr*. 132, 1235-1241.

Proell, J.M., E.E. Mosley, G.L. Powell, and T.C. Jenkins. 2002. Isomerization of stable isotopically labeled elaidic acid to *cis* and *trans* monoenes by rumincl microbes. *J. Lipid Res*. 43, 2072-2076.

Ryder, J.W., C.P. Portocarrero, X.M. Song, L. Cui, and M.Yu. 2001. Isomer specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action and UCP-2 gene expression. *Diabetes* 50, 1149-1157.

Shingfield, K.J., S. Ahvenjarvi, V. Toivonen, A. Arola, K.V.V. Nurmela, P. Huhtanen, and J.M. Griinari. 2003. Effect of fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. *Anim. Sci*. 77, 165-179.

Van Pelt, C.K. and J.T. Brenna. 1999. Acetonitrile chemical ionization tandem mass spectrometry to locate double bonds in polyunsaturated fatty acid methyl esters. *Anal. Chem*. 71, 1981-1989.

Van Nevel, C. and D.I. Demeryer. 1996. Influence of pH on lipolysis and biohydrogenation of soybean oil by rumen contents *in vitro*. *Reprod. Nutr. Dev.* 36, 53-63.

Wahle, K.W., S.D. Heys, and D. Rotondo. 2004. Conjugated linoleic acids: Are they beneficial or detrimental to health? *Prog. Lipid Res*. 43, 553-587.

Wu, Z., O.A. Ohajuruka, and D.L. Palmquist. 1991. Ruminal synthesis, biohydrogenation, and digestibilities of fatty acids by dairy cows. *J. Dairy Sci*. 74, 3025-3034.